Check for updates

RESEARCH ARTICLE

Maize phyllosphere microbial community niche development across stages of host leaf growth [version 1; peer review: 1 approved with reservations]

Heather C. Manching^{1,2}, Kara Carlson^{2,3}, Sean Kosowsky², C. Tyler Smitherman^{2,4}, Ann E. Stapleton ²

¹Department of Plant and Soil Sciences, University of Delaware, Newark, DE, USA

²Department of Biology and Marine Biology, University of North Carolina Wilmington, Wilmington, NC, USA

³North Carolina Museum of Science, Raleigh, NC, USA

⁴Nestlé, Inc., Danville, NC, USA

V1 First published: 18 Sep 2017, 6:1698
<https://doi.org/10.12688/f1000research.12490.1>
 Second version: 13 Dec 2017, 6:1698
<https://doi.org/10.12688/f1000research.12490.2>
 Latest published: 18 Jan 2018, 6:1698
<https://doi.org/10.12688/f1000research.12490.3>

Abstract

Background: The phyllosphere hosts a variety of microorganisms, including bacteria, which can play a positive role in the success of the host plant. Bacterial communities in the phylloplane are influenced by both biotic and abiotic factors, including host plant surface topography and chemistry, which change in concert with microbial communities as the plant leaves develop and age.

Methods: We examined how the *Zea mays* L. leaf microbial community structure changed with plant age. Ribosomal spacer length and scanning electron microscopic imaging strategies were used to assess microbial community composition across maize plant ages, using a novel staggered experimental design.

Results: Significant changes in community composition were observed for both molecular and imaging analyses, and the two analysis methods provided complementary information about bacterial community structure within each leaf developmental stage.

Conclusions: Both taxonomic and cell-size trait patterns provided evidence for niche-based contributions to microbial community development on leaves.

Keywords

epiphytes, plant host, community assembly, *Zea mays* L., ecological niche, scanning electron microscopy

Open Peer Review

Approval Status

	1	2	3
version 3
(revision)		view	view
18 Jan 2018			
version 2
(revision)
13 Dec 2017		view	
version 1
18 Sep 2017	view		

1. **Kathryn M. Docherty**, Western Michigan University, Kalamazoo, USA
2. **Mitja N.P. Remus-Emsermann** , University of Canterbury, Christchurch, New Zealand
Erica Boyd, University of Canterbury, Christchurch, New Zealand
Michal Bernach, University of Canterbury, Christchurch, New Zealand
Rudolf Schlechter, University of Canterbury, Christchurch, New Zealand

Simisola Oso, University of Canterbury,
Christchurch, New Zealand

3. Kiwamu Minamisawa, Tohoku University,
Sendai, Japan

Any reports and responses or comments on the
article can be found at the end of the article.

Corresponding author: Ann E. Stapleton (stapletona@uncw.edu)

Author roles: **Manching HC:** Formal Analysis, Investigation, Methodology, Supervision, Writing – Review & Editing; **Carlson K:** Formal Analysis, Investigation, Methodology, Writing – Review & Editing; **Kosowsky S:** Formal Analysis, Software, Writing – Review & Editing; **Smitherman CT:** Data Curation, Formal Analysis, Investigation, Methodology, Resources, Writing – Review & Editing; **Stapleton AE:** Conceptualization, Data Curation, Methodology, Project Administration, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: The author(s) declared that no grants were involved in supporting this work.

Copyright: © 2017 Manching HC *et al.* This is an open access article distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Data associated with the article are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

How to cite this article: Manching HC, Carlson K, Kosowsky S *et al.* **Maize phyllosphere microbial community niche development across stages of host leaf growth [version 1; peer review: 1 approved with reservations]** F1000Research 2017, 6:1698 <https://doi.org/10.12688/f1000research.12490.1>

First published: 18 Sep 2017, 6:1698 <https://doi.org/10.12688/f1000research.12490.1>

Introduction

The phyllosphere, the microbial community inhabiting the surface of plant leaves¹, can play important positive roles for the plant partner. For example, disease resistance is increased when supportive bacteria are present^{2,3} and increased host plant growth in the presence of specific community members has been documented⁴⁻⁶, along with a positive effect of overall increased microbial diversity⁷. Leaf habitats are also extremely important for the microbial community, as this habitat is quite large in surface area globally. In temperate regions seasonal growth and annual agricultural plantings lead to repeated opportunities for colonization by adapted microbes^{8,9}.

Determinants of community assembly/succession in microbial systems include biotic and abiotic factors, often classified into different community assembly rules¹⁰. Typically random or neutral stochastic models are tested against abiotic effect models, which are called environmental, or, more recently, habitat filtering rules. Environmental filtering can produce different patterns depending on scale¹¹. As recommended by Kraft *et al.* (2015) we will refer to environmental filtering as habitat filtering when there is no separate test for abiotic and biotic effects. A second class of assembly rules addresses the interactions of organisms, by either facilitation or competitive exclusion. These niche interactions are often modeled separately from abiotic effects, or abiotic effects are assumed to happen first.

Community assembly and succession processes are typically inferred from diversity patterns in community samples¹². The number of different taxa (alpha-diversity), the difference in the composition of taxa between replicates (beta-diversity) and the proportion of co-occurring taxa all provide evidence for these different community-generating mechanisms. Niche construction is inferred from a pattern of decreasing beta-diversity, increasing co-occurrence, and increasing richness, while habitat filtering is characterized by decreasing beta-diversity with no trends in co-occurrence or richness^{13,14}. In contrast, neutral community assembly would exhibit increased richness (provided immigration rates are maintained), with unchanged beta-diversity and co-occurrence levels. Once we have measurements of the various components of diversity, we can compare observed trends to these expectations. The combination of increasing/decreasing trends would then support or fail to support a particular mechanism of community succession. Both trait-based (morphological, in our study) and taxonomic measures of diversity can be compared to the expected trends^{15,16}. For microbial systems, cell size is one of the few traits that is measurable at the microscopic scale; other functional traits, such as metabolic activity, are more often examined at the molecular scale, by whole-community DNA sequencing, proteome analysis, or metabolite profiling.

Previous analyses of controlled inoculation phyllosphere community development indicate that habitat filtering is important, with niche effects less often detected across time, though all previous studies have used seasonal or through-time designs that confound exposure time and plant development. Pre-colonization reduces immigrant success^{8,17}, suggesting biotic competition is

key, though high beta-diversity in replicate samples from both pre-colonized and newly inoculated communities suggested that there are many options for species that arrive first to succeed. Several crop species have been tested for seasonal patterns of phyllosphere community development. Microbial populations increased more rapidly on younger leaves of lettuce¹⁸, and alpha-diversity increased over the first few weeks then plateaued¹⁹, which provides evidence that habitat filtering was a contributor to community succession later in the season. In apple flowers, species richness increased early in the season and beta-diversity dropped, then rose²⁰. About half the apple flower microbial species had significant co-occurrence relationships and the numbers of co-occurrences increased and decreased over time. This pattern supports niche construction as a key mechanism for apple flower colonization. As in apple flowers, across-season dicot crop phyllosphere samples became less diverse (alpha-diversity went up then declined). In the crop samples, more shared and plant-specific taxa were found later in the season²¹, again supporting development of specific community structure. In contrast, *Arabidopsis* epiphyte beta-diversity increased with time²², providing stronger support for stochastic effects in this system. The history of annual opportunities for new colonization is reflected in the processes for dispersal and succession on plant leaves. Colonization of new growth in the presence of older leaves may proceed differently than colonization from distant source materials. The contributions of abiotic changes and host leaf age are confounded when sampling occurs through time, as in seasonal sampling, thus necessitating alternative experimental designs and/or additional phylogenetic or functional trait information to examine the roles of these assembly processes. Sampling serially across time, as all prior studies have done, could confound short and long-term trends and does not enable differences in plant host to be distinguished from inoculation processes^{19,20}. Therefore, for this study host plant seeds were planted at different times and sampled once.

