Chapter 3: ITS data analysis using the R package PhyloSeq

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Load the relevant images, then save this chapter's image as a separate image to retain environment data specific to the ITS processing and analysis workflow.

```
# Set the name for this chapter's image:
chptImage    <- "ecobiomics_ITS_analysis.RData"

# Save this chapter's image:
save.image(paste(imageDirPath, chptImage, sep = ""))
```

When re-starting a session, you can quickly load up the image by running the chunk below:

```
sharedPath <- "/isilon/cfia-ottawa-fallowfield/users/girouxeml/PIRL_working_directory/"
analysis <- "ecobiomics/"
sharedPathAn <- paste(sharedPath, analysis, sep = "")
imageDirPath <- "/home/CFIA-ACIA/girouxeml/GitHub_Repos/r_environments/ecobiomics/"
chptImageA <- "ecobiomics_ITS.RData"
load(paste(imageDirPath, chptImageA, sep = ""))
chptImage    <- "ecobiomics_ITS_analysis.RData"
save.image(paste(imageDirPath, chptImage, sep = ""))
```

Let's get familiar with our phyloseq object created at the end of our sequencing sample processing chapter:
Below I am using the ps, rather than phySeq objects. Recall from the last chunks of Chapter 2, the phySeq object is a phyloseq object without the fitGTR$tree info, while the ps object was created leveraging the fitGTR$ information. The phySeq object can be used instead of the ps object if the optim.pml command wasn't run.

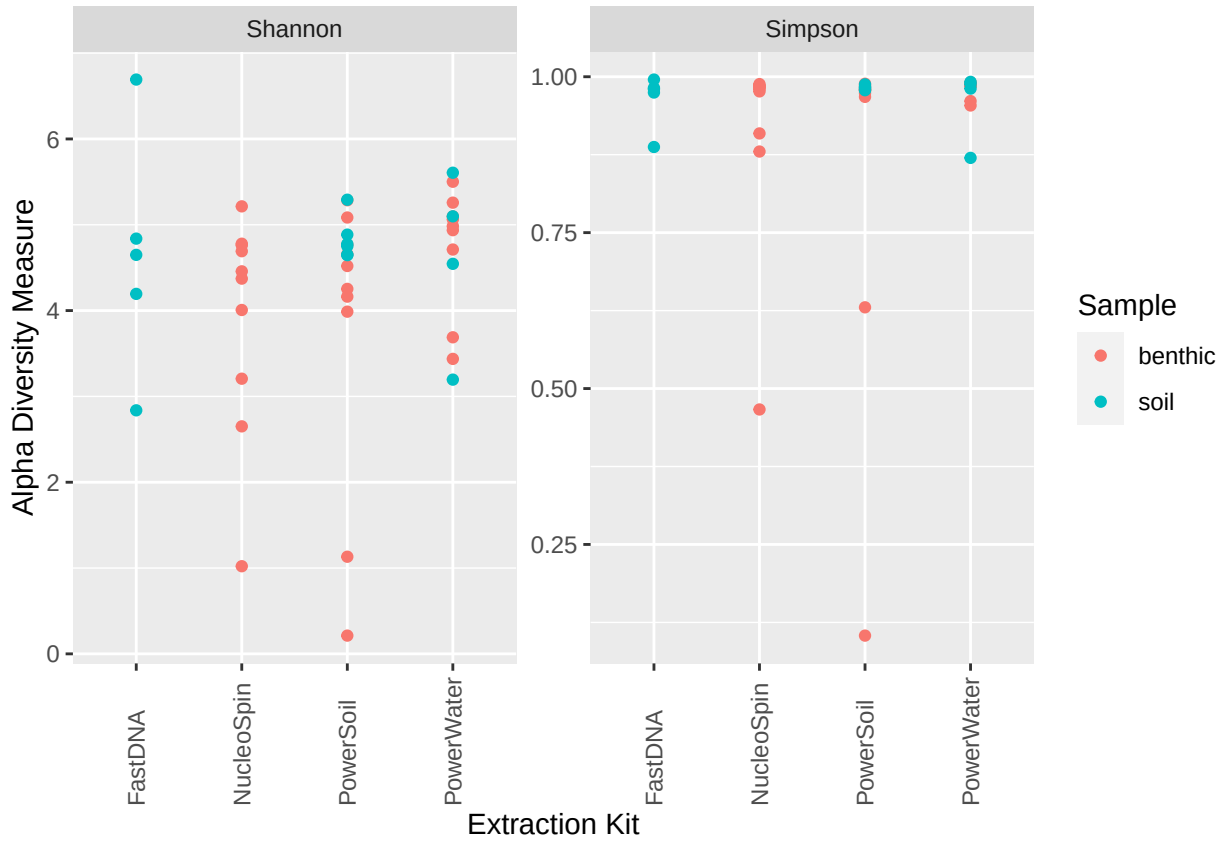
Here is if we filter based on having to know the species:

```
library("phyloseq")
# table(tax_table(ps)[, "Genus"], exclude = NULL)
t <- table(tax_table(ps)[, "Genus"], exclude = NULL)
# head(t[order(-t)])
t[order(-t)][2:10]
```

```
     g__Acidea       g__Bannoa  g__Mortierella  g__Trichoderma     g__Venturia
          1429             512             197              99              60
g__Malassezia g__Tetracladium g__Myrmecridium   g__Alatospora
            57              50              46              41
```

