Microbial Community Analysis Using Colony-based Sequencing Database

Student Handout

Objectives

- Manipulate large datasets to conduct community-level ecological analyses
- Use community-level data to address questions about insect microbiomes
- Use Excel or RStudio programs to calculate community ecology variables
- Compare microbial community using community ecology variables

Introduction

Microbiomes are the communities of microbes (bacteria, viruses, fungi and archaea) living symbiotically with all metazoans. In the past decade, both interest and research on microbiomes, including their implications for human health, have increased dramatically (Christian *et al.* 2015, Costello *et al.* 2012, McFall-Ngai *et al*. 2013, The Human Microbiome Consortium 2012, Young 2016). Insects have been used as model species to study the importance of microbiomes, because of their ease of use and the fact that microbial communities play diverse roles in insects (Engel and Moran 2013).

The data that are collected in any microbiome study consists of lists of the taxonomic units identified and their abundance. The same types of data are evaluated in an ecological community analysis, but now the communities are the collections of microbes that constitute different microbiomes. The community variables, "species" richness and relative abundance, are the same and the statistical methods used to compare communities, diversity and difference indices, also are the same. Perhaps the simplest measure of community structure used by ecologists is "species" or taxon richness, a count of the number of unique taxa in a sample. However, species richness does not consider the relative abundance of species in a community. Imagine two communities with five different species. In one community, all of the species have the same relative abundance. In the other community, one species dominates comprising 95% of individuals in the community. The other four species are very rare. Based on species richness as a measure of community structure, these two communities are the same, although they are clearly very different. As a result, ecologists use other species diversity indices that consider both the number of species and the relative abundance of species in a community. Two common indices are the Simpson Index and the Shannon-Weaver Index. Communities with greater numbers of species and higher evenness (i.e., similar relative abundance of species within a community) are considered more diverse. Finally, measures of species richness and species diversity do not consider the identity of species in a community. So, communities could have the same level of species diversity, but have completely different species. Measure of community similarity, such as the Bray-Curtis Index, compare the similarity (or dissimilarity) between two communities based on the identity of species in the communities, as well as their relative abundances. For more information on indices of species diversity and measures of community similarity, see Krebs (1999).

In this study, bean beetle gut microbiome data were collected by undergraduate students using the protocols developed by Cole *et al*. (2018). Three types of data were collected: colony phenotypes from cultured bacteria, 16s rRNA gene sequencing of specific bacterial colonies, and whole community 16s rRNA gene sequencing, but we will limit our analyses to the colony phenotype and colony-based 16s data.

Questions

Using data from the colony-based sequencing database and the analyses described below, answer the following questions.

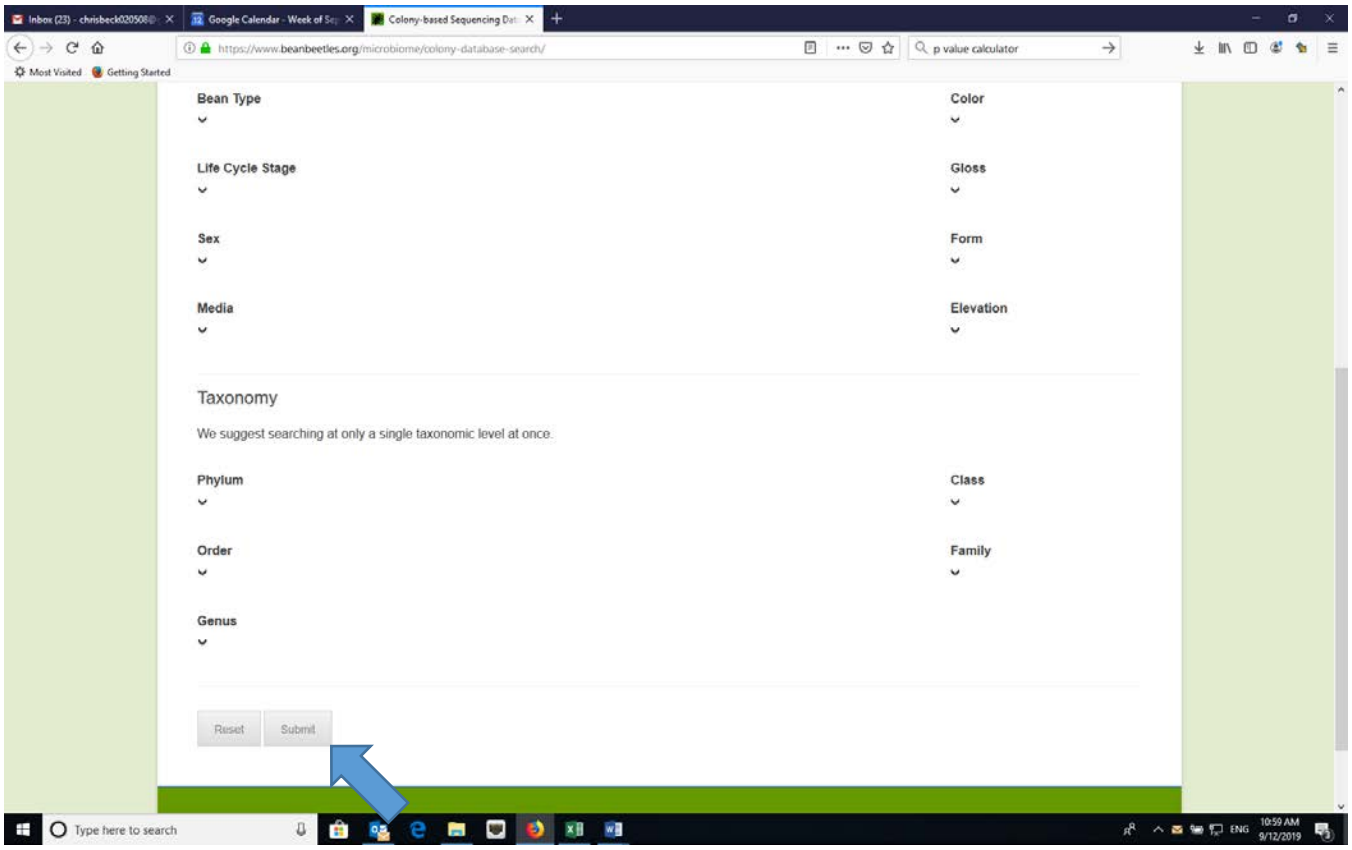
1. Which taxa are most prevalent in the bacterial communities in bean beetles?
2. Do the most prevalent taxa vary based on host bean type?
3. Based on the diversity indices that you calculated, which treatment had the highest (lowest) diversity?
4. Does the answer depend on the measure of species (taxon) diversity that you use?
5. Is there a relationship between number of samples and taxonomic diversity? If so, what might explain this?
6. Which communities are most similar (different)?
7. Do your answers to the questions above depend on the taxonomic level of analysis?

Database description

This database contains data for the microbial community of bean beetles based on 16s rRNA sequencing of individual bacterial colonies cultured from bean beetle homogenates plated on different media. Since only a small number of colonies are sequenced from each plate, the data do not represent the entire microbial community for a particular sample. However, qualitative comparisons can be made based on bean host species, sex of beetle, and other variables.

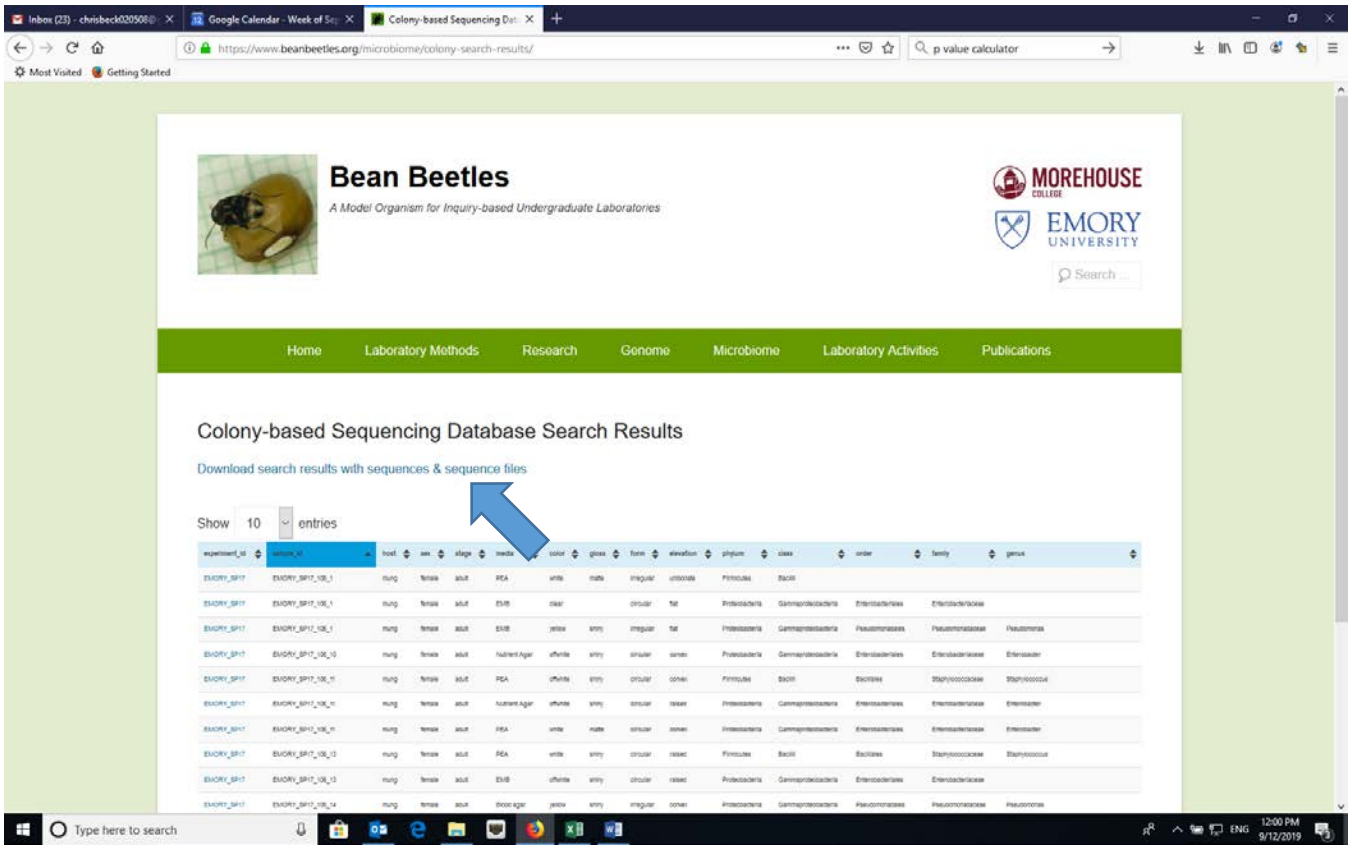
Access the database at https://www.beanbeetles.org/microbiome/colony-database-search/.