How can leaf microbial communities be measured? Molecular methods for detection of species using ribosomal fragments or sequences have become increasingly common^{20,21,23}. Microscopy can be used to detect bacteria (and fungi) on corn leaf surfaces²⁴, and surface bacteria can be followed with reporter genes²⁵ as well as by direct enumeration. Microscopic or particle-based enumeration methods are especially useful for microbial communities where we have limited information on traits and horizontal gene transfer is likely²⁶. For this study we used both a molecular approach to measure relatedness-based (taxonomic) microbial community composition, and direct enumeration of scanning electron micrograph (SEM) images to measure cell-size trait changes.

Our specific questions concerned the pattern of community development – how community succession varies with plant age, and whether the microbial community was primarily shaped by microbe-host interactions (environmental or more generally, habitat filtering) or by microbe-microbe interactions (niche construction). We found complex patterns in the maize leaf microbial community structure even across the short series we sampled, with niche construction and stage-specific increasing-decreasing diversity trends observed.

Methods

Experimental design and sample collection

Zea mays L. B73 inbred seeds obtained from the Maize Genetics Cooperation Stock Center (<http://maizecoop.cropsci.uiuc.edu/>) were planted using a hand held planter between April 26th and June 15th 2009 at the Central Crops Research Station in Clayton, NC (<http://www.ncagr.gov/research/ccrs.htm>). All necessary permits were obtained for the described study, which complied with all relevant regulations; permits were managed by the research station staff. Approximately twenty seeds were planted each date, with plantings between four and eleven days apart in a single field. All plants within a developmental stage were planted in the same row, bordered on either side by older and younger stages, then by additional crops, grass borders, and tree-shrub hedgerows.

All ten replicate leaf samples were collected at once on July 15, 2009. Both scanning electron microscope (SEM) and ribotype (ARISA) samples were obtained from tips of separate central leaves from ten plants at each developmental stage. SEM samples were approximately five centimeters square and were cut along the leaf mid-rib near the tip (Figure 1). Sections were immediately fixed, by immersion in a solution of 2.5 percent glutaraldehyde in 0.1 M Sorenson's phosphate buffer at a pH of 7.1.

Leaf wash samples for ribosomal DNA analysis were then collected from the same ten plants, using adjacent leaf numbers and a similar portion of the leaf tip (Figure 1). Approximately 10 cm of leaf was cut, submerged in buffer (0.2mM Tris pH 7.5, 0.02 mM EDTA, 0.00012X Triton X100), agitated vigorously and removed using the method described in 27. Leaf washes were stored on dry ice while in the field then placed in a -70 degrees C freezer for long-term storage. One mock control (where no leaves were placed in the tubes) and one wash control

(SEM sample from washed leaf to check for washing effectiveness) was taken for each stage.

SEM sample processing

Excess glutaraldehyde was removed from leaf SEM samples by rinsing for 30 minutes in 0.1 M Sorenson's phosphate buffer followed by an additional 30 minutes in distilled water. Sections were then dehydrated in an acetone series (50, 70, 90, 100 percent), each lasting 30 minutes, followed by a 30-minute treatment in hexamethyldisilazane. Sections were then allowed to air dry before being attached by the lower leaf surface to stubs using double-sided sticky tape. One leaf section chosen arbitrarily from each plant was sputter coated with 10 nm of platinum/palladium and observed in the SEM at 5 kV with a spot size of 3. The SEM procedure was tested prior to sampling using young (approximately 25 cm tall), greenhouse grown maize plants and *Pseudomonas aureginosa*²⁸.

SEM image acquisition

Using the SEM stage control, five evenly dispersed random images (quadrants) per plant were taken at 1000x and 2000x for 35 sections (35 plants) each to give two viewpoints by which to count bacteria. All images are available from the image repository BisQue via the iPlant/CyVerse Data Commons, at doi: [10.7946/P2WC77](https://doi.org/10.7946/P2WC77).

Ribosomal spacer amplification and separation (ARISA)

For the DNA analysis, leaf washes were syringe filtered using previously developed methods²⁹ (videos of the method are available upon request) and microbial DNA was extracted using an UltraClean Microbial kit according to the manufacturer's instructions (MoBio Laboratories, Carlsbad, CA). An equal volume of each DNA sample was amplified to ensure fair representation of the epiphytes collected.

Figure 1. Conceptual diagram of the leaf sampling procedure. Each plant in the drawing represents 10 plants from one of seven developmental stages; the experimental design had $n=10$. Small squares represent SEM sampling areas and large squares represent ARISA sampling areas. All samples were taken from center leaves within each developmental group, and both samples per plant were taken from leaves of comparable position based on height.

Two separate, nested PCR reactions were performed on experimental samples as described by Cardinale *et al.*³⁰, along with four positive controls from a mixture of DNAs from cultured bacteria (Carlson, 2015) and four negative (nuclease-free water) controls. For the initial PCR step, a master mix was made to include: Q5 master mix (New England Biolabs, Ipswich, MA), ITSf primer (5'-GTCGTAACAAGGTAGCCGTA-3'), LD primer (5'-CCGGGTTTCCCCATTCGG-3'), and nuclease-free ultrapure water. Ten microliters of master mix was added to sterile PCR tubes followed by experimental and sample controls. The samples and controls were then run on an iCycler (BioRad, Hercules, CA) PCR machine with the following settings: 1X 95C for 30 seconds, 35X (95C for 30 seconds; 61.5C for 30 seconds; 72C for 1 minute, 30 seconds), 1X 72C for 10 minutes. For the second PCR step, a second master mix was made with the following: Q5 master mix, ITSreub primer (5'-GCCAAGGCATCCACC-3'), which contained a fluorescent HEX marker for fragment analysis in a Genetic Analyzer, SD primer (5'-TGCGGCTGGATCCCCTCCTT-3'), and nuclease-free water.

Samples were run on the Applied Biosystems Genetic Analyzer (ABI, Inc, Carlsbad, CA) and analysis of PCR product length was carried out using the instrument software. For each sample, two microliters of PCR product and 13 microliters of GS500-Rox plus HiDi (ABI, Inc, Carlsbad, CA) were combined. The samples were denatured at 94C for ten minutes, placed on ice for two minutes, then placed into the analyzer and the program started.

SEM image analysis

For each SEM quadrant image, Adobe Photoshop was used to manually outline each bacterial organism present (examples are shown in Figure 2). All single bacteria present in the image were outlined; bacteria were identified by size and smoothness of circumference, as originally described by Davis²⁴. Bacterial areas were measured using Image Pro Plus (Media Cybernetics, Inc., Rockville, MD, USA) and recorded for each quadrant picture that was taken. Image Pro Plus sized bacteria that were touching other bacteria as one large organism, therefore as many bacteria as possible were outlined in the clusters without outlining touching bacteria. Each image was individually measured using calibration settings based on the magnification used to take the image. Each image analyst completed analysis of a training image set and the coefficient of variation of their analyses was checked to ensure that it was less than 5 percent.

Bacterial sizes acquired from all images were binned using methods described by Shimazaki *et al.*³¹. Bin sizes were assigned to the microbial samples as size-class units (SCU) and a microbial community profile was created for the samples using the RiboSort package in R³².

ARISA data processing

Files from the Genetic Analyzer were opened in Peak Scanner 2 software (<http://www.appliedbiosystems.com>) using Analysis Method=Sizing Standard-PP, and Size standard=GS500. Only the green (hex-primer-labeled) peaks were used to determine level of bacterial abundance. The size and heights of the green peaks were calculated using the ROX GS500 standard. The individual output

files were assembled into a single microbial profile file using the RiboSort package³² using the script, RiboSort(data,dataformat="standard",dye="G", output="proportions",zerorows=FALSE, repeats=1, mergerepeats="presentinall"). This program automatically bins the peaks to generate abundances; we used the default threshold of 50 fluorescence units as recommended by the program authors.