Visualize alpha-diversity, phylum:

```
library("phyloseq")
library("ggplot2")
library("cowplot")
plot_richness(ps,
              x = "ExtractionKit",
              measures = c("Shannon", "Simpson"),
              color = "Sample") +
                theme(axis.text.x = element_text(angle = 90)) +
  scale_x_discrete(name = "Extraction Kit")
```



Prevalence evaluation for species:

```
library("phyloseq")
prevDf <- apply(X = otu_table(ps),
                MARGIN = ifelse(taxa_are_rows(ps),
```

```
                                yes = 1, no = 2),
               FUN = function(x){sum(x>0)})

prevDf <- data.frame(Prevalence = prevDf,
                     TotalAbundance = taxa_sums(ps),
                     tax_table(ps))

prevalenceTblSpp <- plyr::ddply(prevDf, "Species",
                                function(df1){
                                  cbind(mean(df1$Prevalence),
                                        sum(df1$Prevalence))})
colnames(prevalenceTblSpp) <- c("Species", "Mean", "Sum")
head(prevalenceTblSpp)
head(prevalenceTblSpp[order(-prevalenceTblSpp[,3]),])
```

```
         Species     Mean Sum
1   s__abeliceae 5.000000   5
2 s__acerophilum 1.000000   1
3     s__acicola 1.000000   2
4   s__aculeatus 2.666667   8
5   s__acuminata 3.400000 102
6      s__adusta 1.000000   4
               Species     Mean  Sum
427              <NA> 1.422086 8834
124        s__extrema 1.000000 1429
271  s__ogasawarensis 1.000000  512
349      s__schulzeri 3.465116  149
172      s__harzianum 1.359551  121
295 s__piceae-abietis 6.562500  105
```

Prevalence evaluation for phyla:

```
library("phyloseq")
prevDf <- apply(X = otu_table(ps),
                MARGIN = ifelse(taxa_are_rows(ps),
                                yes = 1, no = 2),
                FUN = function(x){sum(x>0)})

prevDf <- data.frame(Prevalence = prevDf,
                     TotalAbundance = taxa_sums(ps),
                     tax_table(ps))

prevalenceTblPhyla <- plyr::ddply(prevDf, "Phylum",
                                  function(df1){
                                    cbind(mean(df1$Prevalence),
                                          sum(df1$Prevalence))})
```

```
colnames(prevalenceTblPhyla) <- c("Phylum", "Mean", "Sum")
prevalenceTblPhyla[order(-prevalenceTblPhyla[,3]),]
```

```
                  Phylum     Mean  Sum
2          p__Ascomycota 1.700448 9480
4       p__Basidiomycota 1.189139 2584
14                  <NA> 1.164004 2186
10  p__Mortierellomycota 1.777228  359
6     p__Chytridiomycota 1.292857  181
7       p__Glomeromycota 1.111111   30
11       p__Mucoromycota 1.230769   16
13      p__Rozellomycota 1.428571   10
1     p__Aphelidiomycota 1.500000    6
12      p__Olpidiomycota 1.000000    6
5  p__Blastocladiomycota 1.666667    5
9  p__Monoblepharomycota 1.000000    4
8     p__Kickxellomycota 1.000000    3
3   p__Basidiobolomycota 1.000000    1
```

From the above calculations, there are a few low-abundance Phylas that appear in less than 10 samples:
Aphelidiomycota
Basidiobolomycota
Blastocladiomycota
Kickxellomycota
Monoblepharomycota
Olpidiomycota
Rozellomycota

Filter entries with unidentified Phylum, or those phyla that appear in less than 10 samples:

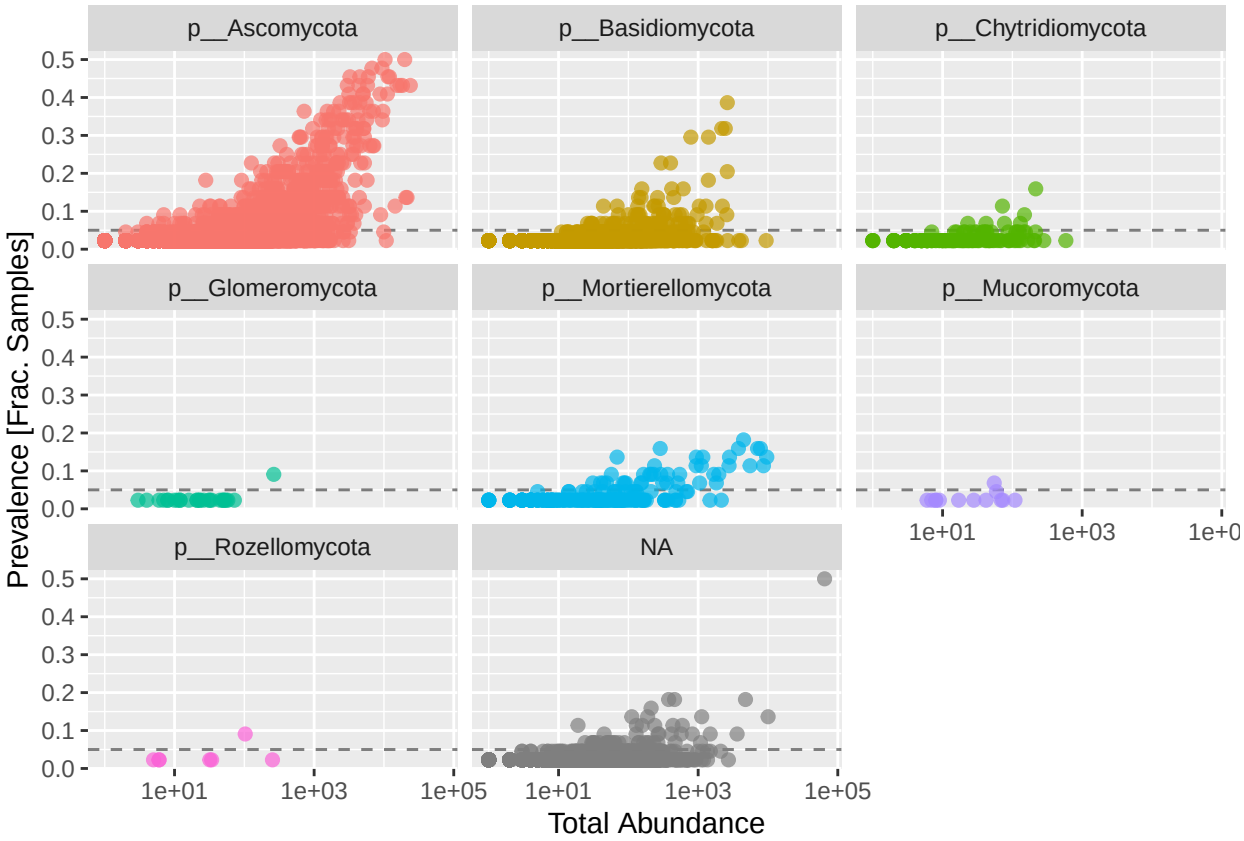
```
library("phyloseq")
phylas <- subset(prevalenceTblPhyla, prevalenceTblPhyla$Sum < 10)
ps1    <- subset_taxa(ps, !Phylum %in% phylas$Phylum)
rank_names(ps1)
head(table(tax_table(ps1)[, "Phylum"], exclude = NULL))
head(table(tax_table(ps1)[, "Species"], exclude = NULL))

t2 <- table(tax_table(ps1)[, "Species"], exclude = NULL)
t2[order(-t2)][2:10]
```

```
[1] "Kingdom" "Phylum"  "Class"   "Order"   "Family"  "Genus"   "Species"

      p__Ascomycota     p__Basidiomycota   p__Chytridiomycota
               5575                 2173                  140
   p__Glomeromycota p__Mortierellomycota      p__Mucoromycota
                 27                  202                   13
```

```
  s__abeliceae s__acerophilum     s__acicola   s__aculeatus   s__acuminata
             1               1              2              3             30
     s__adusta
             4

      s__extrema s__ogasawarensis     s__harzianum   s__minutissima
            1429              512               89               49
    s__schulzeri      s__elongata       s__hyalina     s__acuminata
              43               33               33               30
    s__aquaticus
              30
```

Plot Phylum:

```
library("phyloseq")
library("ggplot2")
prevDf1 <- subset(prevDf, Phylum %in% get_taxa_unique(ps1, "Phylum"))
ggplot(prevDf1, aes(TotalAbundance, Prevalence / nsamples(ps1),
                    color = Phylum)) +
  geom_hline(yintercept = 0.05, alpha = 0.5, linetype = 2) +
  geom_point(size = 2, alpha = 0.7) +
  scale_x_log10() + xlab("Total Abundance") + ylab("Prevalence [Frac. Samples]") +
  facet_wrap(~Phylum) + theme(legend.position = "none")
```