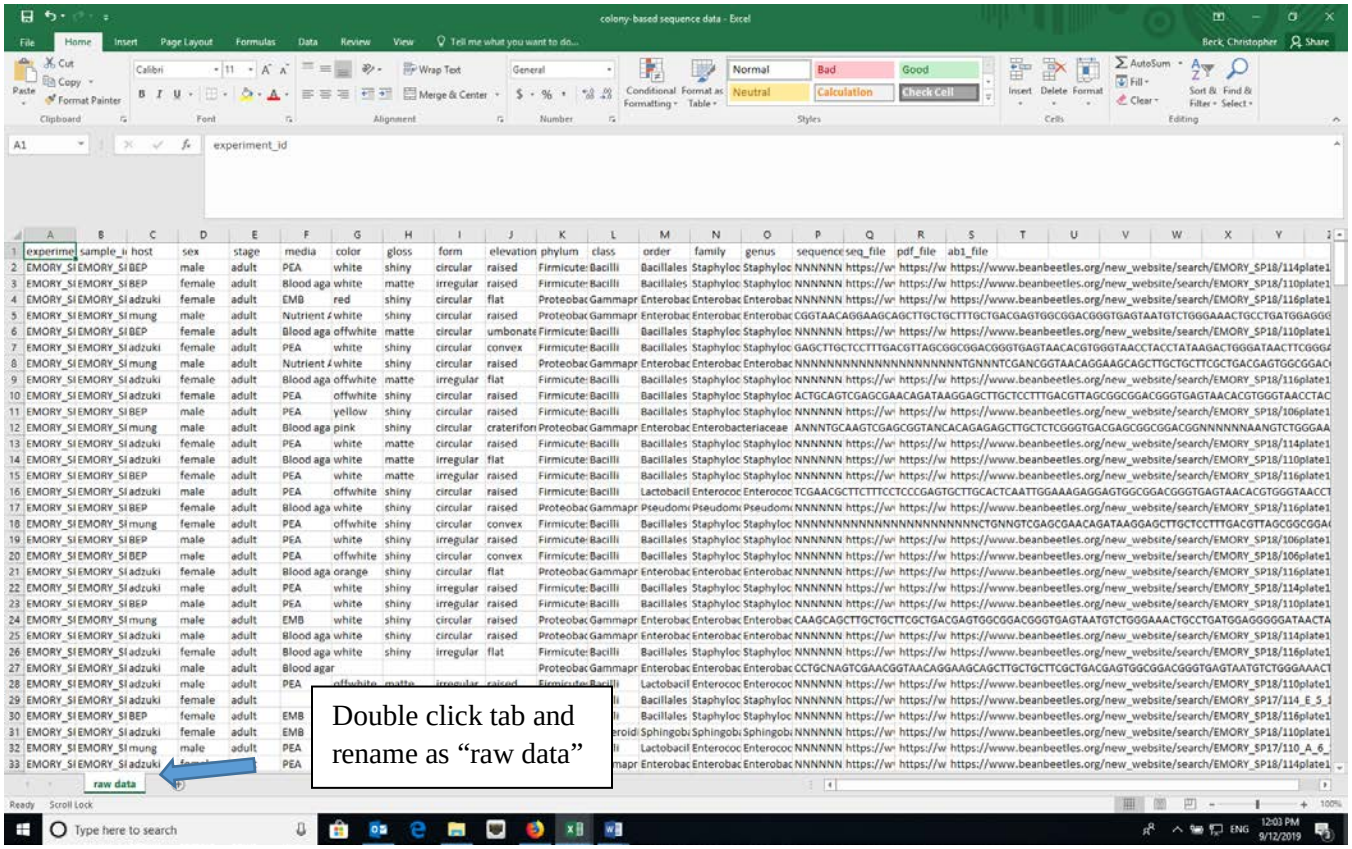
The database allows you to limit your search by bean host type, sex, life cycle stage, media on which bacteria were grown, colony phenotype, and bacterial taxonomy. Since we are interested in making comparisons between bacterial communities based on host species and sex, we want to download the entire database. Clicking “Submit” without limiting the search will allow you to view all of the data.



Downloading Data

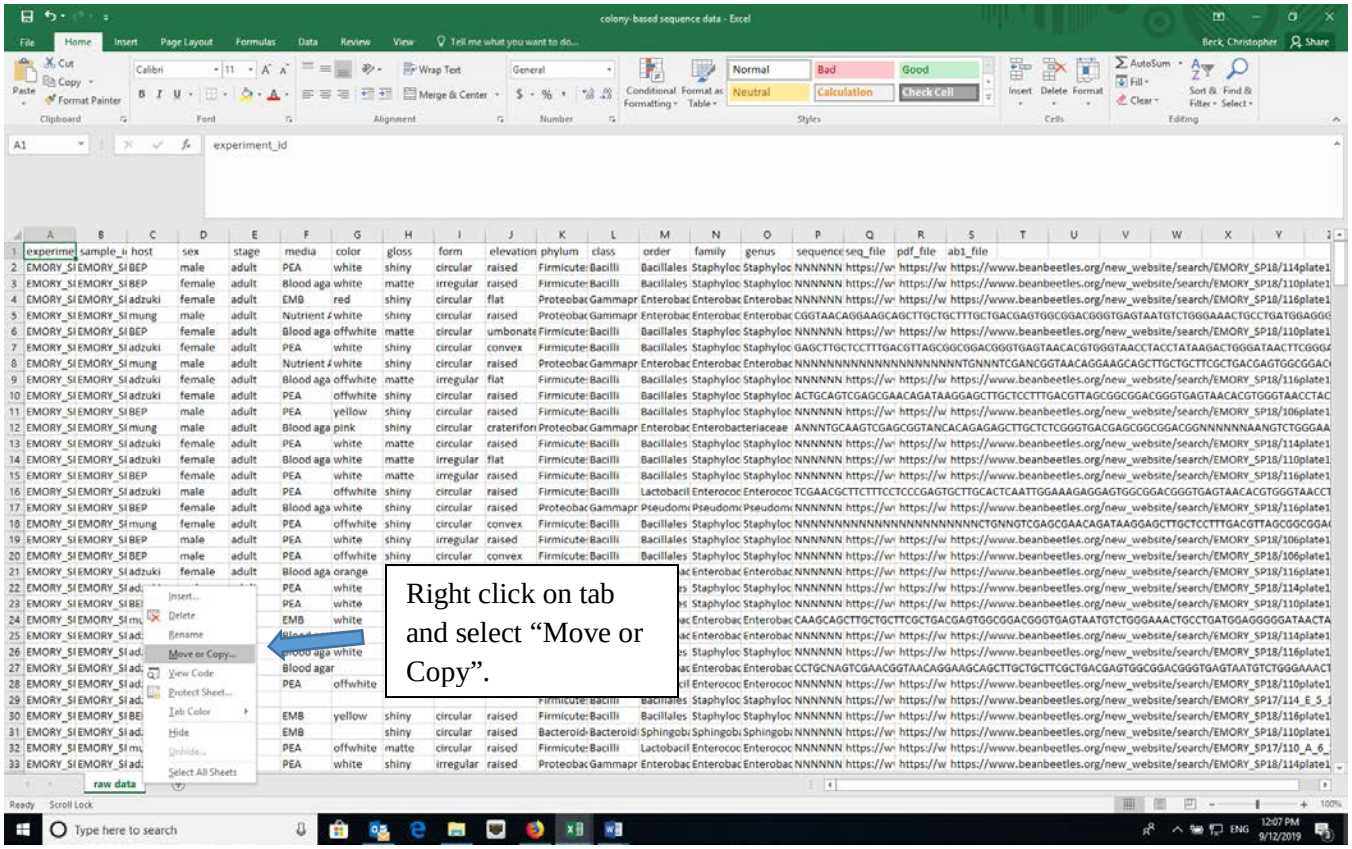
While we can view the data on the website, we want to download the data to manipulate. Click the download link to download a csv file with the data. Then, save the file as an Excel file (name the file "colony-based sequence data") and rename the tab "raw data."

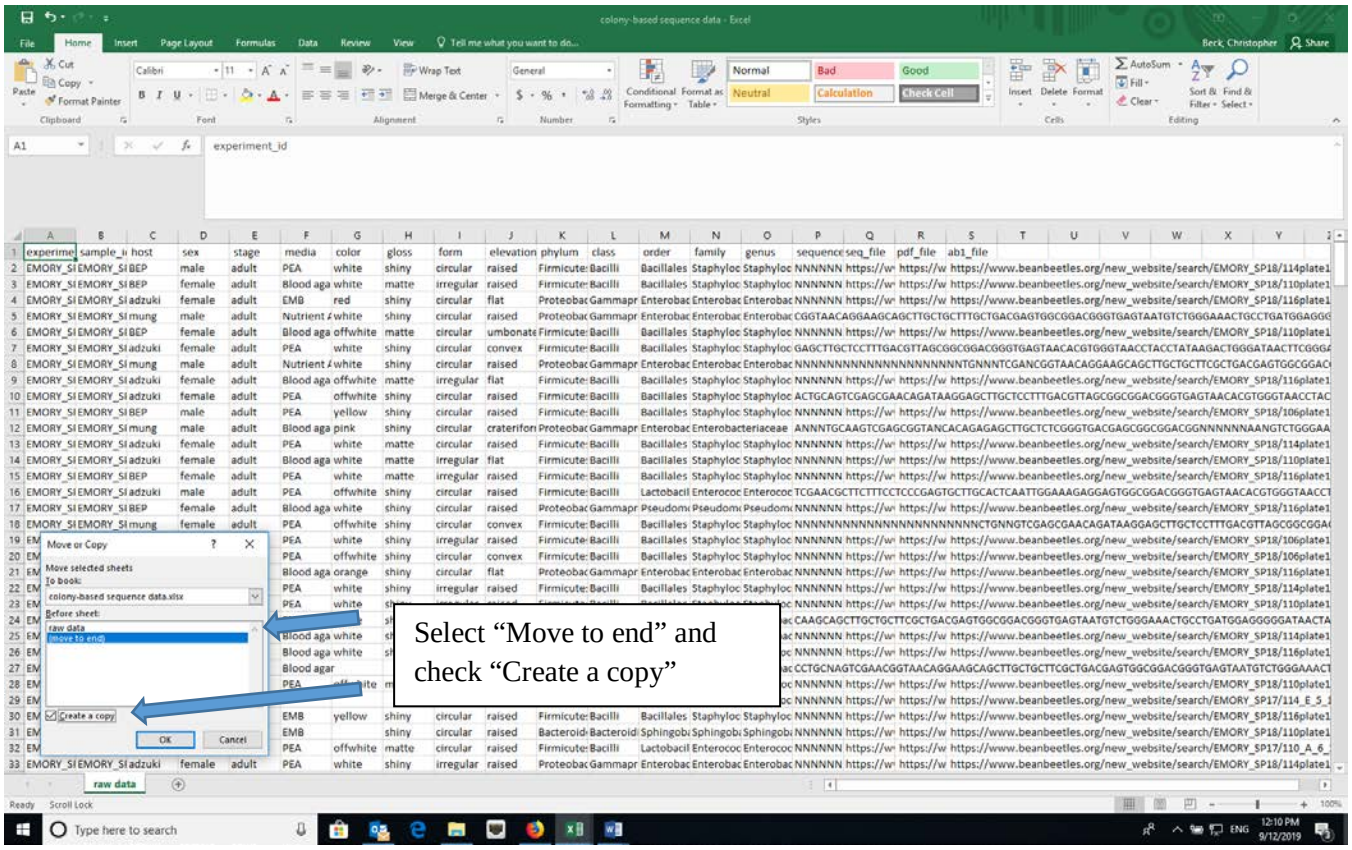

Bean Beetles
A Model Organism for Inquiry-based Undergraduate Laboratories
MOREHOUSE COLLEGE
EMORY UNIVERSITY
Home
Laboratory Methods
Research
Genome
Microbiome
Laboratory Activities
Publications
Colony-based Sequencing Database Search Results
Download search results with sequences & sequence files
Show 10 entries


Double click tab and rename as “raw data”
raw data

Data Reduction

1. Make a copy of the raw data in a new sheet using the sheet copy function in Excel (right click on the tab and select “Move or copy” and rename the tab (“reduced raw data”).