Statistical analysis of presence-absence matrices

The OTU (operational taxonomic unit) abundance matrices output from Ribosort were analyzed by Permdisp and Permanova³³⁻³⁵ using Primer-E software v6³⁶. Overall square root transformation and Bray Curtis similarity resemblance were applied to the initial abundance data within Primer-E before Permanova and Permdisp tests were run. The P-values from the program were Bonferroni-corrected. Primer-E SIMPER analysis was used to compute the number of OTU contributing to differences between stages. Primer-E functions were used to compute diversity measures and taxa numbers for each sample (Supplementary Dataset).

The evenness (Pielou's J') of OTU and SCU for each sample was computed using Primer-E v6³⁶. The values of J' for OTU and SCU were compared across plant stages by rank-transformed analysis of means using the routines in JMP Pro v11 (SAS Inc, Cary, NC) with P-values adjusted for multiple comparisons. For the ranktransformed analysis of means the P-value threshold was set at the Bonferroni-corrected value of P less than 0.007.

This rank-transformed analysis of means is not sensitive to heteroschedasticity and is more powerful than other non-parametric tests when the data distribution is heavy-tailed³⁷. Nonparametric pairwise comparisons of evenness between stages were done using the Steel-Dwass multiple-comparison method. The distributions of SCU areas in each stage of plant growth were also computed with JMP Pro v11.

Co-occurrence data analysis

Pairwise analysis was completed using the R package Co-Occur³⁸; this package allows determination of the number of OTU/SCU that occur together in the same sample day at a probability above random, along with the number of taxa that are not found together (negatively correlated taxa) above the random-pair threshold. The analysis code, input and output files are available at <https://github.com/skosowsky/Cooccur>. We created network visualizations of the co-occurring OTU and SCU with Cytoscape (v. 3.4) and compared networks using the Cytoscape plug-in app DyNet (v1.0)³⁹. Intensity of node connection change across the seven stages is reflected in the intensity of the color of the DyNet-analyzed nodes.

Statistical analysis of presence-only data

The number of OTU and SCU present within each sample was computed using Primer-E v6 (abbreviated as S, also known as Chao1). The number of OTU and SCU compared to the overall mean across plant stages was analyzed using rank-transformed analysis of means via routines in JMP Pro v11 (SAS Inc, Cary, NC), with P-values adjusted for multiple comparisons. For the rank-transformed analysis of means the P-value threshold was set at

a) 30 days plant 4 image 3

b) 72 days plant 2 image 3

c) 80 days plant 2 image 5

Figure 2. Example SEM (left panel) and bacterial overlay images (right panel). A) Typical image after 30 days of growth in the field. **B)** 72-day image, with leaf hair and stomata clearly visible. **C)** Typical image after 80 days of growth in the field.

the Bonferroni-corrected value of $P=0.007$. This rank-transformed analysis of means is not sensitive to heteroschedasticity and is more powerful than other nonparametric tests when the data distribution is heavy-tailed. Nonparametric pairwise comparisons between stages were carried out with the Steel-Dwass multiple-comparison method within JMP.

Results

Our experimental design allowed for a single microbial sampling that was not confounded by differential exposure to recent weather events, with sampling for molecular and microscopic analyses in each plot (Figure 1). Molecular analysis of community ribotype composition (ARISA) and image analysis of microbial community

size cell assemblage size classes (by scanning electron microscopy, SEM) provided two complementary views of community assembly processes.

Presence-absence matrix comparisons

ARISA community composition and beta-diversity. Microbial community composition changed over time in bacterial ribosomal taxa (Permanova P-value of 0.026), as measured by differences in operational taxonomic units (OTU). The Permanova pairwise results for bacterial ribosomal OTU summarized in Table 1 and with additional details in Supplementary Table 1 and Supplementary Table 2 indicate that, specifically, day 30 and day 80 were significantly different from each other in community composition. Thus we conclude there was a difference in either the richness or arrangement of the communities' ribotypes in the earliest and latest stages of community development. To determine which aspect of community structure contributed to the difference detected by Permanova, we examined the dispersion as a measure of beta-diversity, i.e. the amount of range in the set of species present across replicates within each time. There was no significant change in the dispersion in the ribosomal OTU datatype across stages (Permdisp P=0.445, details in Supplementary Results). To further analyze the ARISA community structure, we compared the evenness of species distributions across stages.

Evenness is a measure of the range of OTU abundances in a sample unit; we used the Pielou's J' measure of sample evenness for comparison of stage evenness values. Evenness values were not normally distributed and were heavy-tailed (with kurtosis less than 3), so nonparametric tests were used for analysis of evenness differences across stages. Evenness was significantly different (P=0.0354) between stages 30 and 80 in the ARISA datatype in nonparametric pairwise tests (see Supplementary Results section 1.b for details), with the evenness value lower in the latest stage (i.e. a few OTU are more abundant in the stage 80 samples, compared to a more similar set of abundances in the first samples), though overall the evenness

averages are not significantly different from the overall mean across all the sample days (Supplementary Results section b). This pairwise-significant evenness difference in important OTU abundance ranges in the earliest and latest sample dates is also visible in a SIMPER analysis of ARISA OTU, with the top contributions to the day 30 OTU more evenly spread (for day 30: OTUs with 31, 19, 11, 11, 8, 6, 4 percent) as compared to day 80 (with OTUs of 50, 19, 10, 9, 5 percent, Supplementary Table 4).

SEM community composition and beta-diversity. In contrast to the ARISA datatype, in the SEM-based size-class unit (SCU) datatype the beta-diversity significantly decreased in the day 41 samples relative to the day 48 samples (Permdisp P=0.004, Table 2, details in Supplementary Table 3). This beta-diversity difference confounds Permanova tests, so we cannot further compare SEM community compositions across stages with that method. The significant SEM beta-diversity community change tells us that there is an important successional process at this specific stage relative to the earlier and later samples. Comparison of the Pielou's J' evenness values in the SCU datatype using nonparametric tests indicated that day 72 had significantly lower evenness of size-classes relative to the overall mean (Supplementary Results section 2), with pairwise tests showing that day 72 was significantly lower in evenness than day 80 (Supplementary Results 2.b). Pairwise comparisons among all other days were not significantly different.

Presence-only comparisons

We compared the number of OTU present in each sample to the overall mean using nonparametric comparisons suitable for non-normal, heavy-tailed distributions (Figure 3). The number of OTU – the richness – increases significantly in the ARISA datatype at day 62 (this mean is above the confidence interval and is thus indicated with a red dot in Figure 3 a), with earlier and later samples showing no significant difference from the overall mean richness (means indicated with green dots) and no pairwise

Table 1. ARISA Permanova Community Structure Comparisons. Significance groups with different letters are significantly different by pairwise test with a Bonferroni multiple-test-correction threshold of P=0.001315.

Stage (day after planting)	Significance group
30	A
41	A,B
48	A,B
58	A,B
62	A,B
72	A,B
80	B

Table 2. SEM Permdisp Community Structure Comparisons. Significance groups with different letters are significantly different by pairwise test with a Bonferroni multiple-test-correction threshold of P=0.001315.

Stage (day after planting)	Average centroid	Significance group
30	61.789	A,B
41	55.974	A
48	64.629	B
58	63.854	A,B
62	62.28	A,B
72	61.929	A,B
80	61.892	A,B

Figure 3. Non-parametric analysis of means of microbial OTU number (richness). The pale blue area is the confidence interval (UDL and LDL) above and below the overall mean. Rank-means for each stage are points at the tip of the drop-down bars. Significant means (adjusted $P < 0.05$) have red line tips, while non-significant means within the confidence interval have green tips. **A)** ARISA mean differences with transformed ranks. **B)** SEM mean difference of transformed ranks of taxa.

differences in means compared to each other with nonparametric tests (Supplementary Results section 1a). In contrast to the ARISA datatype pattern, the number of different SCU present in the size-class datatype is significantly lower at day 48 (Figure 3b), while all other stages are within the confidence limit for the

overall mean richness. Nonparametric pairwise comparisons of SCU indicated that day 48 was significantly lower than all later-stage samples and that days 72 and 80 had significantly higher SCU means present than day 30 (Supplementary Results section 2a).