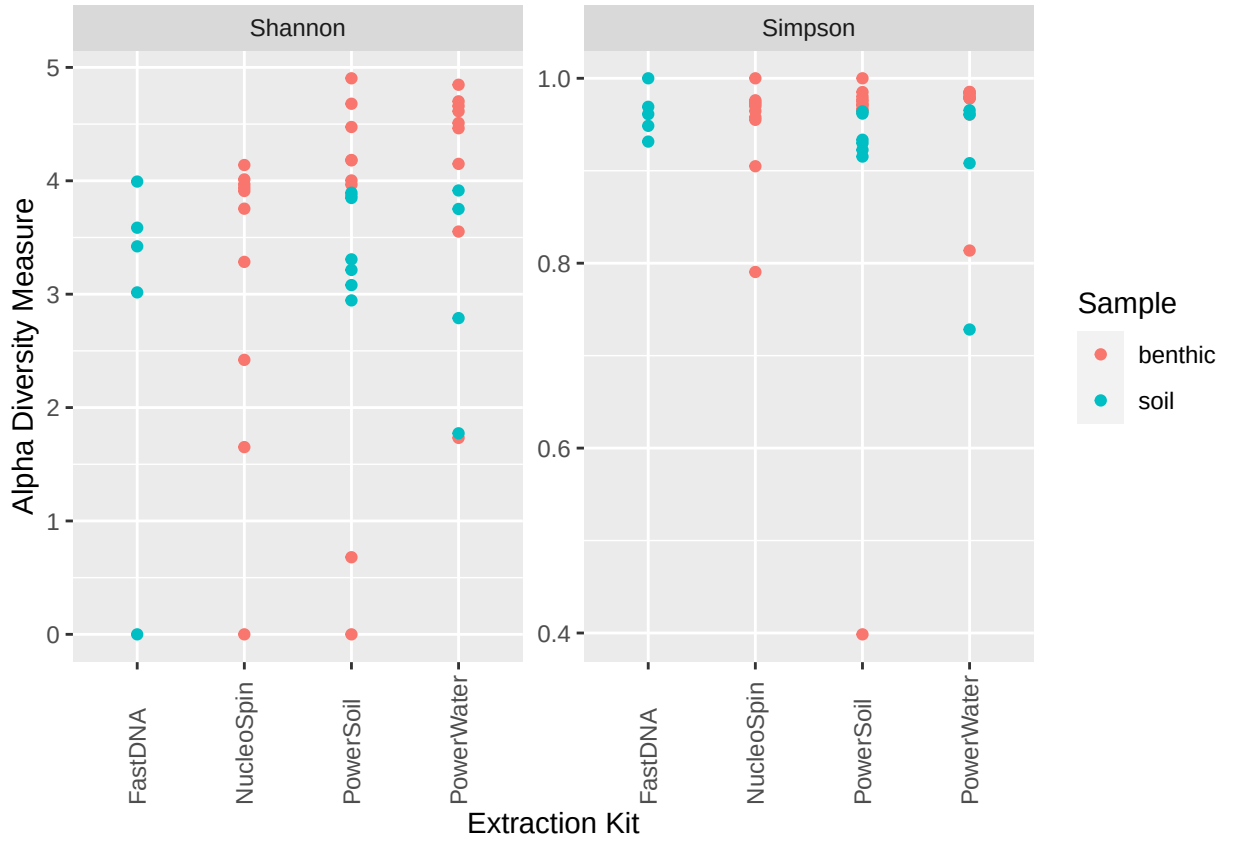
Each point in the above plots is a different taxa, Phylum.

```
prevalenceThreshold = 0.05*nsamples(ps1)
prevalenceThreshold
```

```
[1] 2.2
```

The taxa with a prevalence threshold less than the one set in the above chunk are removed using prune_taxa and put into a new phyloseq object, ps2, and we look at the resulting richness plot:

```
library("phyloseq")
keepTaxa <- rownames(prevDf1)[(prevDf1$Prevalence >= prevalenceThreshold)]
ps2 <- prune_taxa(keepTaxa, ps1)
rank_names(ps2)
# table(tax_table(ps2)[, "Phylum"], exclude = NULL)
# table(tax_table(ps2)[, "Species"], exclude = NULL)
plot_richness(ps2,
              x = "ExtractionKit",
              measures = c("Shannon", "Simpson"),
              color = "Sample") +
                theme(axis.text.x = element_text(angle = 90)) +
  scale_x_discrete(name = "Extraction Kit")
```




```
[1] "Kingdom" "Phylum"  "Class"   "Order"   "Family"  "Genus"   "Species"
```

Phylum - curiosity:
Curious about the mean and sum prevalence after keeping only taxa passing prevalenceThreshold:

```
library("phyloseq")
prevDf2 <- apply(X = otu_table(ps2),
                 MARGIN = ifelse(taxa_are_rows(ps2), yes = 1, no = 2),
                 FUN = function(x){sum(x > 0)})

prevDf2 <- data.frame(Prevalence = prevDf2,
                      TotalAbundance = taxa_sums(ps2),
                      tax_table(ps2))

prevPhylatblThreshold <- plyr::ddply(prevDf2, "Phylum",
                                     function(df1){
                                       cbind(mean(df1$Prevalence),
                                             sum(df1$Prevalence))})
colnames(prevPhylatblThreshold) <- c("Phylum", "Mean", "Sum")
prevPhylatblThreshold
```

```
                Phylum      Mean  Sum
```

```
1        p__Ascomycota 6.277439 4118
2     p__Basidiomycota 4.890411  357
3   p__Chytridiomycota 4.000000   28
4     p__Glomeromycota 4.000000    4
5 p__Mortierellomycota 4.450000  178
6      p__Mucoromycota 3.000000    3
7     p__Rozellomycota 4.000000    4
8                 <NA> 4.510638  212
```

Note: I am assuming that the mean is the mean number of times the phylum was seen in a sample for all samples in which it was seen, while the sum is the total times it was seen across all samples. Ascomycota was seen a total of 1,857 times, with about 6 occurances per sample, while Mucoromycota was seen 3 times total ans the mean is simply 3 because when it was seen, it was all three in one sample.