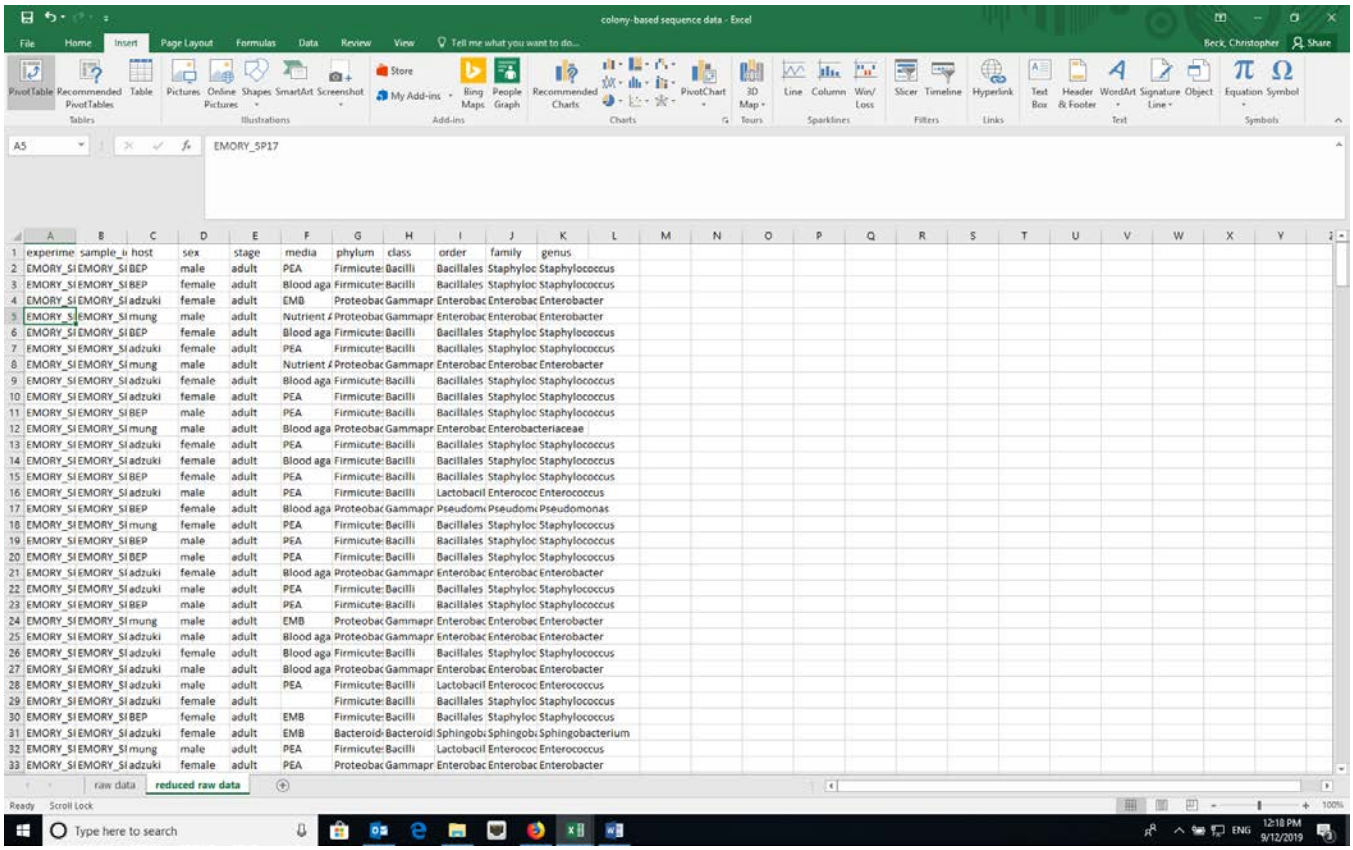


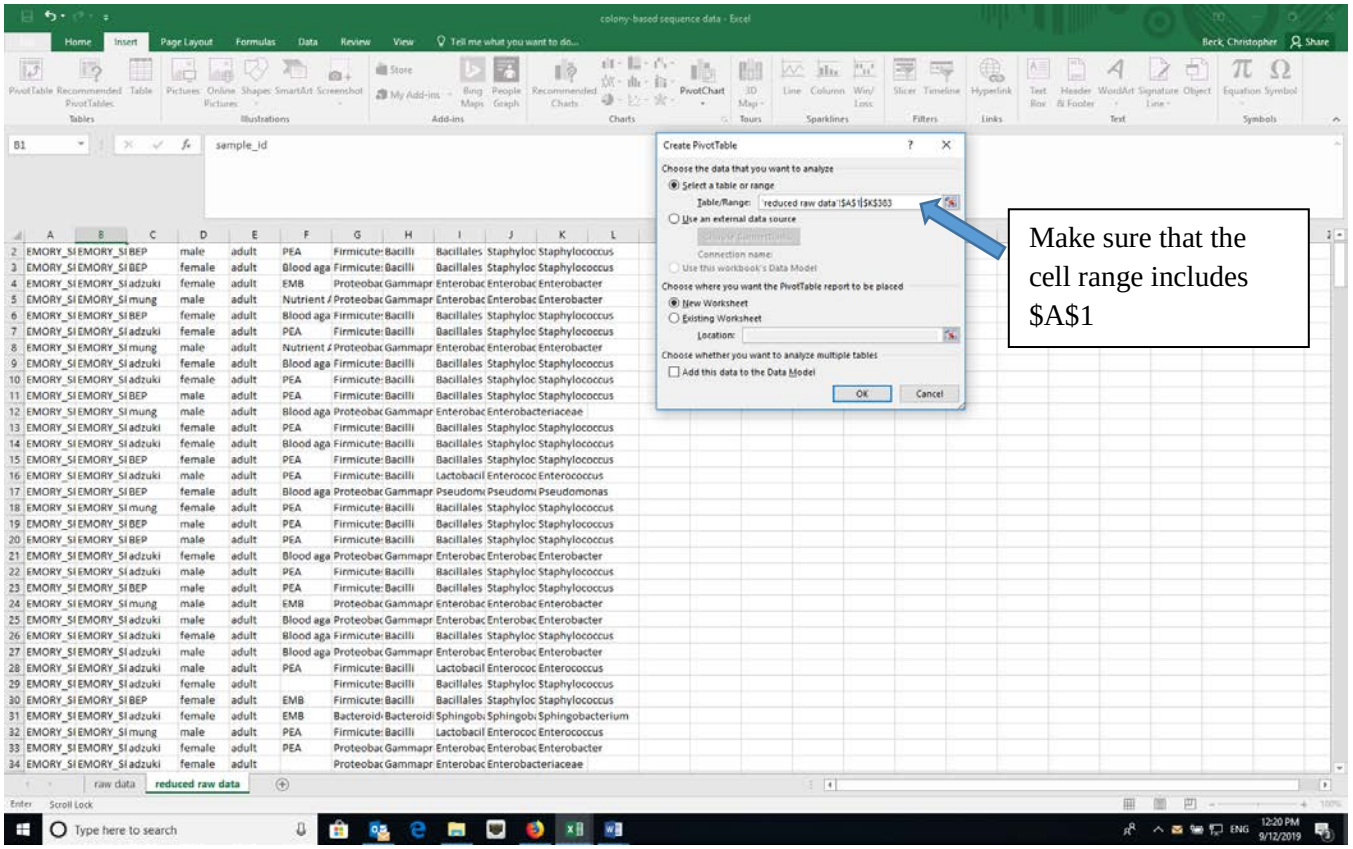


2. In the “reduced raw data” sheet, delete any columns that we don’t need, such as the colony phenotype (color, gloss, form, elevation) and sequence data columns. The “reduced raw data” sheet is the data source if you choose to analyze these data in RStudio. Additional data manipulation and formatting (below) is required if you choose to analyze these data in Excel.

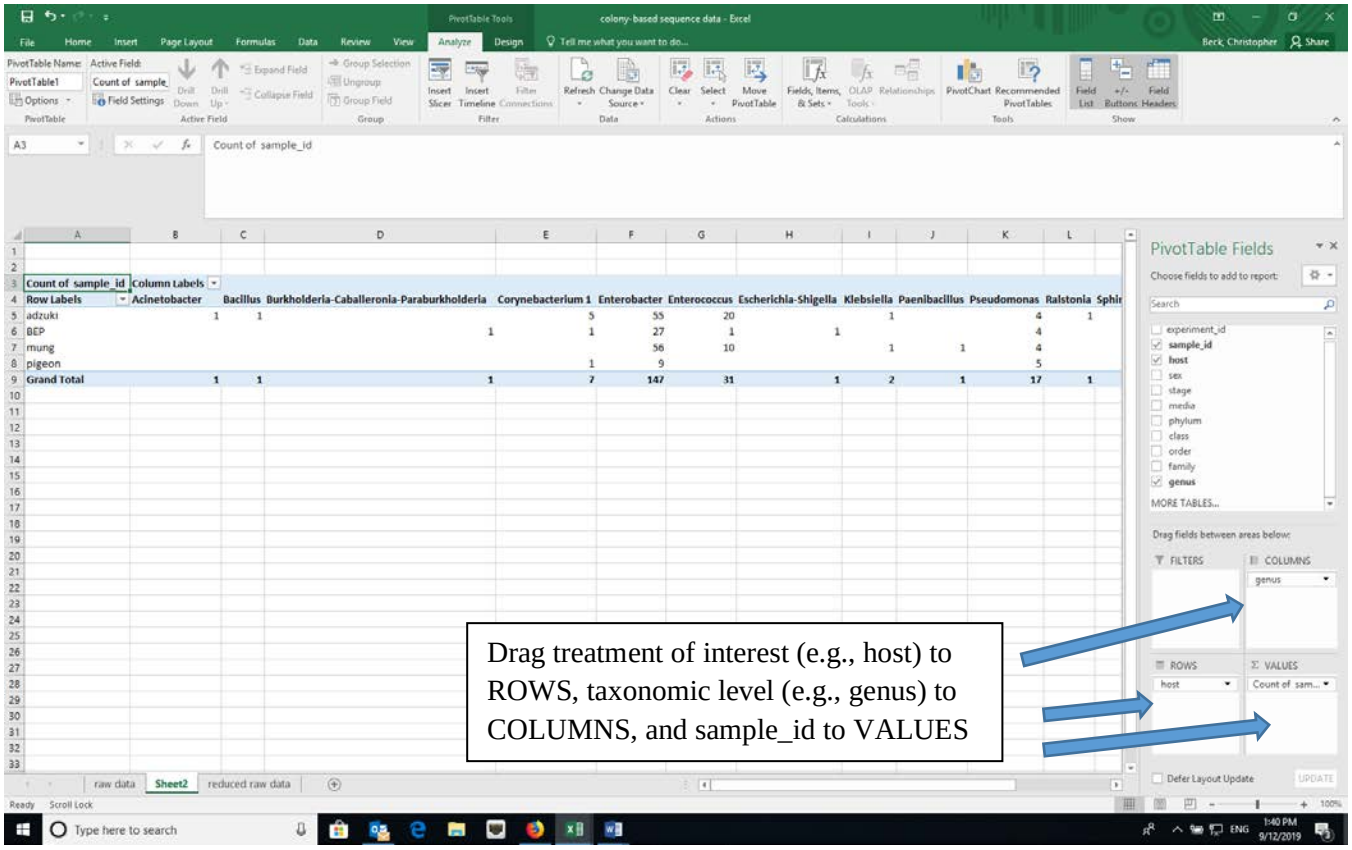
Data manipulation

1. We need to consolidate the data for each host species, each sex, or the combination of the two by the bacterial taxa. The easiest way to do this is with the Pivot Table function in Excel.
2. When clicked on a cell within the data, create a Pivot Table (Insert -> Pivot Table OR Data -> Summarize with Pivot Table). Make sure that the data source includes the top row (the cell range should include “$A$1”), which has the column headings. Click OK to create the pivot table in a new worksheet and label the tab “pivot table”.