Core community members

For the ARISA datatype, there were three OTU found in all stages of community development; these were OTU9, OTU22, and OTU24, which comprised 1.5 percent of the total number of OTU. For the SEM datatype, there were 13 SCU (5.7 percent of the total); a visual display of the proportions of all 13 across the seven sample dates is shown in [Supplementary Figure 3](#).

Co-occurrence pattern

To further examine community assembly processes we looked at trends in the co-occurrence of pairs of species in both phyllosphere datatypes. We examined these patterns in our data to identify ways that community composition could differ. Positive

co-occurrence is defined as species occurring together more often than random (and thus having potential mutualistic interactions), while negative co-occurrence values indicate that the species pair is found together less often than the random expectation (potential antagonistic interactions). Co-occurrence patterns changed across plant stages in both ARISA ([Figure 4](#)) and SEM ([Figure 5](#)). Participation of certain OTU and SCU in varying interactions across multiple stages is indicated by the red color intensity and number of overall edge interactions in the DyNet networks ([Figure 4b](#) and [Figure 5b](#)). In the ARISA datatype, highly variable co-occurring OTU (intense red color in [Figure 4b](#)) had both positive and negative interactions with other community members. In contrast, the SEM datatype exhibited more positive

Figure 4. Network visualization of significantly co-occurring ribotype OTU pairs at each plant stage, with the identifier number for each OTU shown with the label “a” plus the number. If there were no significant co-occurring OTU, then that sample stage was not shown. Negative interactions between OTU nodes are shown as edges with a barred arrow tip and positive correlations between OTU are indicated with pointed arrows. **a)** Significant co-occurring OTU from each sampled stage. **b)** Results of DyNet analysis of co-occurring OTU most changed across stages. Only significant co-occurring OTU (as shown in part a) were analyzed using DyNet. Intensity of the red color of nodes indicates amount of change across stages, with darker color indicating the most ‘rewiring’ across stages.

Figure 5. Network visualization of significantly co-occurring SCU pairs at each plant stage, with the identifier number for each SCU shown with the node label “s” plus the number. If there were no significant co-occurring SCU, then that day was not shown. Negative interactions between SCU nodes are shown as edges with a barred arrow tip and positive correlations between SCU are indicated with pointed arrows. **a)** Significant co-occurring SCU from each sampled stage. **b)** Results of DyNet analysis of co-occurring SCU most changed across stages. Only significant co-occurring SCU (as shown in part **a**) were analyzed using DyNet. Intensity of the red color of nodes indicates amount of change across stages, with darker color indicating the most ‘rewiring’.

interactions connecting highly dynamic nodes (intense red nodes in Figure 5b). There were more positive than negative significant co-occurrences in both the ARISA and SEM datatypes (Figure 4 and Figure 5, edge arrows with points). The number of co-occurrences increased with increasing plant growth time in each datatype, though not at the same stage; as seen in Figure 4, ARISA had the largest number of network nodes at day62 and for SEM (Figure 5) the largest number of nodes was day72. The overall proportion of co-occurring pairs was similar in each datatype (Table 3), with a minority of microbial types found to have significant co-occurrence partners. Specific OTU and SCU identifiers are individually displayed in stacked plots that show details about which OTU occurred in more than one pair within Supplementary Figure 1 and Supplementary Figure 2.

Dataset 1. ARISA and SEM taxonomic abundance data

<http://dx.doi.org/10.5256/f1000research.12490.d177516>

Table 3. Proportion of Co-occurring Pairs within the ARISA and SEM Community Measures.

Datatype	Total number of OTU or SCU	Percent that significantly co-occur at least once	Percent with no interaction
ARISA OTU	195	14	93
SEM OTU	229	28	88

Dataset 2. Detailed description of column headers, row labels and cell content for Dataset 1 and Supplementary materials

<http://dx.doi.org/10.5256/f1000research.12490.d177517>

Discussion

Our experimental design was selected to avoid confounding by differential exposure to recent weather events. This design, which has recently been recommended by Shade *et al.* (2013) and by Williams *et al.* (2013), allowed us to examine the contributions to community assembly due to dispersal, filtering and niche-related processes. Comparisons of presence-absence patterns to presence-only richness within a datatype are complementary analyses, as the presence-absence matrix allows for overall tests of community difference (Permanova-based, 33) and tests for beta-diversity difference as difference in homogeneity of variance (Permdisp, 34). Presence-only lists of taxa or size-classes allow examination of the richness dimension of the microbial communities, abstracted away from other structural differences.

Are two datatypes better than one? We measured a morphological trait (microbe size) and a relatedness (ribosomal length difference) metric, and the two datatypes were not especially similar at specific time points, making them complementary rather than redundant; we infer that predictive power from one datatype to the other would be low. The differences between datatypes suggest that size-classes may include different taxa, in other words our size-classes may not have a phylogenetic signal. Some recent work in phytoplankton and soil microbial communities contrasts with our results, showing that traits can be conserved across taxonomic groupings^{26,40}; generally conservation of traits and evolutionary relatedness is likely to depend on the genetic architecture of the phenotypic trait⁴¹. The relationship between metabolic and functional traits and phylogenetic signal is as yet unclear for microbial communities that have substantial levels of gene transfer^{42,43}. Complementary information provided by two (or more) datatypes is thus recommended for future phyllosphere experiments.

Misclassification of ribosomal spacer lengths to different taxa when they are in fact due to population differences, and misclassification of large bacterial cells in a size-class separate from their smaller offspring, would have consequences for trends across samples if the misclassification probability were different at the different stages of community development. In our study, the underlying assumption is that our probability of misclassification is the same across stages. For size-classes, misclassification could increase with time due to growth of cells, resulting in a continuous increase in the number of SCU over time. We do not see this trend in our data, so from our study there is no evidence of confounding misclassification. We cannot rule out misclassification that occurs only at specific stages using our experimental design. We suggest that sample processing for future

experiments include inline size separation so that size-classes, ribosomal data, and molecular/metabolic data are collected as true multivariate data in each sample. Image information such as our scanning electron micrographs or fluorescence-based microbial images could also be analyzed for patterns that could illuminate resource use traits and specific cell-shape-based mutualisms⁴⁴.

Trends in richness, co-occurrence trends, and beta-diversity changes together define overall community assembly mechanistic contributions, with habitat filtering characterized by unchanged richness, unchanged co-occurrence frequency, and decreasing beta-diversity. Niche development would be observed as a combination of increasing richness, increasing co-occurrence, and lowered beta-diversity. Finally, a neutral pattern – with no change in immigration probability over time – would exhibit trends toward increasing richness, unchanged co-occurrence, and unchanged beta-diversity. Comparison of recent phyllosphere analyses and our work using this framework emphasizes the role of niche development as a mechanism of community assembly.

First we compare our results with recent work in apple flower microbial communities, where beta-diversity was initially at relatively high levels, decreased, then increased over the remainder of the season; richness (measured as phylogenetic diversity in the apple flower work) increased then leveled off, and finally co-occurrences showed early increase then decline and rebound toward higher levels over the season²⁰. To summarize, early in the apple flowering season the diversity patterns support niche development mechanisms, and later in the season both habitat filtering and niche development patterns were seen. In our study of maize leaf microbial epiphytes we also found that there was no overall temporal trend. Our leaf ARISA pattern of diversity change was not especially similar to the apple flower changes; for example, we found no significant change in beta-diversity, while beta-diversity declined then rose over the later part of the season in apple flowers. Our ARISA richness and the apple flower phylogenetic diversity also differed, with apple flower phylogenetic diversity increasing then leveling off, while on maize leaves richness increased only in one later growth stage. The evenness in both our ARISA and apple flower phylogenetic diversity varied; in apple flower the ‘mid’ stage had a lower evenness while in our ARISA dataset the day 80 evenness was lower than in day 30. Co-occurrences in our study increased in mid-season, as did the apple flower communities in the early flower stage. Overall, the early season patterns in the across-season apple flower microbial community analysis were the most similar in community assembly to our maize leaf staggered-stage analysis. In addition to the apple flower study, in a recent study of three different crop leaf phyllosphere seasonal patterns, Copeland *et al.*²¹ found that alpha-diversity increased then decreased across the season, with more shared species later in the season. In all three crops, beta-diversity (as measured as Unifrac distance for leaf-specific microbes) decreased across season, though not evenly; the beta-diversity decrease was more prominent later in season and at one time-point was higher during the later phase.