Number of unique phyla, genera and species, across all samples:

```
library("phyloseq")
uniqueClasses <- c("Phylum", "Genus", "Species")
for(i in unique(uniqueClasses))
  cat(cat(i), length(phyloseq::get_taxa_unique(ps2, taxonomic.rank = i)), "\n")
```

```
Phylum 8
Genus 139
Species 132
```

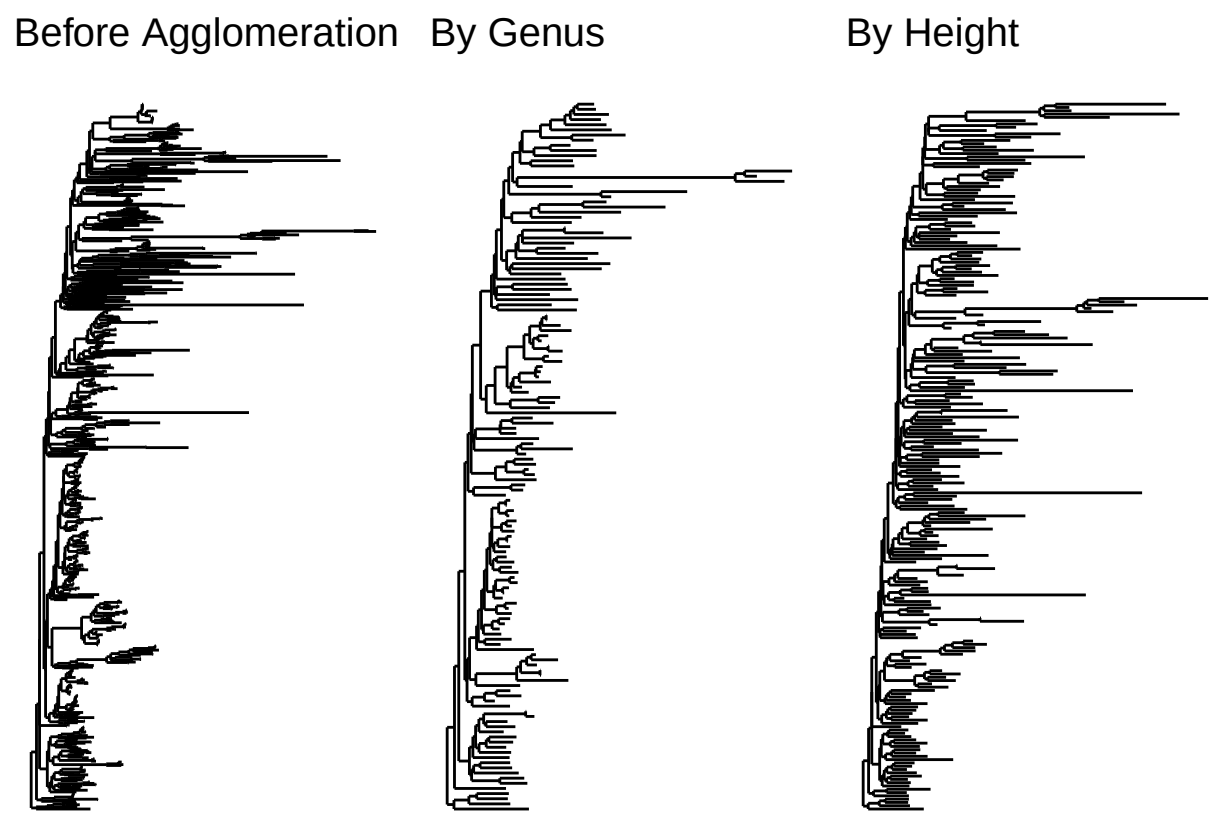
The tax_glom function of phyloseq merges species that have the same taxonomy at certain taxonomic rank, using categorical data. The tip_glom function agglomerates tree tips into a single taxa if they are separated by less than a height specified by `h`.

```
library("phyloseq")
ps3 <- phyloseq::tax_glom(ps2, "Genus", NArm = TRUE)
h1 = 0.4
ps4 <- phyloseq::tip_glom(ps2, h = h1)
```

Below we will look at plots of our trees before agglomeration, with agglomeration using tax_glom, and with agglomeration by tip separation using tip_glom:

```
library("phyloseq")
library("ggplot2")
library("gridExtra")
multiPlotTitleTextSize = 15
p2Tree <- phyloseq::plot_tree(ps2, method = "treeonly",
                              ladderize = "left",
                              title = "Before Agglomeration") +
  ggplot2::theme(plot.title = element_text(size = multiPlotTitleTextSize))
```