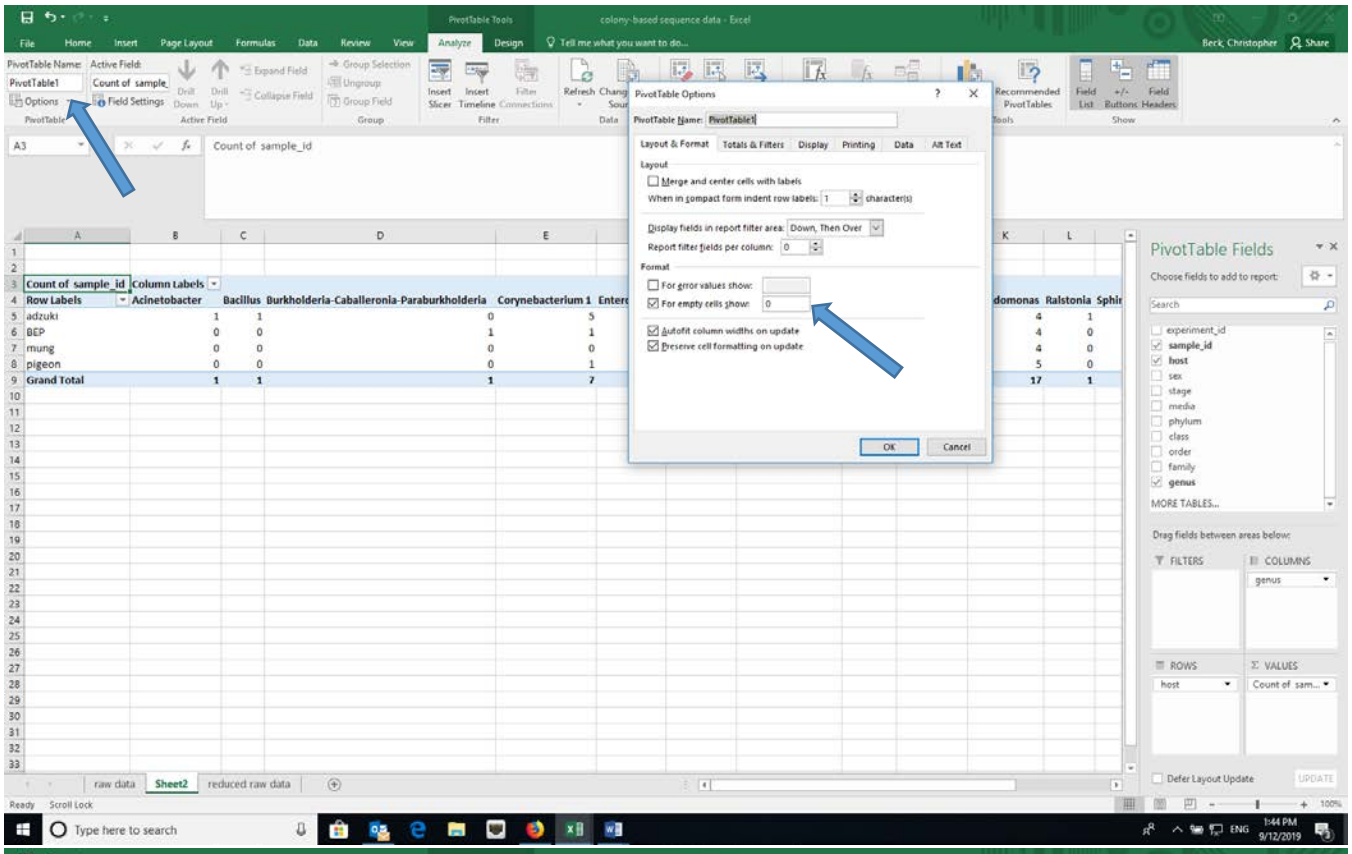

colony-based sequence data - Excel
EMORY_SP17
raw data
reduced raw data

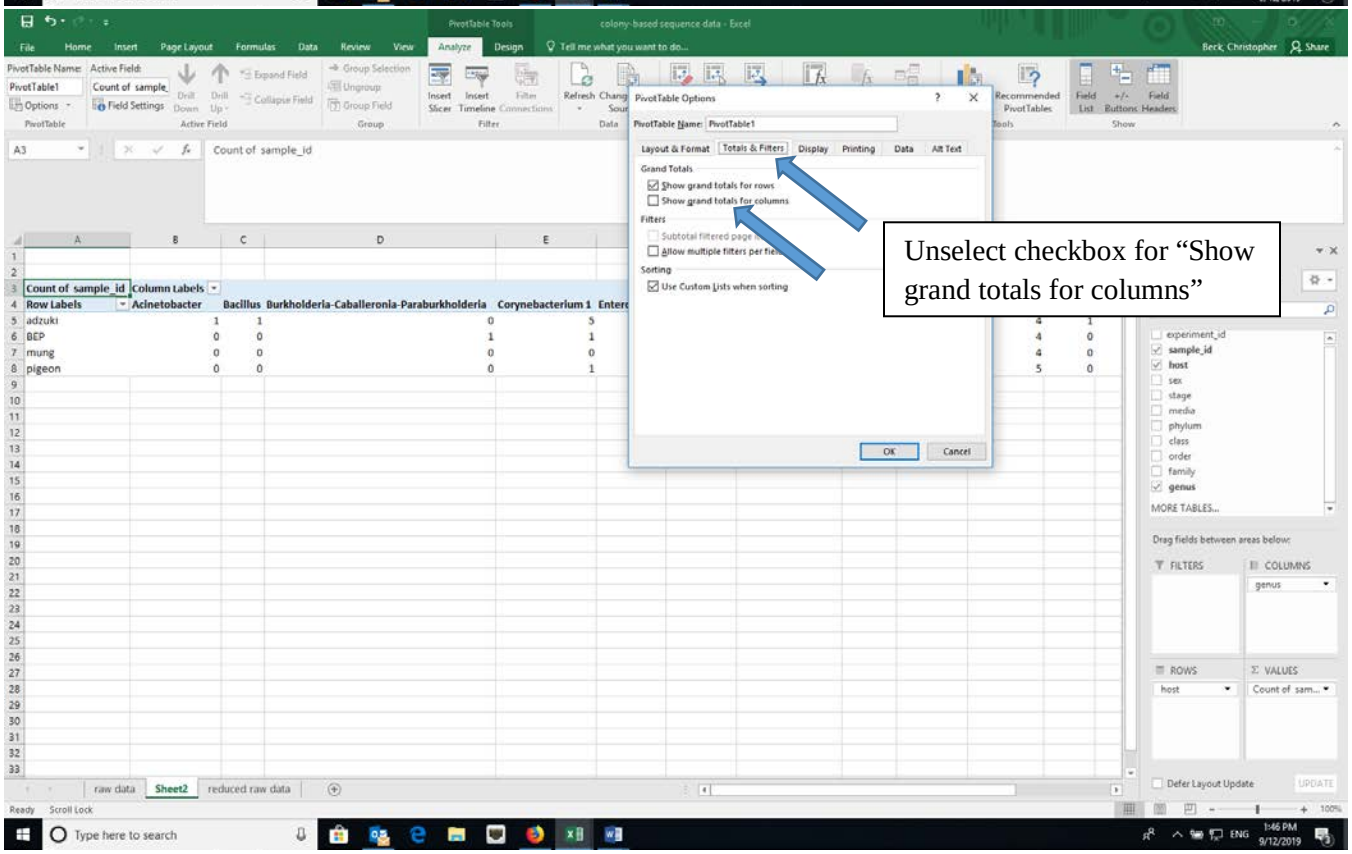

colony-based sequence data - Excel
sample_id
Create PivotTable
Choose the data that you want to analyze
Select a table or range
Table/Range: reduced raw data!$A$1:$K$303
Use an external data source
Choose where you want the PivotTable report to be placed
New Worksheet
Existing Worksheet
Location:
Choose whether you want to analyze multiple tables
Add this data to the Data Model
OK
Cancel
Make sure that the cell range includes $A$1
raw data
reduced raw data

3. Set the treatment(s) that you are interested (for example, host species) in as the rows and the bacterial taxonomic level you are interested in as the columns. The Values should be a COUNT of the sample_id, as each row in the dataset represents a single sample.

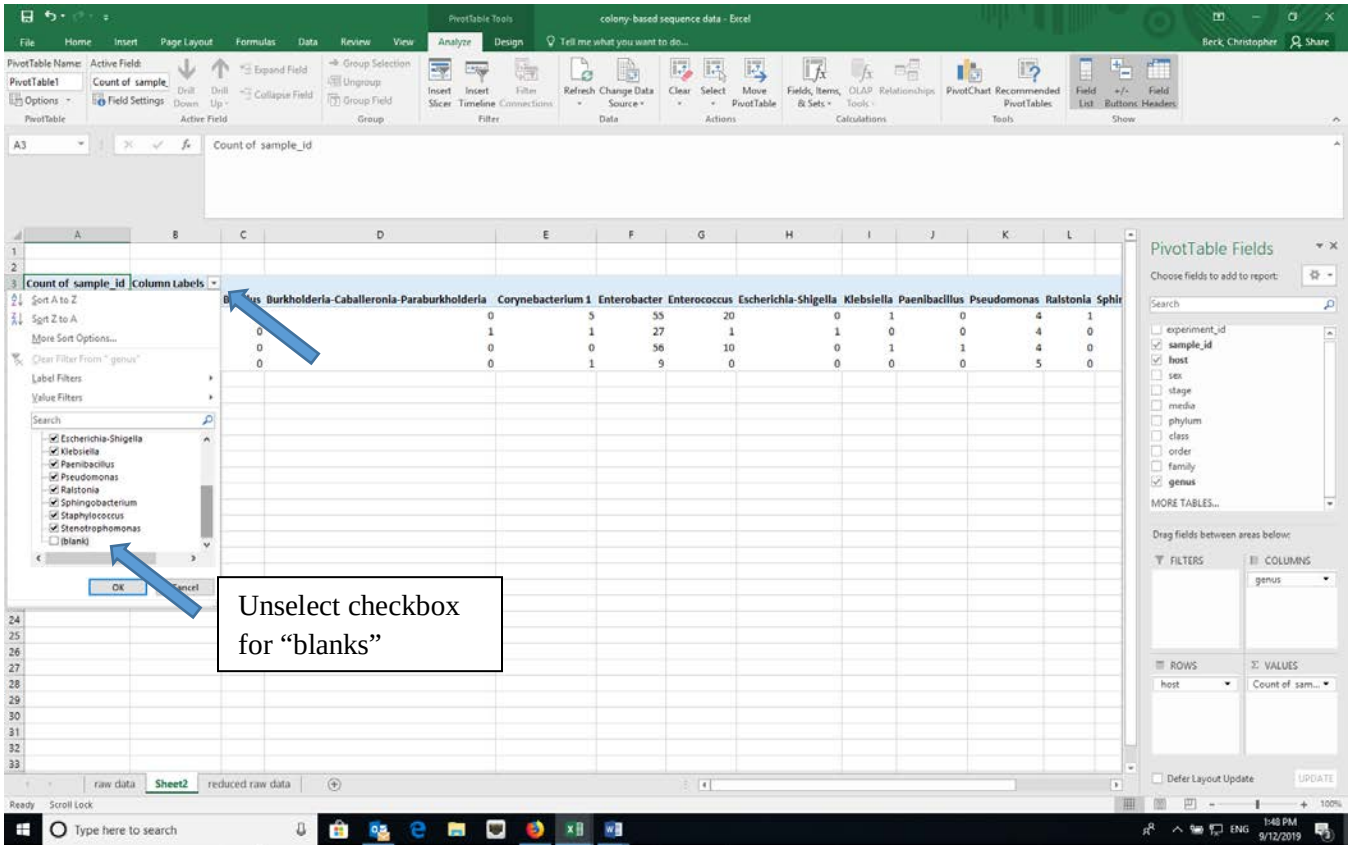


4. You can add zeros to all of the empty cells in the Pivot Table using the Options menu and remove the Grand totals for Columns using the Options menu or the Design tab depending of your version of Excel. (You want to keep the Grand totals for rows to calculate diversity indices.)