Co-occurrences were not analyzed in this study, so these crop study results are consistent with niche and/or habitat mechanisms.

Core microbial communities in crops are those that are either recruited early and retained, or continually input across the season. These are often found by presence⁴⁵, as in our analysis of ARISA and SEM core microbial community members. We found a relatively small proportion of core OTU and SCU; other phyllosphere studies such as on apple flowers²⁰ found more (for apple flowers the generalist category was 14 percent, for example). Our core set should be considered as a first description, as we did not consider absences or compare our core set to air or soil samples. Instead, our core set focused on microbial community members that colonized leaves of all developmental stages.

Conclusions

Our ARISA taxonomic diversity patterns and microbial cell size trait datatypes exhibited different specific patterns of diversity, but there was similarity in trend overall, as both datatypes support niche process contributions to community assembly. Niche (deterministic) processes have recently been found to be important in soil microbial communities^{46,47}. In lakes, microbial generalists found at multiple spatially separated sites had a better fit to neutral stochastic models, with specialists exhibiting a more deterministic pattern⁴⁸. As leaf surface communities are quite different than air or soil communities⁹, and the number of core species in our inventory is low, we suggest that many of the maize leaf microbial community member that we detect are specialists. Our staggered experimental design highlighted the role of microbe-microbe interactions in leaf microbial community development. We thus suggest that future experiments use staggered experimental designs and multivariate metabolic, genic, and size-trait data collection.

Data availability

Dataset 1: ARISA and SEM taxonomic abundance data. DOI, [10.5256/f1000research.12490.d177516](https://doi.org/10.5256/f1000research.12490.d177516)⁴⁹

Dataset 2: Detailed description of column headers, row labels and cell content for [Dataset 1](#) and [Supplementary materials](#). DOI, [10.5256/f1000research.12490.d177517](https://doi.org/10.5256/f1000research.12490.d177517)⁵⁰

Supplementary material

Supplementary Figure S1: a-n Arisa Plots of Significant Co-occurring OTU Pairs.

[Click here to access the data.](#)

Supplementary Figure S2: a-n Plots of SEM datatype co-occurrence at each growth stage.

[Click here to access the data.](#)

Supplementary Figure S3: SEM Core SCU.

[Click here to access the data.](#)

Files in the CyVerse/iPlant Data Commons repository are available from http://datacommons.cyverse.org/browse/iplant/home/shared/commons_repo/curated/Stapleton_MaizeLeafMicrobesByLeaf-Age_2015, with DOI, [10.7946/P2WC77](https://doi.org/10.7946/P2WC77). This repository includes the following individual files:

SupplementarySEMfiles Folder

binnedSEMSizes.csv

readmeSEMfolderfilemetadata.txt

SEMImages Folder and Subfolders

SEMOverlays Folder

SEMRibosortinputfiles Folder

SEMRiboSortoutputfiles Folder

SupplementaryARISAFiles Folder

ARISAFilenamemetadata.csv

ARISARibosortinput Folder

ARISARibosortoutput Folder

ARISARuns Folder and SubFolders

readmeARISAFolderandfilemetadata.txt

Source data files formatted for CytoScape visualization are available at <https://github.com/AEStapleton/plantstage>.

Competing interests

No competing interests were disclosed.

Grant information

The author(s) declared that no grants were involved in supporting this work.

Acknowledgements

We are grateful to A. Ulloa-Bacillio, UNCW and J. Geyer, UNCW for assistance with SEM image analysis, to the UNCW microscopy facility for training and equipment support, and to the staff of the Central Crops research station for experimental field management.

Supplementary Results.

[Click here to access the data.](#)

Supplementary Table 1.

[Click here to access the data.](#)

Supplementary Table 2.

[Click here to access the data.](#)

Supplementary Table 3.

[Click here to access the data.](#)

Supplementary Table 4.

[Click here to access the data.](#)

References

- Vacher C, Hampe A, Porté AJ, *et al.*: **The Phyllosphere: Microbial Jungle at the Plant-Climate Interface.** *Annu Rev Ecol Evol Syst.* 2016; **47**(1): 1–24.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Bulgarelli D, Schlaeppi K, Spaepen S, *et al.*: **Structure and functions of the bacterial microbiota of plants.** *Annu Rev Plant Biol.* 2013; **64**(1): 807–838.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Newton AC, Gravelle C, Fountaine JM: **Managing the ecology of foliar pathogens: ecological tolerance in crops.** *Ann Appl Biol.* 2010; **157**(3): 343–359.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Berlec A: **Novel techniques and findings in the study of plant microbiota: Search for plant probiotics.** *Plant Sci.* 2012; **193–194**: 96–102.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Glick BR: **Bacteria with ACC deaminase can promote plant growth and help to feed the world.** *Microbiol Res.* 2014; **169**(1): 30–39.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Kim YC, Leveau J, McSpadden Gardener BB, *et al.*: **The multifactorial basis for plant health promotion by plant-associated bacteria.** *Appl Environ Microbiol.* 2011; **77**(5): 1548–1555.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Laforest-Lapointe I, Paquette A, Messier C, *et al.*: **Leaf bacterial diversity mediates plant diversity and ecosystem function relationships.** *Nature.* 2017; **546**(7656): 145–147.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Leveau JHJ: **Life of Microbes on Aerial Plant Parts.** In Ben Lugtenberg, editor, *Principles of Plant-Microbe Interactions*, Springer International Publishing, 2015; 17–24.
[PubMed Abstract](#)
- Lindow SE, Brandl MT: **Microbiology of the phyllosphere.** *Appl Environ Microbiol.* 2003; **69**(4): 1875–1883.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Götzenberger L, de Bello F, Bräthen KA, *et al.*: **Ecological assembly rules in plant communities—approaches, patterns and prospects.** *Biol Rev Camb Philos Soc.* 2012; **87**(1): 111–127.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Kraft NJB, Adler PB, Godoy O, *et al.*: **Community assembly, coexistence and the environmental filtering metaphor.** *Funct Ecol.* 2015; **29**(5): 592–599.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Nemergut DR, Schmidt SK, Fukami T, *et al.*: **Patterns and processes of microbial community assembly.** *Microbiol Mol Biol Rev.* 2013; **77**(3): 342–356.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Chase JM, Leibold MA: **Ecological Niches: Linking Classical and Contemporary Approaches.** University of Chicago Press, 2003.
[Reference Source](#)
- Dini-Andreote F, Stegen JC, van Elsland JD, *et al.*: **Disentangling mechanisms that mediate the balance between stochastic and deterministic processes in microbial succession.** *Proc Natl Acad Sci U S A.* 2015; **112**(11): E1326–E1332.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Kraft NJ, Cornwell WK, Webb CO, *et al.*: **Trait evolution, community assembly, and the phylogenetic structure of ecological communities.** *Am Nat.* 2007; **170**(2): 271–283.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Weihner E, Freund D, Bunton T, *et al.*: **Advances, challenges and a developing synthesis of ecological community assembly theory.** *Philos Trans R Soc Lond B Biol Sci.* 2011; **366**(1576): 2403–2413.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Remus-Emsermann MN, Lückner S, Müller DB, *et al.*: **Spatial distribution analyses of natural phyllosphere-colonizing bacteria on *Arabidopsis thaliana* revealed by fluorescence *in situ* hybridization.** *Environ Microbiol.* 2014; **16**(7): 2329–40.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Brandl MT, Amundson R: **Leaf Age as a Risk Factor in Contamination of Lettuce with *Escherichia coli* O157:H7 and *Salmonella enterica*.** *Appl Environ Microbiol.* 2008; **74**(8): 2298–2306.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Williams TR, Moyne AL, Harris LJ, *et al.*: **Season, Irrigation, Leaf Age, and *Escherichia coli* Inoculation Influence the Bacterial Diversity in the Lettuce Phyllosphere.** *PLoS One.* 2013; **8**(7): e68642.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Shade A, McManus PS, Handelsman J: **Unexpected diversity during community succession in the apple flower microbiome.** *mBio.* 2013; **4**(2): pii: e00602-12.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Copeland JK, Yuan L, Layeghifard M, *et al.*: **Seasonal Community Succession of the Phyllosphere Microbiome.** *Mol Plant Microbe Interact.* 2015; **28**(3): 274–285.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Maignien L, DeForce EA, Chafee ME, *et al.*: **Ecological succession and stochastic variation in the assembly of *Arabidopsis thaliana* phyllosphere communities.** *mBio.* 2014; **5**(1): e00682–13.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Ikeda S, Tokida T, Nakamura H, *et al.*: **Characterization of leaf blade- and leaf sheath-associated bacterial communities and assessment of their responses to environmental changes in CO₂, temperature, and nitrogen levels under field conditions.** *Microbes Environ.* 2015; **30**(1): 51–62.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Davis DG, Meola SM, Mullins JS: **Extraneous Material on Plant Surfaces: An SEM Study.** *Weed Science.* 1976; **24**(3): 341–347.
[Reference Source](#)
- Remus-Emsermann MN, Tecon R, Kowalchuk GA, *et al.*: **Variation in local carrying capacity and the individual fate of bacterial colonizers in the phyllosphere.** *ISME J.* 2012; **6**(4): 756–765.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Portillo MC, Leff JW, Lauber CL, *et al.*: **Cell size distributions of soil bacterial and archaeal taxa.** *Appl Environ Microbiol.* 2013; **79**(24): 7610–7617.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Kadivar H, Stapleton AE: **Ultraviolet radiation alters maize phyllosphere bacterial diversity.** *Microb Ecol.* 2003; **45**(4): 353–361.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Smitherman CT: **The Zea mays phyllosphere exhibits increases in abundance and decreases in diversity of bacteria through plant development.** M S University of North Carolina Wilmington, 2010.