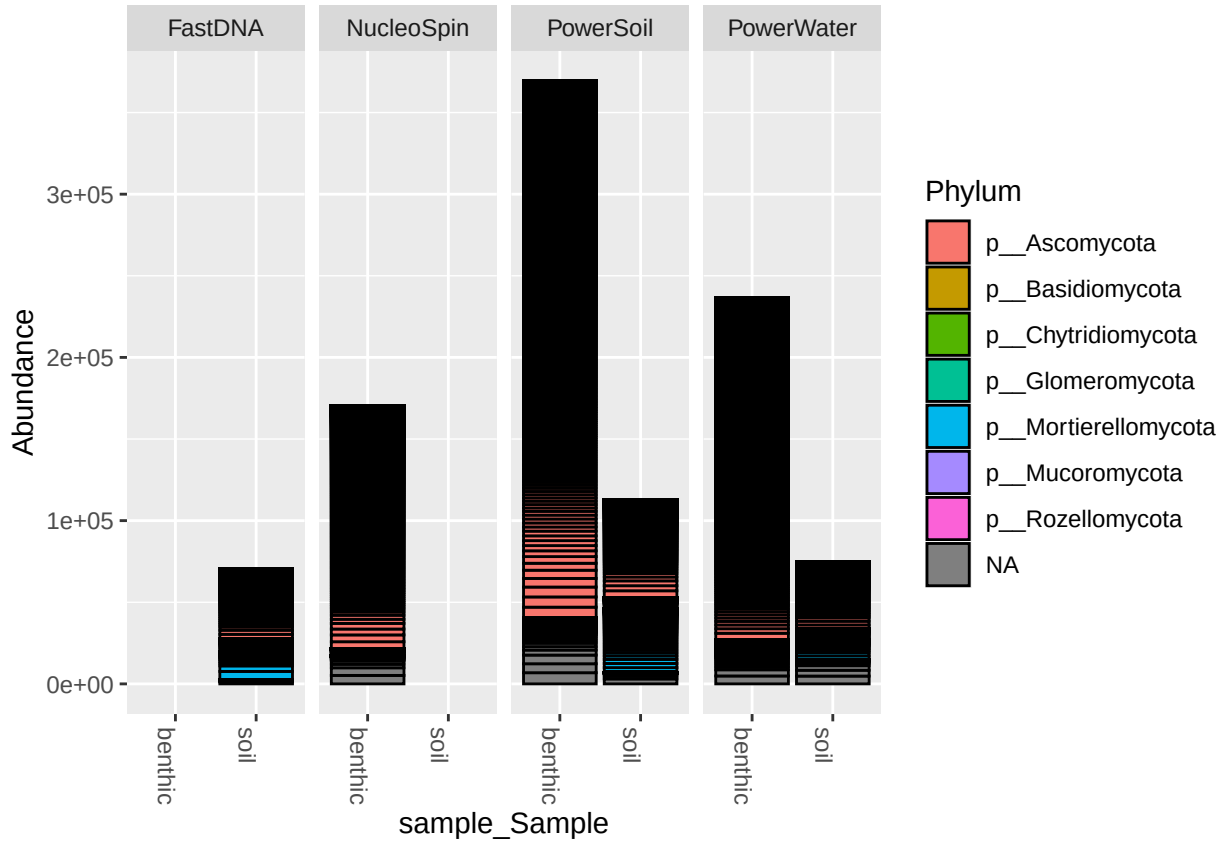
```
p3Tree <- phyloseq::plot_tree(ps3, method = "treeonly",
                              ladderize = "left", title = "By Genus") +
  ggplot2::theme(plot.title = element_text(size = multiPlotTitleTextSize))

p4Tree <- phyloseq::plot_tree(ps4, method = "treeonly",
                              ladderize = "left", title = "By Height") +
  ggplot2::theme(plot.title = element_text(size = multiPlotTitleTextSize))
gridExtra::grid.arrange(nrow = 1, p2Tree, p3Tree, p4Tree)
```



From here on we will continue using the ps2 phyloseq object, that has ahd the 'NA', low-abundance, and prevalence threshold filters applied.

```
library("phyloseq")
phyloseq::plot_bar(ps2,
                   x = "sample_Sample",
                   fill = "Phylum",
                   facet_grid = ~ExtractionKit)
```

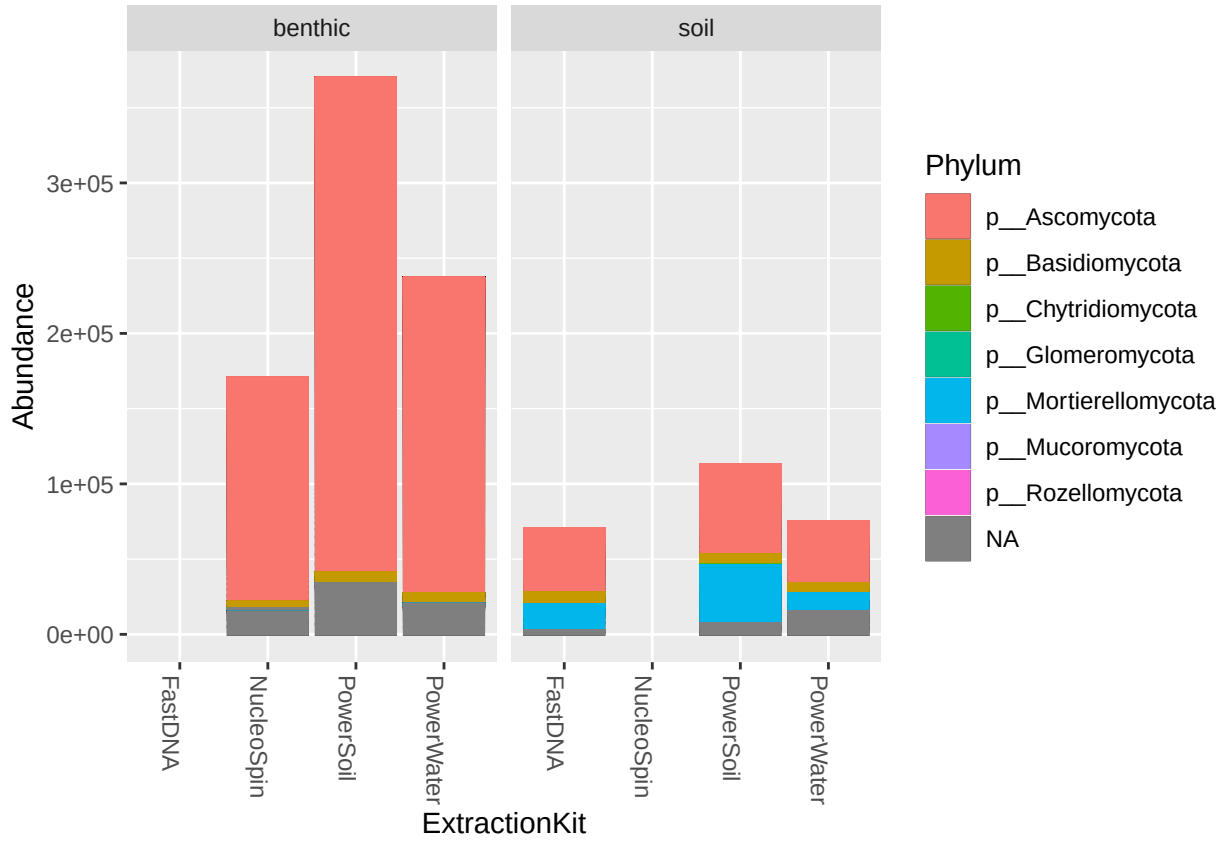
```
Warning in psmelt(physeq): The sample variables:
Sample
 have been renamed to:
sample_Sample
to avoid conflicts with special phyloseq plot attribute names.
```




```
library("phyloseq")
library("ggplot2")
plotPhylum <- phyloseq::plot_bar(ps2, x = "ExtractionKit", fill  = "Phylum",
                                 facet_grid = ~sample_Sample) +
  ylab("Abundance") +
  geom_bar(aes(color = Phylum, fill = Phylum),
           stat = "identity", position = "stack")
```