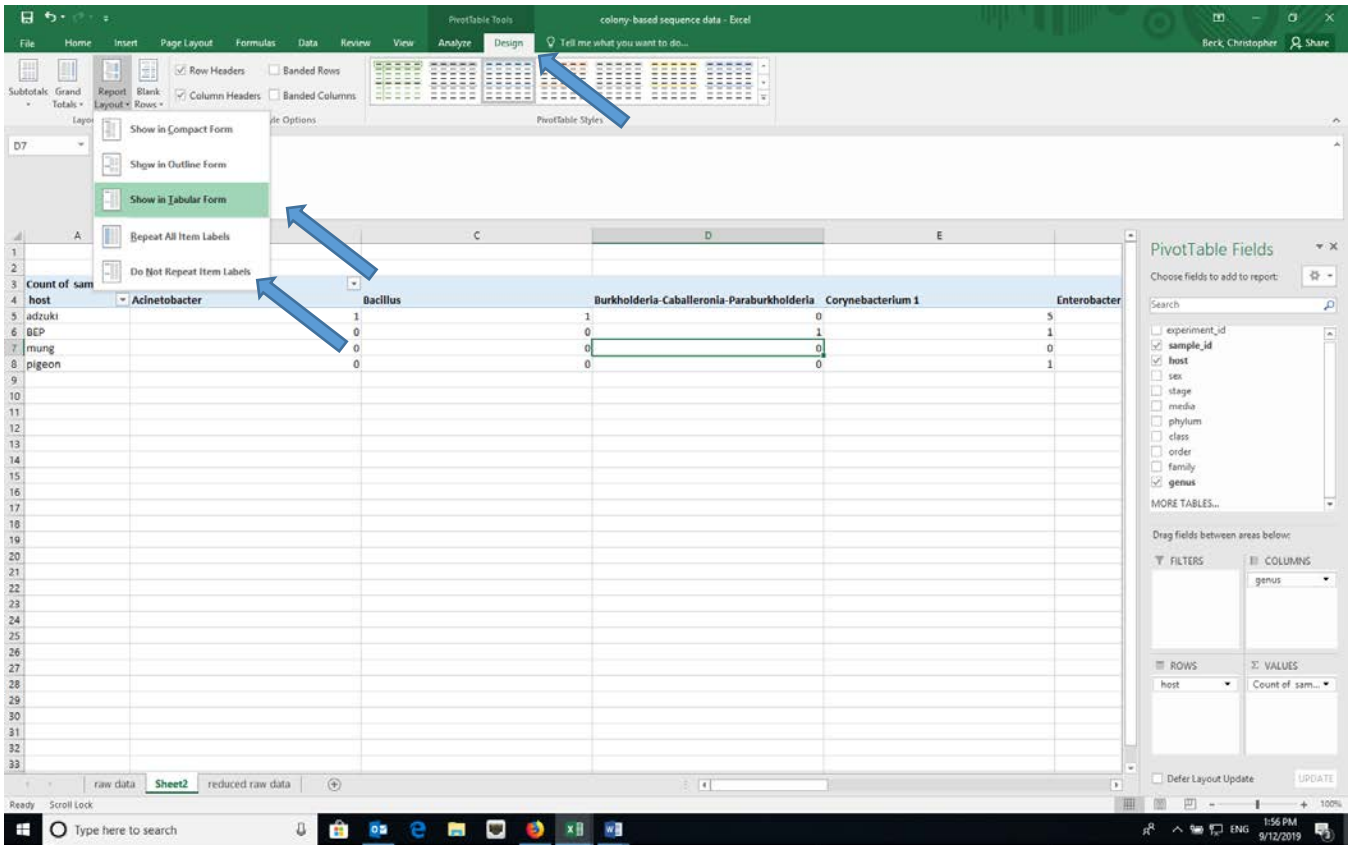

PivotTable Options
Count of sample_id
Column Labels
Row Labels
Acinetobacter
Bacillus
Burkholderia-Caballeronia-Paraburkholderia
Corynebacterium 1
adzuki
BEP
mung
pigeon
Grand Total
PivotTable Fields
raw data
Sheet2
reduced raw data


PivotTable Options
Totals & Filters
Grand Totals
Show grand totals for rows
Show grand totals for columns
Unselect checkbox for “Show grand totals for columns”
PivotTable Fields
raw data
Sheet2
reduced raw data

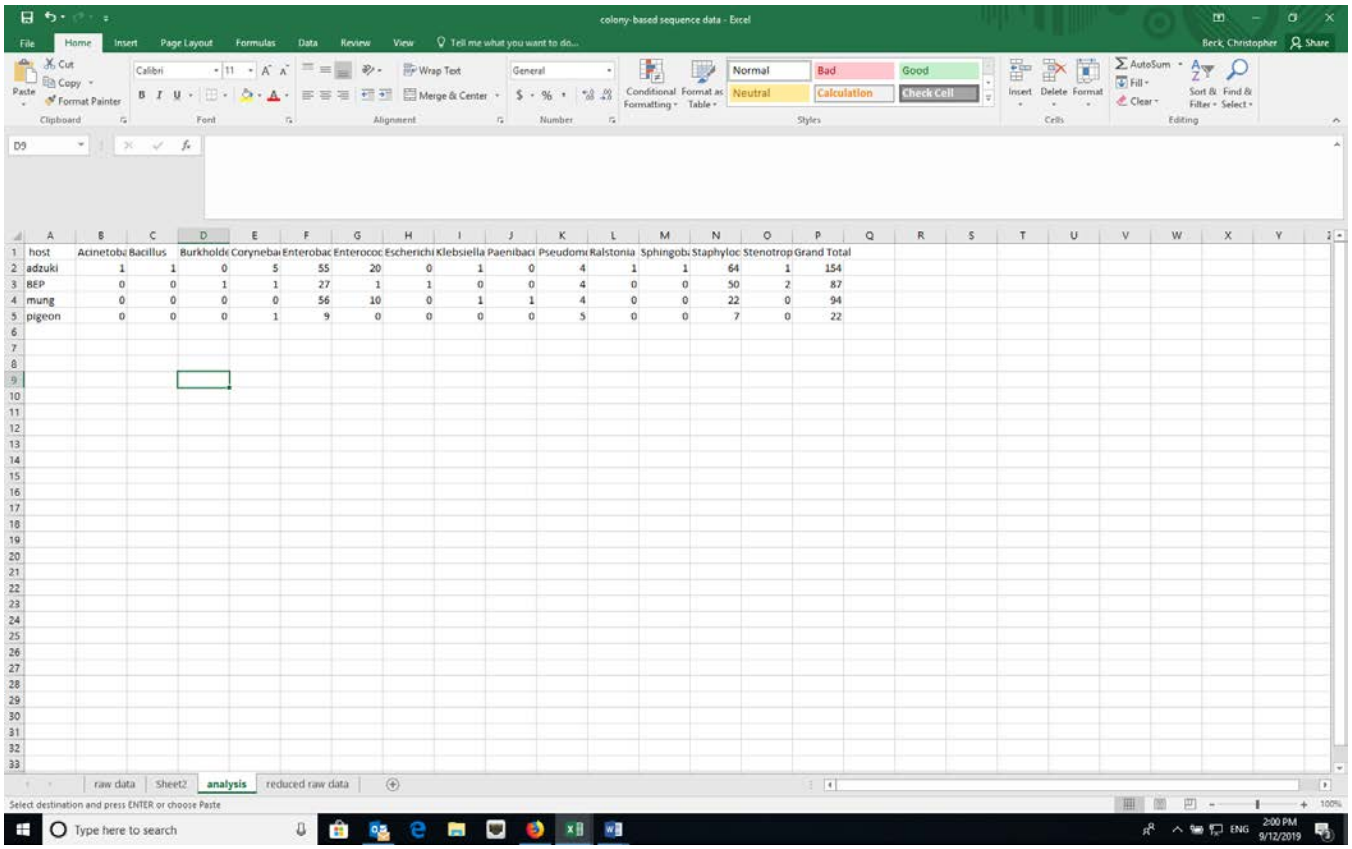
5. You can remove the “blanks” column using the Column labels dropdown (located at upper left of the sheet) and unselecting “blank”.



6. If you selected more than one treatment for the rows, you can get the treatment data to repeat for each sample. In the Design tab, select “Report Layout” and choose “Show in Tabular form” and “Repeat All Item Labels”.

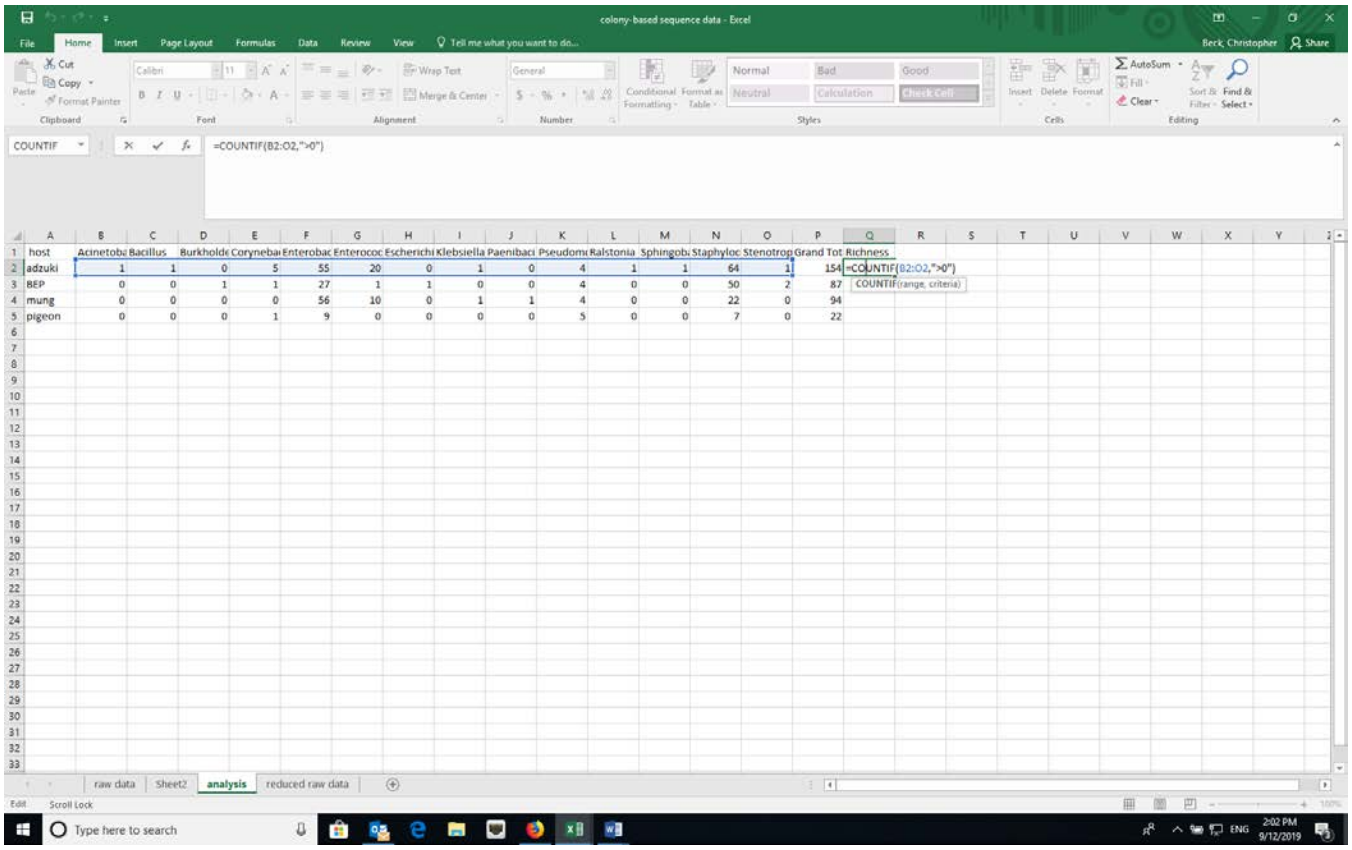


7. Copy and paste (as values) the pivot table to a new worksheet and remove any extra rows at the top. The top row should now have the taxa names. Name this tab “analysis”. Conduct the community ecology analyses that follow in Excel on the “analysis” sheet.