29. Stapleton AE, Simmons SJ: **Plant control of phyllosphere diversity: genotype interactions with ultraviolet-B radiation.** In Bailey MJ, Lilley AK, Timms-Wilson TM, and Spencer-Phillips PTN, editors, *Microbial Ecology of Aerial Plant Surfaces*. CAB International, Wallingford, 2006; 223–239.
[Publisher Full Text](#)
30. Cardinale M, Brusetti L, Quatrini P, *et al.*: **Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities.** *Appl Environ Microbiol.* 2004; **70**(10): 6147–6156.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
31. Shimazaki H, Shinomoto S: **A method for selecting the bin size of a time histogram.** *Neural Comput.* 2007; **19**(6): 1503–1527.
[PubMed Abstract](#) | [Publisher Full Text](#)
32. Scallan U, Liliensiek A, Clipson N, *et al.*: **ribosort: a program for automated data preparation and exploratory analysis of microbial community fingerprints.** *Mol Ecol Resour.* 2008; **8**(1): 95–8.
[PubMed Abstract](#) | [Publisher Full Text](#)
33. Anderson MJ: **A new method for non-parametric multivariate analysis of variance.** *Austral Ecol.* 2001; **26**(1): 32–46.
[Publisher Full Text](#)
34. Anderson MJ: **Distance-based tests for homogeneity of multivariate dispersions.** *Biometrics.* 2006; **62**(1): 245–253.
[PubMed Abstract](#) | [Publisher Full Text](#)
35. Anderson MJ, Ellingsen KE, McArdle BH: **Multivariate dispersion as a measure of beta diversity.** *Ecol Lett.* 2006; **9**(6): 683–693.
[PubMed Abstract](#) | [Publisher Full Text](#)
36. Clarke KR, Gorley RN: **User manual/tutorial.** PRIMER-E, Plymouth, 2006.
37. Nelson P, Wludyka P, Copeland K: **The Analysis of Means: A Graphical Method for Comparing Means, Rates, and Proportions.** SIAM Press, Philadelphia, PA, 2005.
[Publisher Full Text](#)
38. Veech JA: **A probabilistic model for analysing species co-occurrence.** *Glob Ecol Biogeogr.* 2013; **22**(2): 252–260.
[Publisher Full Text](#)
39. Shannon P, Markiel A, Ozier O, *et al.*: **Cytoscape: a software environment for integrated models of biomolecular interaction networks.** *Genome Res.* 2003; **13**(11): 2498–2504.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
40. Bruggeman J: **A Phylogenetic Approach to the Estimation of Phytoplankton Traits (1).** *J Phycol.* 2011; **47**(1): 52–65.
[PubMed Abstract](#) | [Publisher Full Text](#)
41. Martiny JB, Jones SE, Lennon JT, *et al.*: **Microbiomes in light of traits: A phylogenetic perspective.** *Science.* 2015; **350**(6261): aac9323.
[PubMed Abstract](#) | [Publisher Full Text](#)
42. Goberna M, Verdú M: **Predicting microbial traits with phylogenies.** *ISME J.* 2016; **10**(4): 959–967.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
43. Boon E, Meehan CJ, Whidden C, *et al.*: **Interactions in the microbiome: communities of organisms and communities of genes.** *FEMS Microbiol Rev.* 2014; **38**(1): 90–118.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
44. Ji Z, Card KJ, Dazzo FB: **CMEIAS JFrad: A Digital Computing Tool to Discriminate the Fractal Geometry of Landscape Architectures and Spatial Patterns of Individual Cells in Microbial Biofilms.** *Microb Ecol.* 2015; **69**(3): 710–720.
[PubMed Abstract](#) | [Publisher Full Text](#)
45. Shade A, Handelsman J: **Beyond the Venn diagram: the hunt for a core microbiome.** *Environ Microbiol.* 2012; **14**(1): 4–12.
[PubMed Abstract](#) | [Publisher Full Text](#)
46. Cao P, Wang JT, Hu HW, *et al.*: **Environmental Filtering Process Has More Important Roles than Dispersal Limitation in Shaping Large-Scale Prokaryotic Beta Diversity Patterns of Grassland Soils.** *Microb Ecol.* 2016; **72**(1): 221–230.
[PubMed Abstract](#) | [Publisher Full Text](#)
47. Powell JR, Karunaratne S, Campbell CD, *et al.*: **Deterministic processes vary during community assembly for ecologically dissimilar taxa.** *Nat Commun.* 2015; **6**: 8444.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
48. Liao J, Cao X, Zhao L, *et al.*: **The importance of neutral and niche processes for bacterial community assembly differs between habitat generalists and specialists.** *FEMS Microbiol Ecol.* 2016; **92**(11): pii: fiw174.
[PubMed Abstract](#) | [Publisher Full Text](#)
49. Manching H, Carlson K, Kosowsky S, *et al.*: **Dataset 1 in: Maize phyllosphere microbial community niche development across stages of host leaf growth.** *F1000Research.* 2017.
[Data Source](#)
50. Manching H, Carlson K, Kosowsky S, *et al.*: **Dataset 2 in: Maize phyllosphere microbial community niche development across stages of host leaf growth.** *F1000Research.* 2017.
[Data Source](#)

Open Peer Review

Current Peer Review Status: ?