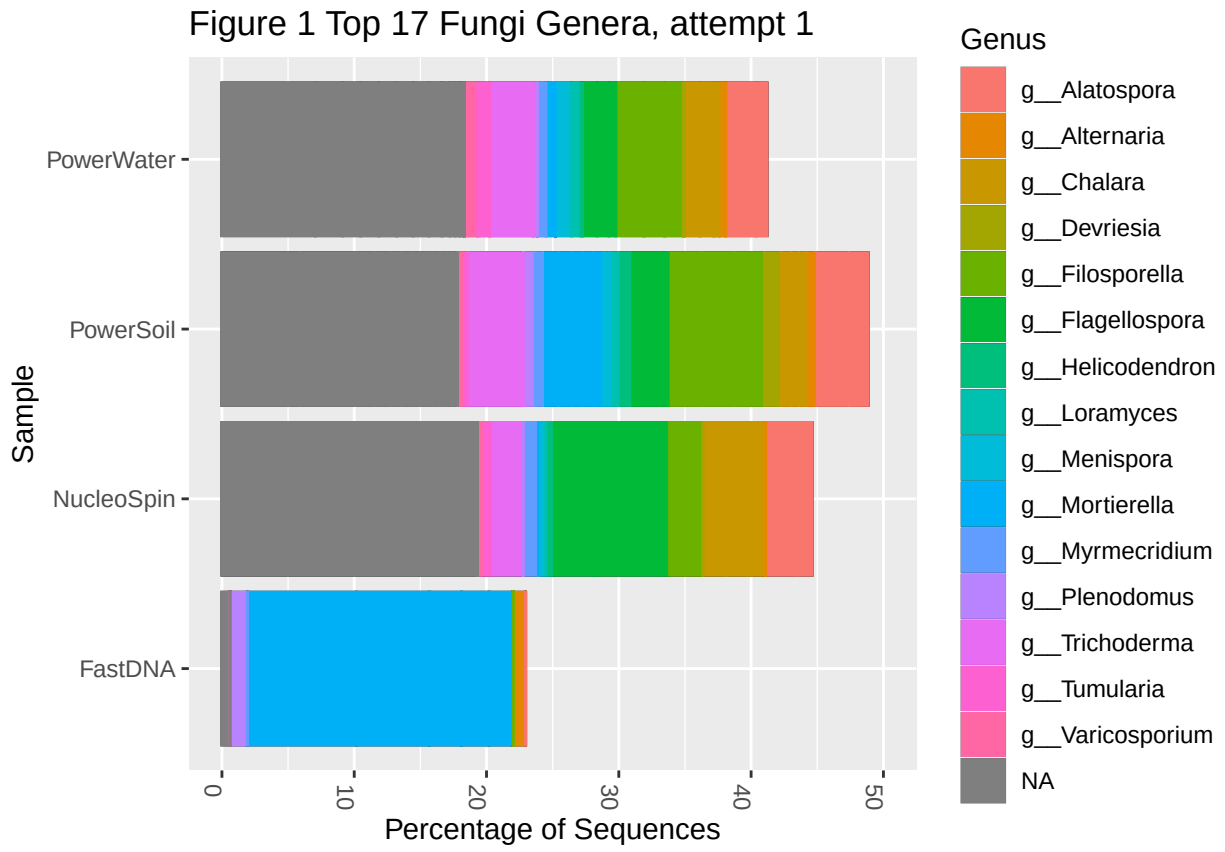
```
Warning in psmelt(physeq): The sample variables:
Sample
 have been renamed to:
sample_Sample
to avoid conflicts with special phyloseq plot attribute names.
```

```
plotPhylum
```




```
library("phyloseq")
library("ggplot2")

topGenus    <- names(sort(phyloseq::taxa_sums(ps2), TRUE)[1:41])
taxTabGenus <- cbind(phyloseq::tax_table(ps2), Genus = NA)
taxTabGenus[topGenus, "Genus"] <- as(tax_table(ps2)[topGenus, "Genus"],
                                     "character")

tax_table(ps2) <- phyloseq::tax_table(taxTabGenus)
ps2m <- merge_samples(ps2, "ExtractionKit")
sample_data(ps2m)$ExtractionKit <- levels(sample_data(ps2)$ExtractionKit)
ps2m <- phyloseq::transform_sample_counts(ps2m, function(x) 100 * x/sum(x))

ps2mTop = prune_taxa(topGenus, ps2m)
title = "Figure 1 Top 17 Fungi Genera, attempt 1"
plotGenus <- plot_bar(ps2mTop,
                      #x = "Sample",
                      fill  = "Genus",
                      title = title) +
  coord_flip() +
  ylab("Percentage of Sequences") + ylim(0, 50) +
  geom_bar(aes(color = Genus, fill = Genus),
```