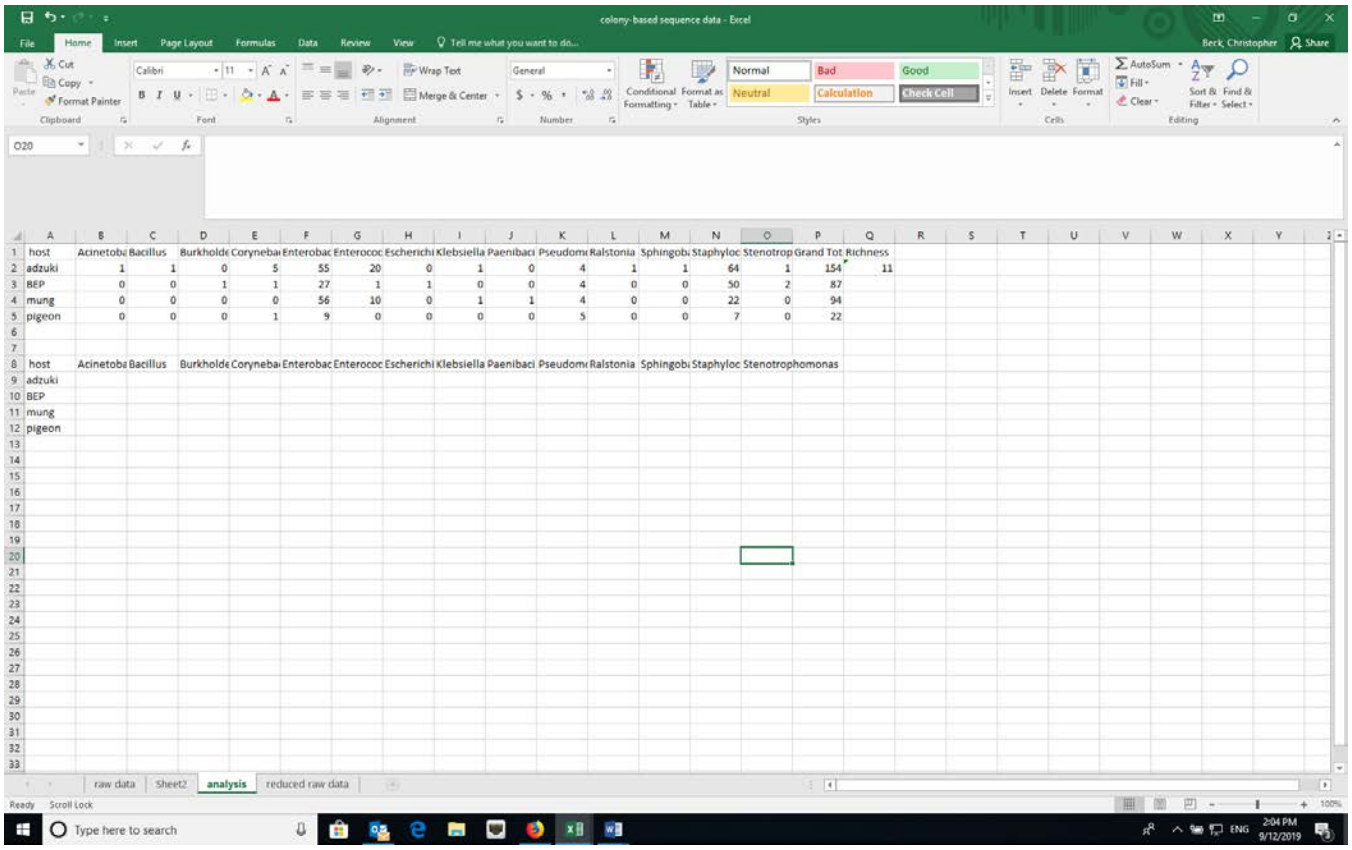
Calculating diversity indices

1. Species (taxon) richness – the number of unique species (taxa) in a sample
    a. Although you could manually count the number of cells with values greater than zero for each treatment, using the COUNTIF formula in Excel is easier (e.g., =COUNTIF(range,”>0”)). Where “range” is the cell range in the datasheet, for example “C2:M2”, a single row or treatment.



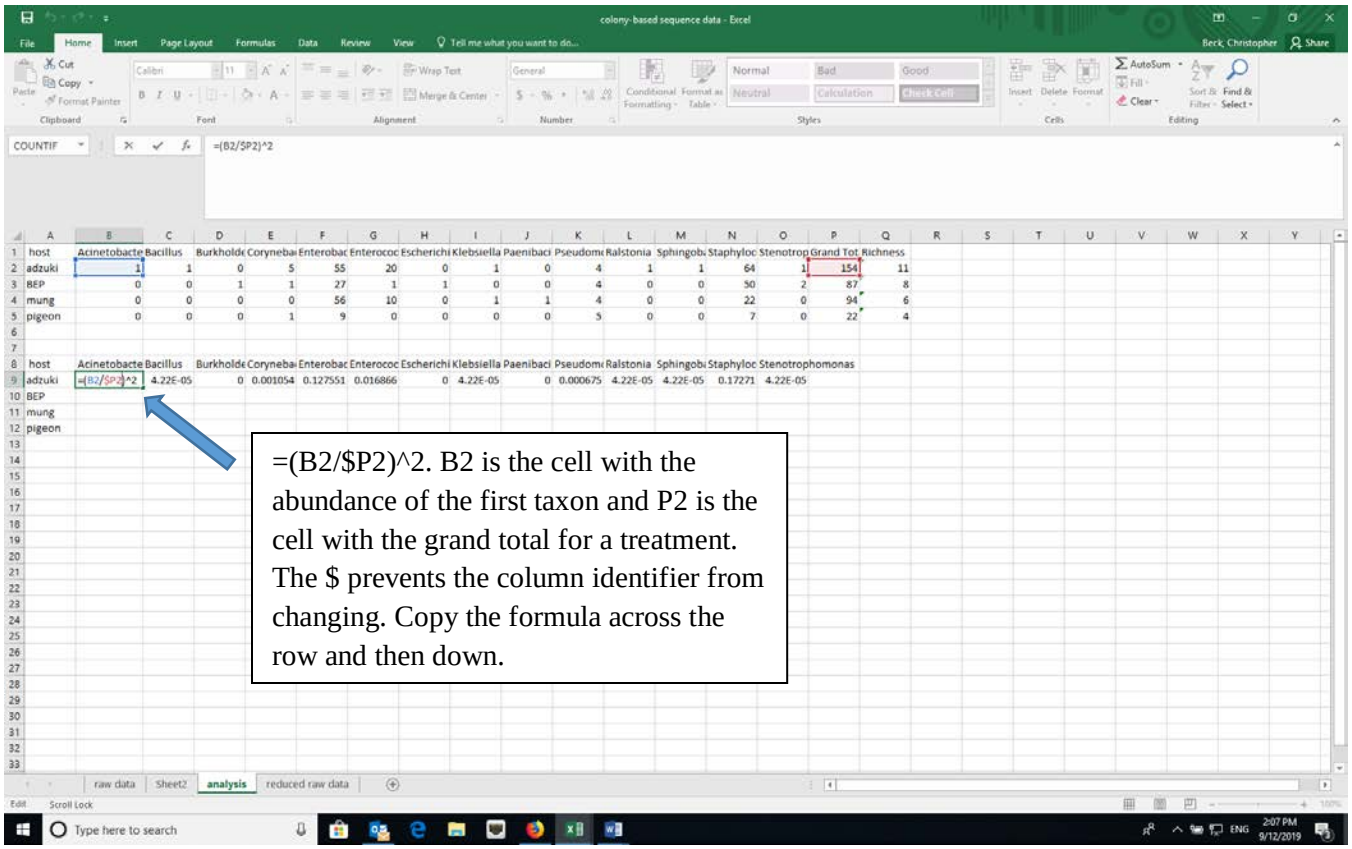
2. Simpson Index – the Simpson Index incorporates both species (taxon) richness and species (taxon) evenness.

    a. D = Σ(n/N)^2, where n=number of individuals of a particular species (taxon) and N=total number of individuals in a sample. D increases as diversity decreases, which is counterintuitive. A reciprocal or inverse index would be more intuitive and are easily calculated.
    b. Reciprocal Simpson = 1/D and scales so the maximum value is the species richness of a community.
    c. Inverse Simpson = 1-D and scales to a maximum value of 1.0.
    d. Create a new data array below the original using the same row labels (treatment variables) and the same column labels (bacterial taxa).

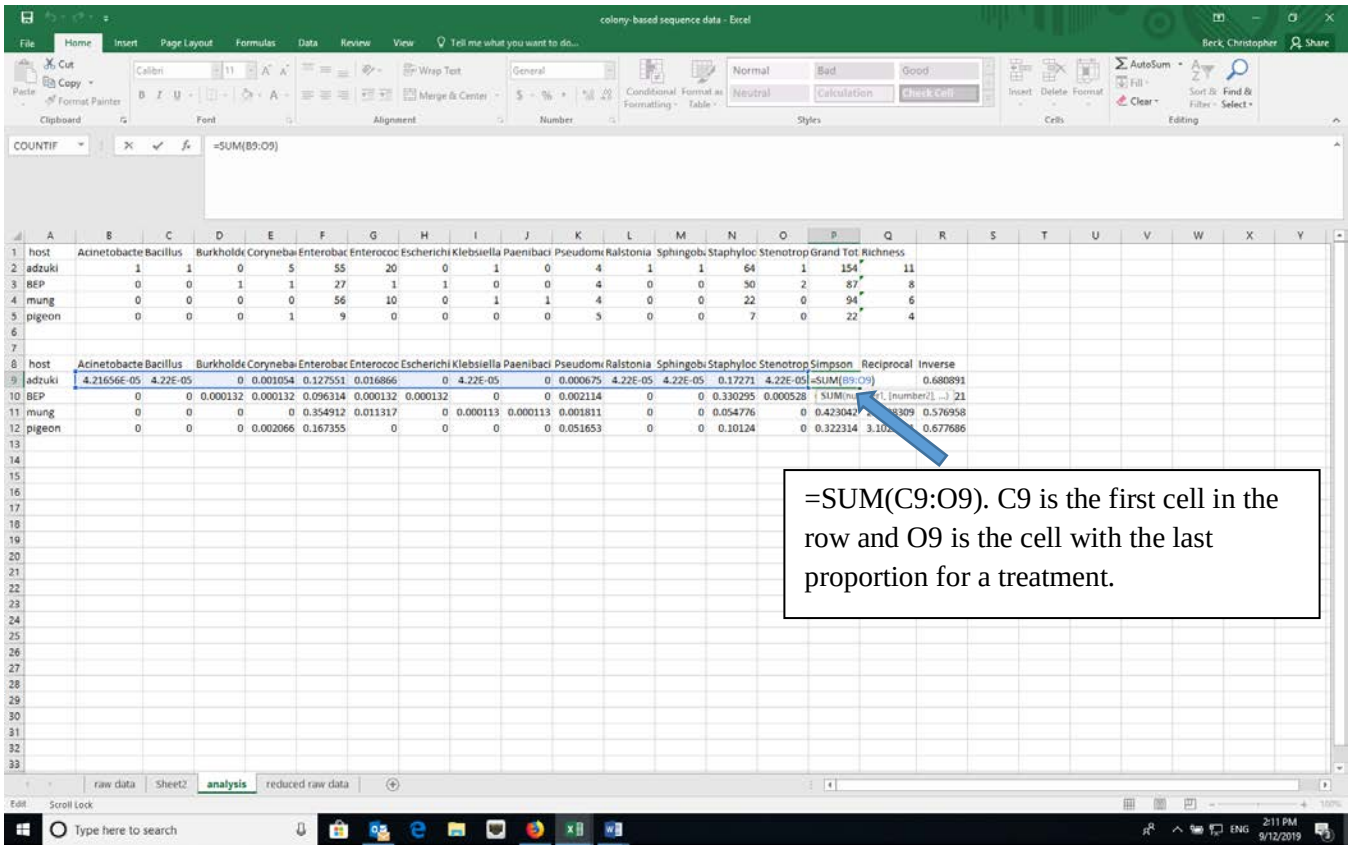


	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	host	Acinetoba	Bacillus	Burkholde	Corynebac	Enterobac	Enterococ	Escherichi	Klebsiella	Paenibaci	Pseudomo	Ralstonia	Sphingobi	Staphyloc	Stenotrop	Grand Tot	Richness
2	adzuki	1	1	0	5	55	20	0	1	0	4	1	1	64	1	154	11
3	BEP	0	0	1	1	27	1	1	0	0	4	0	0	50	2	87	
4	mung	0	0	0	0	56	10	0	1	1	4	0	0	22	0	94	
5	pigeon	0	0	0	1	9	0	0	0	0	5	0	0	7	0	22	
6																	
7																	
8	host	Acinetoba	Bacillus	Burkholde	Corynebac	Enterobac	Enterococ	Escherichi	Klebsiella	Paenibaci	Pseudomo	Ralstonia	Sphingobi	Staphyloc	Stenotrophomonas		
9	adzuki																
10	BEP																
11	mung																
12	pigeon																

e. To calculate the proportion squared for each taxa, use the grand totals for each treatment. Using the Excel trick that $ before a column or row prevents Excel from iterating when copying a formula makes this easy. For example, =(C2/$P2)^2. Copy the formula across the row and then down.