Version 1

Reviewer Report 18 October 2017

<https://doi.org/10.5256/f1000research.13524.r26099>

© 2017 Docherty K. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Kathryn M. Docherty

Department of Biological Sciences, Western Michigan University, Kalamazoo, MI, USA

I reviewed the manuscript "Maize phyllosphere microbial community niche development across stages of host leaf growth" by Manching et al. published in *F1000Research*. I rated this article "Approved with Reservations".

Summary: The researchers used a staggered experimental design to examine microbial community assembly processes on leaves of *Zea mays*. That is, they planted seeds at different times, and then collected samples on one date from 7 different growth stages of maize (Days 30, 41, 48, 58, 62, 72, 80). They examined leaf samples from 10 plants at each growth stage using two approaches. First, they examined microbial communities using SEM and categorized microorganisms into different size class units (SCUs). Second, they examined microbial communities using ARISA and categorized taxa into different operational taxonomic units (OTUs). Using microscopic and molecular approaches they found different results. Size-classes varied on days 41 and 48, first dropping in average size and then increasing in average size. On the rest of the dates, the size classes did not differ. OTUs were different between days 30 and 80, but for the rest of the time period, the communities did not differ significantly. To explore this further, the researchers used a network analysis approach to elucidate how either specific SCUs or OTUs were related to changes (positive or negative) in other SCUs or OTUs, respectively. They found that there were more positive co-occurrences than negative co-occurrences in both datasets, but that most individual data points did not have a significant co-occurrence pattern with other members of the dataset. They found a relatively small number of core SCUs and OTUs across the different time points of the dataset. They suggest that this may indicate that there are a number of specialists in the maize phyllosphere microbial community, and conclude that their results provide evidence for niche-based contributions to microbial community assembly on maize leaves.

Overall, the manuscript provides novel information about changes in phyllosphere microbial communities during development stages of an important agricultural crop. The idea that plant leaves can be used for basic studies of microbial community assembly is interesting and the use of complementary datasets (size and composition) was a good approach, because it gets at 2 aspects of the community. There were a few issues with the manuscript that I believe need to be

addressed before it is acceptable.

- **Work is discussed appropriately in the context of the current literature**

No, unfortunately the literature review needs to be updated. There are several important publications in this field that should be included, and this will require a re-write of the introduction and discussion sections.

See:

Redford et al.¹

Kembel et al.²

- **Whether suitable methods have been used**

There are several things that are unclear about the choices for the experimental design. Please address:

- Why were the specific day-intervals and developmental stage-dates chosen? Do they relate to specific physiological processes in maize development or were they chosen at random? Explain this choice further in the methods/results.

- Planting all seeds for a particular treatment (i.e. development stage) in the same row sets the experiment up for statistical issues that are usually dealt with using a randomized block. In the current experimental design, one can interpret the results that (for example) day 30 and day 80 plant samples differ in OTU composition simply because the 30 day plants are subject to a different environmental variable than the 80 day plants (maybe soil nitrogen or light or water). Please re-do analyses using a randomized block and provide any information to suggest that there is no gradient that may account for significant differences across treatments.

- The samples were collected and analyzed in 2009. At that time ARISA was a suitable method for this study, but since then, several other methods have been developed that could address the hypotheses more fully. For example, using predicted metagenomics pathways might indicate that there is a connection between community structure and cell size that was not demonstrated with ARISA. The authors need to address that this could be the case in the discussion and that further studies using these techniques could yield different results.

I liked that the investigators included control DNA extractions. Were there any OTUs associated with the controls? How were those dealt with in ARISA processing?

I thought that the network visualization approach was interesting, but it is really unclear what the authors are showing with Figure 5b. If it is just that the interconnections between SCUs gets really complicated, then I do not think a figure is necessary.

- **Sufficient information and source data have been provided to allow others to repeat every step of the work**

Yes, I think there is sufficient information to repeat the work.

- **The conclusions are supported by the findings.**

No, I think that there are a few things that need to be addressed in the discussion. First, please refer to the references I listed above and the articles that have cited them since, and re-think the discussion in that new framework. Second, the statistical issues of having all of 1 treatment in 1 row need to be addressed, otherwise all statistical results could be considered related to some unknown environmental gradient.

Some minor comments:

The introduction is mostly about community assembly. However, to me, the really interesting thing about this study is that it is done using a major agricultural crop. Little is discussed about why it would be interesting to know about bacterial community assembly in this plant. Perhaps there is a context for plant productivity or agricultural disease that could be expanded to make the intro more specific to the study?

I found the organization of the manuscript difficult to follow. Please reorder so that all SEM-related sections are together and all ARISA sections are together in the methods. Then follow whatever order you have chosen for the results.

The supplementary data tables are simply raw R output. Can you summarize the raw output in a supplementary table and then provide the raw output so that it is easier for the reader to interpret?

It would be easier to interpret Tables 1 and 2 if the significances were just included in Figure 3 instead of in a separate table. (i.e. put the letters above each of the averages shown in Figure 3 to indicate significances)

Figure 3 - the red/green dots are difficult to see (and I am not red/green color blind, so it would be even harder for 10% of our colleagues). Please adjust these so that they are more obviously different or have different shapes.

Supplementary Figure 3 needs a legend that identifies what each of the SCU_IDs are and more information about the graph.

On page 9, the authors state that "In the ARISA datatype, highly variable co-occurring...". Please provide more of a definition of what you mean by "highly variable". Is this quantifiable?

Discussion Section - there are 3 different referencing styles used. Change for consistency.

References

1. Redford AJ, Bowers RM, Knight R, Linhart Y, et al.: The ecology of the phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves. *Environ Microbiol.* 2010; **12** (11): 2885-93 [PubMed Abstract](#) | [Publisher Full Text](#)
2. Kembel SW, O'Connor TK, Arnold HK, Hubbell SP, et al.: Relationships between phyllosphere bacterial communities and plant functional traits in a neotropical forest. *Proc Natl Acad Sci U S A.* 2014; **111** (38): 13715-20 [PubMed Abstract](#) | [Publisher Full Text](#)

Is the work clearly and accurately presented and does it cite the current literature?

No

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

No

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microbial ecology, soil, airborne, biodegradation

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 01 Dec 2017

Ann Stapleton, Division of Allergy & ID, University of Washington, USA

Reply to Reviewer 1

Thank you for your insightful comments and suggestions! We have addressed each point in the revised manuscript, and we explain each below, with review comments in italic and author replies in regular font.

I reviewed the manuscript "Maize phyllosphere microbial community niche development across stages of host leaf growth" by Manching et al. published in F1000Research. I rated this article "Approved with Reservations".

Summary: *The researchers used a staggered experimental design to examine microbial community assembly processes on leaves of Zea mays. That is, they planted seeds at different times, and then collected samples on one date from 7 different growth stages of maize (Days 30, 41, 48, 58, 62, 72, 80). They examined leaf samples from 10 plants at each growth stage using two approaches. First, they examined microbial communities using SEM and categorized microorganisms into different size class units (SCUs). Second, they examined microbial communities using ARISA and categorized taxa into different operational taxonomic units (OTUs). Using microscopic and molecular approaches they found different results. Size-classes varied on days 41 and 48, first dropping in average size and then increasing in average size. On the rest of the dates, the size classes did not differ. OTUs were different between days 30 and 80, but for the rest of the time period, the communities did not differ significantly. To explore this further, the researchers used a network analysis approach to elucidate how either specific SCUs or OTUs were related to changes (positive or negative) in other SCUs or OTUs, respectively. They found that there were more positive co-occurrences than negative co-occurrences in both datasets, but that most individual data points did not have a significant co-occurrence pattern with other members of the dataset. They found a relatively small number of core SCUs and OTUs across the different*

time points of the dataset. They suggest that this may indicate that there are a number of specialists in the maize phyllosphere microbial community, and conclude that their results provide evidence for niche-based contributions to microbial community assembly on maize leaves.