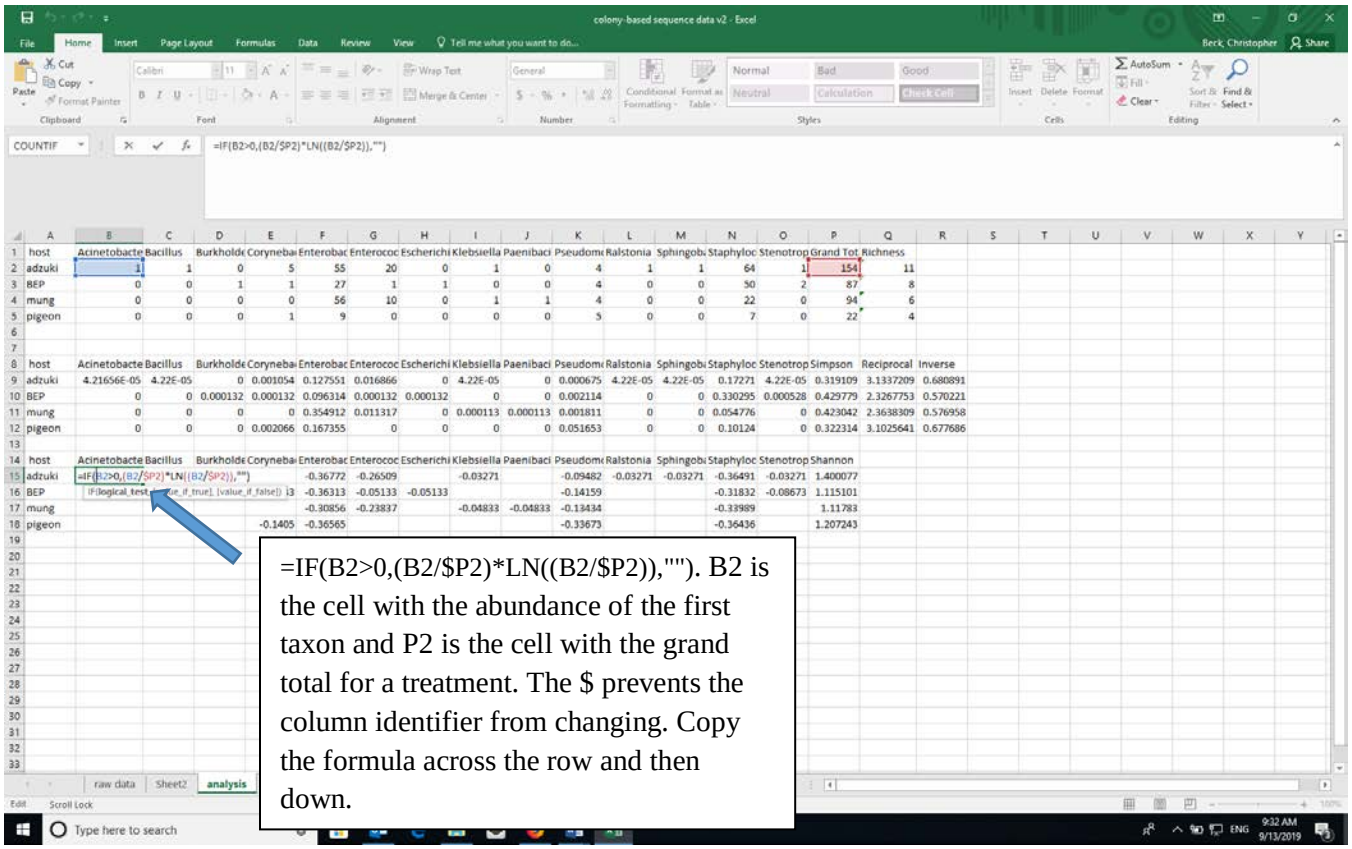


f. Calculate the sum of the proportions squared (=SUM in Excel) to calculate the Simpson Index.

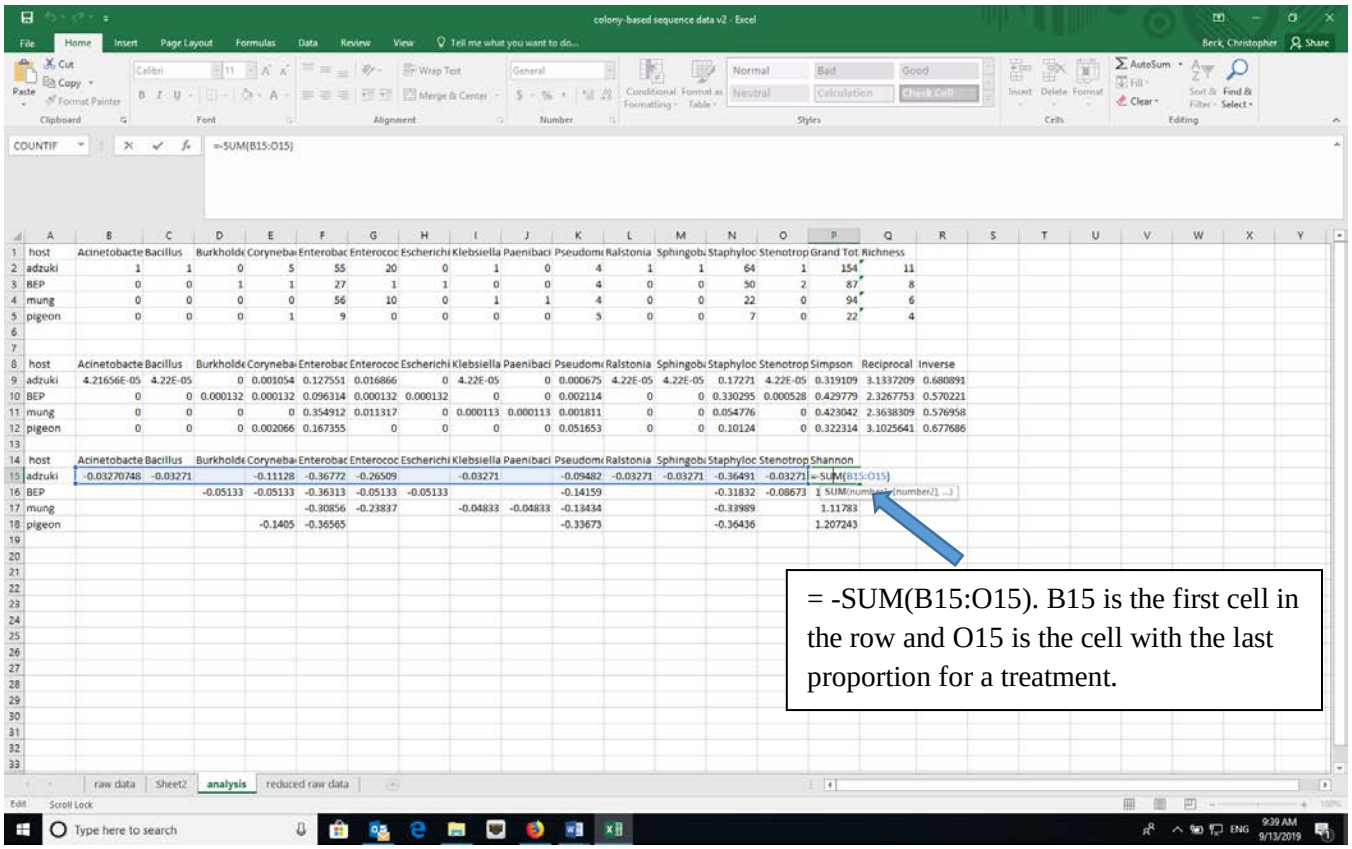


g. Calculate the reciprocal (e.g., =1/P9) and inverse Simpson (e.g., =1-P9) using formulas in Excel.

3. Shannon-Weaver (Shannon-Weiner) Index – also incorporates species (taxon) richness and species (taxon) evenness
    a. $H=-\Sigma p \ln p$, where p is the proportion of individuals of each bacterial taxon in a community (i.e., n/N).
    b. Create a new data array below the original using the same row labels (treatment variables) and the same column labels (species).
    c. Using the grand totals for each treatment, calculate the proportions ($p \ln p$). Using the Excel trick that $ before a column or row prevents Excel from iterating when copying a formula makes this easy.
    d. Note that $\ln p$ *is* undefined if $p=0$, so you can use an "IF" statement in Excel. For example, =IF(B2>0,(B2/$P2)*LN((B2/$P2)),"")

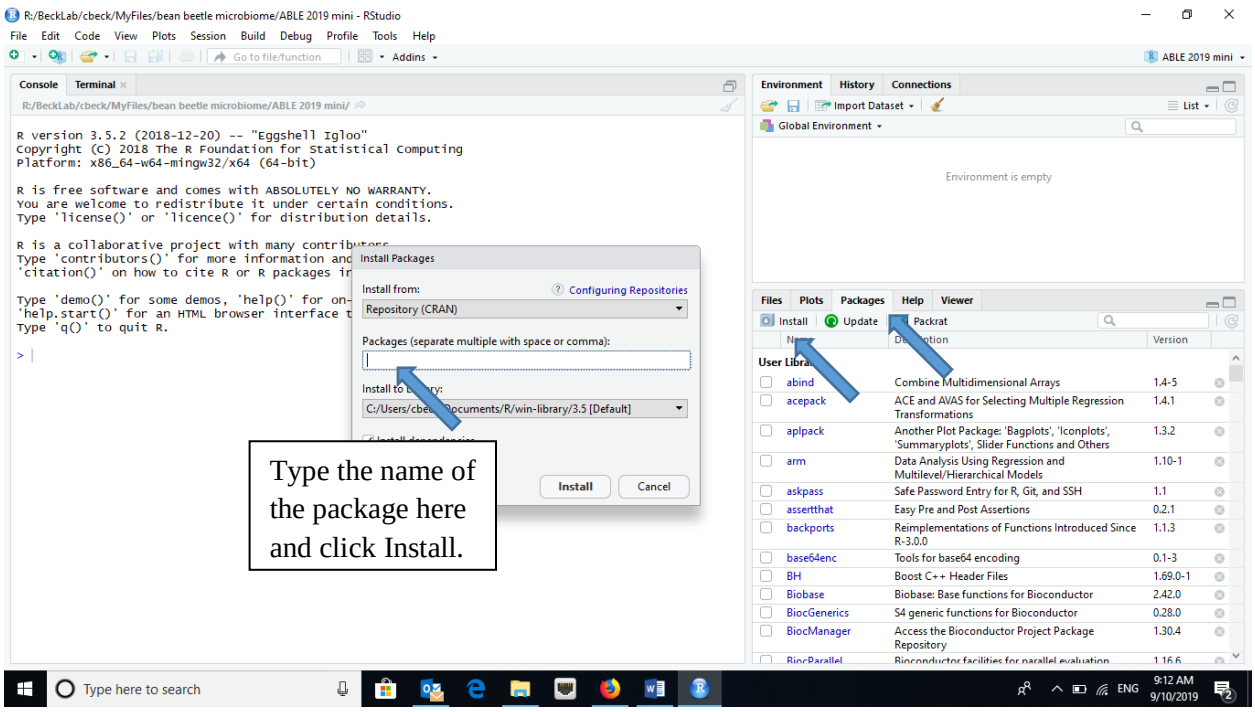


e. Calculate the negative sum of the proportions (*p*ln*p*) (=-SUM in Excel for each row, a different microbial community) to calculate the Shannon-Weaver Index.