Overall, the manuscript provides novel information about changes in phyllosphere microbial communities during development stages of an important agricultural crop. The idea that plant leaves can be used for basic studies of microbial community assembly is interesting and the use of complementary datasets (size and composition) was a good approach, because it gets at 2 aspects of the community. There were a few issues with the manuscript that I believe need to be addressed before it is acceptable.

- **Work is discussed appropriately in the context of the current literature**

No, unfortunately the literature review needs to be updated. There are several important publications in this field that should be included, and this will require a re-write of the introduction and discussion sections.

See:

Redford et al.¹

Kembel et al.²

These references discuss across-taxa patterns, which is not our focus (we use one genotype of the domesticated crop *Zea mays*), so we did not include them in our manuscript. We have added two additional sources (Redford 2009 and Dees 2015) to the introduction section to expand our coverage of seasonal pattern literature.

- **Whether suitable methods have been used**

There are several things that are unclear about the choices for the experimental design. Please address:

- Why were the specific day-intervals and developmental stage-dates chosen? Do they relate to specific physiological processes in maize development or were they chosen at random? Explain this choice further in the methods/results.

These sampling intervals were chosen to capture maize leaf developmental stages (classically described as V(1 to n) for vegetative growth); each stage occurs at intervals of approximately one week and our sampling dates reflect this timing. We added a statement to the methods section explaining this.

- Planting all seeds for a particular treatment (i.e. development stage) in the same row sets the experiment up for statistical issues that are usually dealt with using a randomized block. In the current experimental design, one can interpret the results that (for example) day 30 and day 80 plant samples differ in OTU composition simply because the 30 day plants are subject to a different environmental variable than the 80 day plants (maybe soil nitrogen or light or water). Please re-do analyses using a randomized block and provide any information to suggest that there is no gradient that may account for significant differences across treatments.

The experimental design was chosen to fit agricultural practice, specifically maize field cultivation practice. This means that seeds are planted in rows with spacing dictated by planting equipment (30 in rows at this field station) and weed control (cultivation), fertilization, and pest control are carried out in those rows. Our experimental seeds were planted in adjacent rows, so nested spatial models that incorporate the physical distance between plants across the plots would be an ideal model to fit. However, spatial multivariate models with zero-inflated distributions for species counts are quite complex and we chose not to add this complexity to our model, as we have no evidence for spatial gradients within these short physical distances; for example, the soil type is the same (within the limits of our measurement instruments), as is the temperature and light regime. Our experimental design controls for field-level effects of environmental history such as recent rainfall, but cannot and does not control for developmental history of exposure to environmental factors in a field setting. Controlled environment chambers would be required to control for past environment effects; however, chamber experiments are much less agronomically relevant, so we chose to carry out field experiments.

- The samples were collected and analyzed in 2009. At that time ARISA was a suitable method for this study, but since then, several other methods have been developed that could address the hypotheses more fully. For example, using predicted metagenomics pathways might indicate that there is a connection between community structure and cell size that was not demonstrated with ARISA. The authors need to address that this could be the case in the discussion and that further studies using these techniques could yield different results.

We agree that functional gene and metabolic pathway information would be valuable, and we suggest some specific hypotheses to test using such information in the discussion section. An automated cell-sorting assay would be required to collect suitable multivariate paired metabolic/pathway and cell size data, and we encourage development of instruments of this type.

I liked that the investigators included control DNA extractions. Were there any OTUs associated with the controls? How were those dealt with in ARISA processing?

After optimization of the nested PCR protocol there were no OTU associated with the controls. We did find that the PCR master mix was important for consistent amplification and low false positive rates, so we included details of the PCR reactions within our methods section.

I thought that the network visualization approach was interesting, but it is really unclear what the authors are showing with Figure 5b. If it is just that the interconnections between SCUs gets really complicated, then I do not think a figure is necessary.

With assistance from the F1000 editorial staff we attempted to create interactive network graphics that allow readers to zoom and highlight, but it was not technically feasible at this time. Future analyses of succession might explore network modularity measures, as the networks we see in Fig. 5 appear to show modularity, especially in conjunction with future metabolic pathway analyses.

- **Sufficient information and source data have been provided to allow others to repeat**

every step of the work

Yes, I think there is sufficient information to repeat the work.

- **The conclusions are supported by the findings.**

No, I think that there are a few things that need to be addressed in the discussion. First, please refer to the references I listed above and the articles that have cited them since, and re-think the discussion in that new framework. Second, the statistical issues of having all of 1 treatment in 1 row need to be addressed, otherwise all statistical results could be considered related to some unknown environmental gradient.

Some minor comments:

The introduction is mostly about community assembly. However, to me, the really interesting thing about this study is that it is done using a major agricultural crop. Little is discussed about why it would be interesting to know about bacterial community assembly in this plant. Perhaps there is a context for plant productivity or agricultural disease that could be expanded to make the intro more specific to the study?

We added more text to the first paragraph of the introduction to incorporate agricultural context. We are pleased that you find this interesting! There is currently substantial investment in the business sector around plant microbiomes and probiotics (several hundred million dollars, and at least four new companies that we know of), but there is relatively little publicly available basic research on agricultural plant microbiomes. We added a citation from one review of this area (Parnell et al., 2016).

I found the organization of the manuscript difficult to follow. Please reorder so that all SEM-related sections are together and all ARISA sections are together in the methods. Then follow whatever order you have chosen for the results.

We moved the SEM image analysis section to the correct place after the SEM image acquisition section in the methods. Thank you for pointing this out!

The supplementary data tables are simply raw R output. Can you summarize the raw output in a supplementary table and then provide the raw output so that it is easier for the reader to interpret?

These are output from the Primer-E program with full details of the model fits and outcomes. We added more explanation to the methods for Tables 1 to 4 to make this clear.

It would be easier to interpret Tables 1 and 2 if the significances were just included in Figure 3 instead of in a separate table. (i.e. put the letters above each of the averages shown in Figure 3 to indicate significances)

The permdisp/permanova and non-parametric richness analyses have different assumptions, so we chose not to place them in the same figure.

Figure 3 - the red/green dots are difficult to see (and I am not red/green color blind, so it would

be even harder for 10% of our colleagues). Please adjust these so that they are more obviously different or have different shapes.

We changed these to different colors (manually). We agree that these are not correctly formatted and we have submitted a request to the company that developed the program asking them to adjust their defaults to color-blind-compatible colors and shapes.

Supplementary Figure 3 needs a legend that identifies what each of the SCU_IDs are and more information about the graph.

We have added the caption to this figure.

On page 9, the authors state that "In the ARISA datatype, highly variable co-occurring...". Please provide more of a definition of what you mean by "highly variable". Is this quantifiable?

We added more explanation to this sentence. Since network interaction patterns are a summary measure (and thus not replicated) we do not discuss them quantitatively.

Discussion Section - there are 3 different referencing styles used. Change for consistency.

Updated to the F1000 style.

- Is the work clearly and accurately presented and does it cite the current literature?

No

- Is the study design appropriate and is the work technically sound?

Partly

- Are sufficient details of methods and analysis provided to allow replication by others?

Yes

- If applicable, is the statistical analysis and its interpretation appropriate?

No

- Are all the source data underlying the results available to ensure full reproducibility?

Yes

- Are the conclusions drawn adequately supported by the results?

Partly

References

1. Redford AJ, Bowers RM, Knight R, Linhart Y, Fierer N: The ecology of the phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves. *Environ Microbiol.* 2010; **12** (11): 2885-93 [PubMed Abstract](#) | [Publisher Full Text](#)
2. Kembel SW, O'Connor TK, Arnold HK, Hubbell SP, Wright SJ, Green JL: Relationships between phyllosphere bacterial communities and plant functional traits in a neotropical forest. *Proc Natl Acad Sci U S A.* 2014; **111** (38): 13715-20 [PubMed Abstract](#) | [Publisher Full Text](#)

Competing Interests: No competing interests were disclosed.

Referee Expertise: Microbial ecology, soil, airborne, biodegradation

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant

reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com

F1000Research