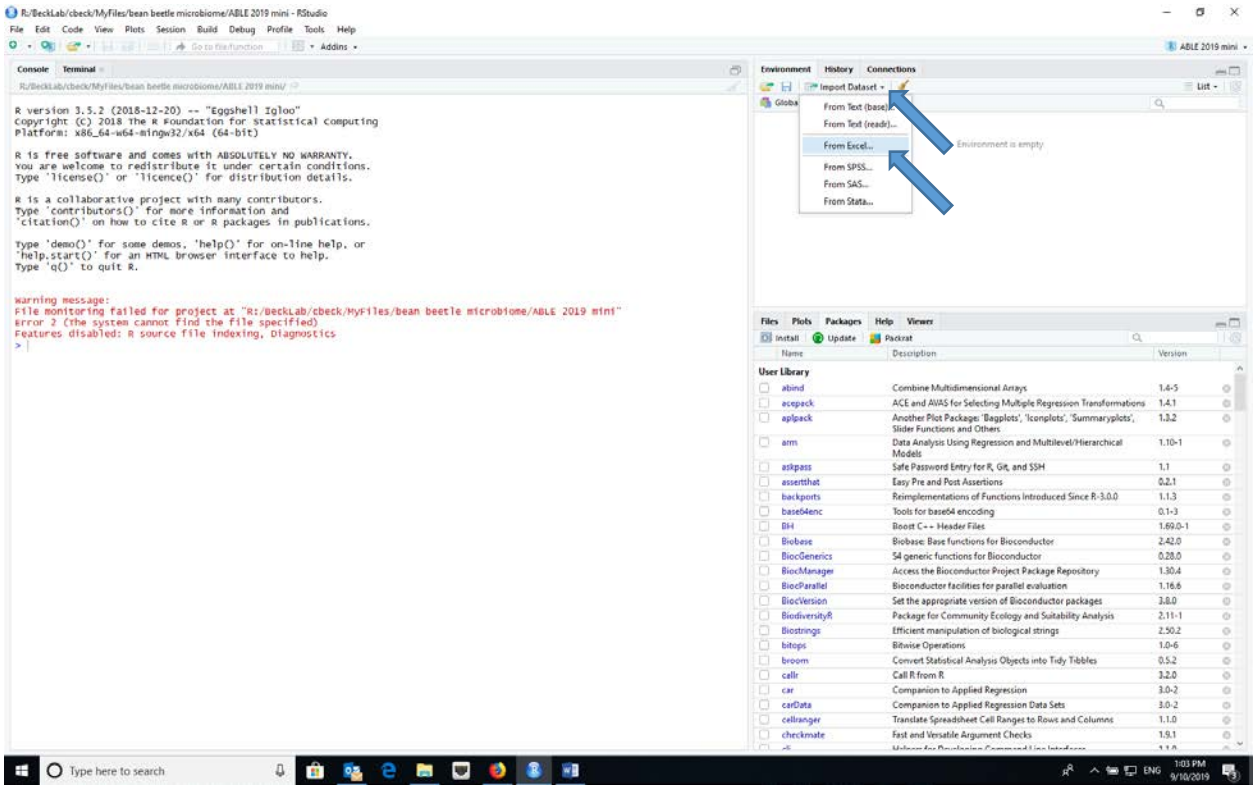


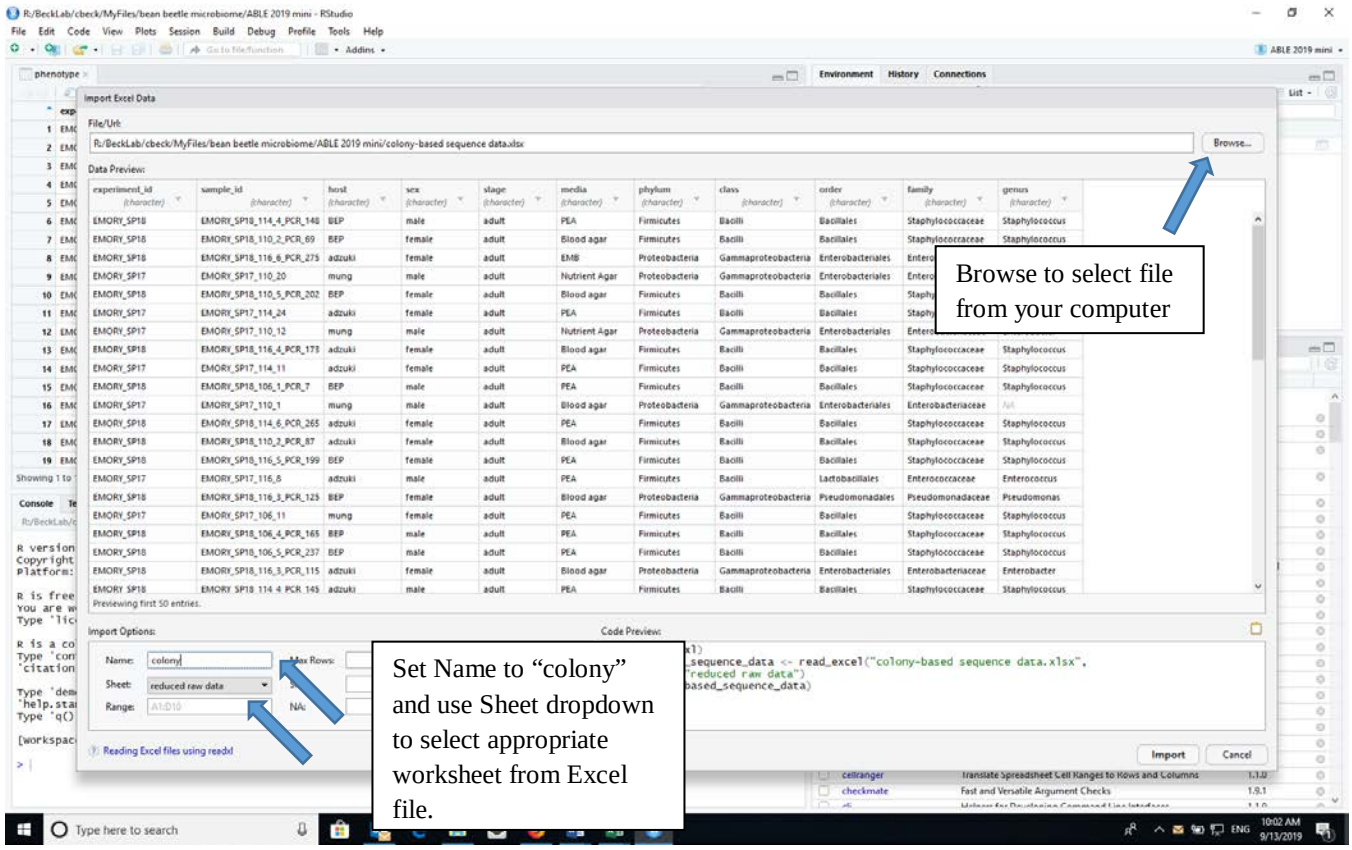
Data Manipulation in R

1. Open RStudio and create a new project using the New Project option under File and select for the new project to be in an existing folder where your data are.
2. Install the following packages either using the Packages tab in RStudio or the command install.packages("name_of_package") in the console. Note that BiodiversityR requires QuartzX on a Mac. If you are using a MacOS and don't have QuartzX, install it first and restart your computer before install these packages.
    a. dplyr
    b. reshape2
    c. vegan
    d. BiodiversityR
    e. ggplot2



3. Load the packages listed above by clicking the checkboxes for the appropriate packages in the Packages tab or the command library("name_of_package") in the console.
4. Import the dataset "reduced raw data" (dataset without the extra metadata that you created in the Excel section) into RStudio.


From Text (base)...
From Text (readr)...
From Excel...
From SPSS...
From SAS...
From Stata...


Browse to select file from your computer
Set Name to “colony” and use Sheet dropdown to select appropriate worksheet from Excel file.

5. Attach the imported dataset ("colony") to the dataframe using the attach command in the console (attach(colony))
6. Create a community matrix (named "community" in this example) for a particular treatment. This example assumes that you are doing the analysis at the genus level. This can be changed to other taxonomic levels using the appropriate variable name

```
>community<-table(host,genus)
```

7. If you want to look at two factors at the same time, creating the community matrix is a little more complicated. The first command calculates the count of each genus by each sex and host combination and drops any missing values. The second command creates a community matrix.

```
> community_2 <- colony %>% count(sex,host,genus) %>% drop_na()
> comm2<-dcast(community_2, sex+host~genus, value.var = "n", fun.aggregate = sum)
```

"genus" in both command lines may be whatever taxon level you wish to evaluate in the dataset. For example, it could be changed to "family" or "order".

Calculating diversity indices

Note: "community" is the name of the community matrix

1. Species Richness

```
> diversityresult(community,index="richness",method="each site")
```

2. Simpson

```
> diversityresult(community,index="Simpson",method="each site")
```

This calculates the inverse Simpson described above

```
> diversityresult(community,index="inverseSimpson",method="each site")
```

This calculates the reciprocal Simpson described above. (confusing that it is called in the inverseSimpson)

3. Shannon

```
> diversityresult(community,index="Shannon",method="each site")
```

Calculating community similarity (distance)

Sometimes we are interested in how similar (or different) two communities are based on what species (taxa) are present and the relative abundance of those species (taxa) in the two communities. One of the most common measures of distance is the Bray Curtis Dissimilarity. Similarity can be measured as 1-BC.

$$BC_{ij} = 1 - \frac{2C_{ij}}{S_i \div S_j}$$

Where:

- i & j are the two samples,
- S_i is the total number of specimens counted in sample i,
- S_j is the total number of specimens counted in sample j,
- C_{ij} is the sum of only the lesser counts for each taxa found in both sites.

Although Bray-Curtis Dissimilarity is often used in community ecology, it is not robust to incomplete sampling of the community (all taxa are not sampled) or unbalanced sampling (all treatments are not equally sampled). An alternative is the Morista-Horn Index of Dissimilarity (1-C_H). Morista-Horn Index of Similarity is

$$C_H = \frac{2\sum_{i=1}^{D_{12}} \frac{X_i}{n}\frac{Y_i}{m}}{\sum_{i=1}^{D_1}\left(\frac{X_i}{n}\right)^2 + \sum_{i=1}^{D_2}\left(\frac{Y_i}{m}\right)^2}$$

Where

- D_1=number of taxa in sample 1
- D_2=number of taxa in sample 2
- D_{12}=number of taxa in shared in both communities
- X_i=number of individuals of taxon *i* in sample 1
- Y_i=number of individuals of taxon *i* in sample 2
- n=total number of individuals in sample 1
- m=total number of individuals in sample 2

So that X_i/n and Y_i/m are proportion of individuals of taxon *i* in each of the samples (communities).

To produce a matrix of all of the pair-wise distances between samples using the Bray Curtis index of dis tance, use the following command.

```
> vegdist(community, method="bray", binary=FALSE, diag=FALSE, upper=FALSE)
```

To produce a matrix of all of the pair-wise distances between samples using the Morista-Horn index of distance.

```
> vegdist(community, method="horn", binary=FALSE, diag=FALSE, upper=FALSE)
```

Cited References

Christian N, Whitaker BK, Clay K. 2015. Microbiomes: unifying animal and plant systems through the lens of community ecology theory. Front. Microbiol. 6:1–15.

Cole MF, Acevedo-Gonzalez T, Gerardo NM, Harris EV, Beck CW. 2018. Effect of diet on bean beetle microbial communities. Article 3 In: McMahon K, editor. Tested studies for laboratory teaching. Volume 39. Proceedings of the 39th Conference of the Association for Biology Laboratory Education (ABLE).

Costello EK, Stagaman K, Dethlefsen L, Bohannan BJM, Relman DA. 2012. The application of ecological theory toward an understanding of the human microbiome. Science. 336:1255–1262

Engel P, Moran NA. 2013. The gut microbiota of insects – diversity in structure and fuction. FEMS Microbiol. Rev. 37:699-735.

Krebs CJ. 1999. Ecological Methodology, 2nd edition. New York: Benjamin Cummings.

McFall-Ngai M, Hadfield MG, Bosch TCG, Carey HV, Domazet-Lošo T, Douglas AE, Dubilier N, Eberl G, Fukami T, Gilbert SF, Hentschel U, King N, Kjelleberg S, Knoll AH, Kremer N, Mazmanian SK, Metcalf JL, Nealson K, Pierce NE, Rawls JF, Reid A, Ruby EG, Rumpho M, Sanders JG, Tautz D, Wernegreen JJ. 2013. Animals in a bacterial world, a new imperative for the life sciences. Proc. Natl. Acad. Sci. U.S.A. 110:3229–3236.

The Human Microbiome Consortium. 2012. Structure, function and diversity of the healthy human microbiome. Nature 486: 207-214.

Young E. 2016. I Contain Multitudes: The Microbes Within Us and a Grander View of Life. New York: HarperCollins Publishers.

This study is based on Blumer LS, Beck CW 2020. **Introducing community ecology and data skills with the bean beetle microbiome project**. Advances in Biology Laboratory Education 41.