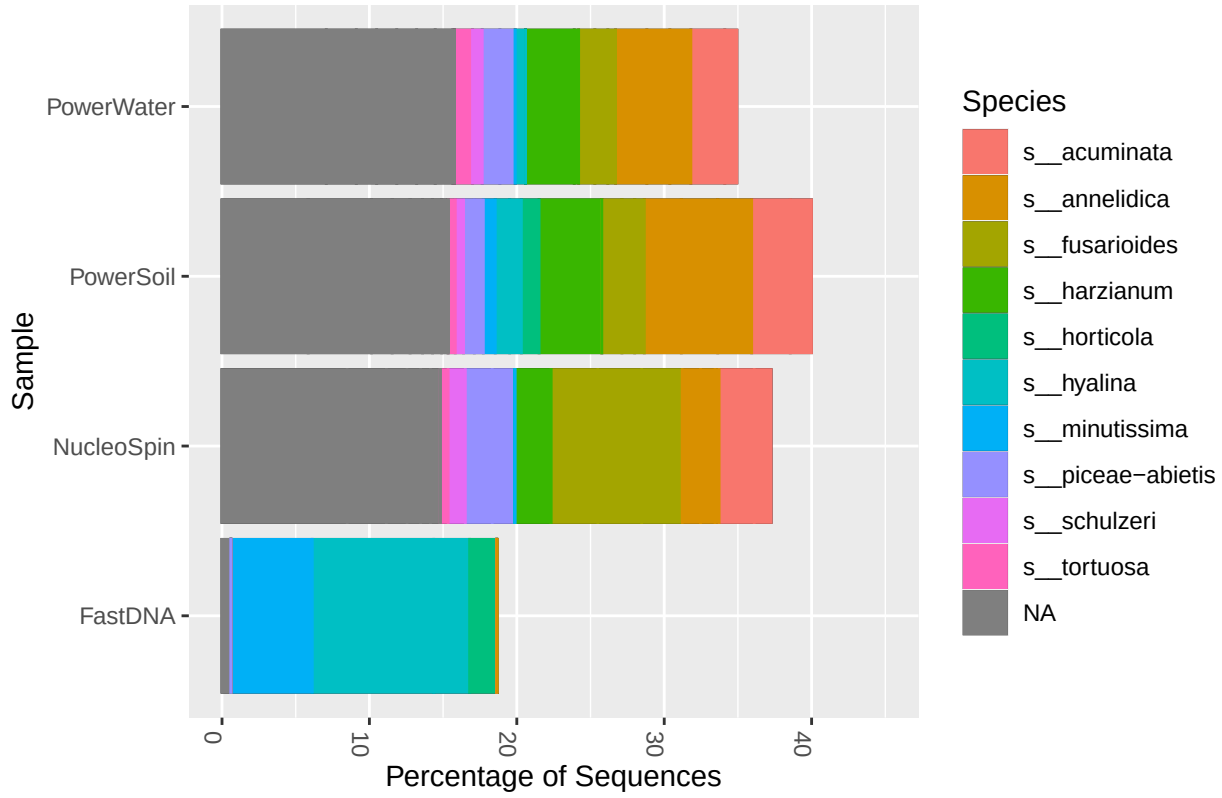
```
            stat = "identity", position = "stack")

plotGenus
```




```
library("phyloseq")
library("ggplot2")

topSpecies <- names(sort(taxa_sums(ps2), TRUE)[1:27])
taxTabSpp  <- cbind(phyloseq::tax_table(ps2), Species = NA)
taxTabSpp[topSpecies, "Species"] <- as(phyloseq::tax_table(ps2)[topSpecies, "Species"],
                                       "character")
tax_table(ps2) <- phyloseq::tax_table(taxTabSpp)
ps2mSpp <- phyloseq::merge_samples(ps2, "ExtractionKit")
sample_data(ps2mSpp)$ExtractionKit <- levels(sample_data(ps2)$ExtractionKit)
ps2mSpp <- phyloseq::transform_sample_counts(ps2mSpp, function(x) 100 * x/sum(x))

ps2mSppTop = prune_taxa(topSpecies, ps2mSpp)
title = "Figure 2 Top 17 Fungal Species, attempt 1"
plotSpecies <- plot_bar(ps2mSppTop,
                        x = "Sample",
                        fill = "Species",
                        title = title) +
```

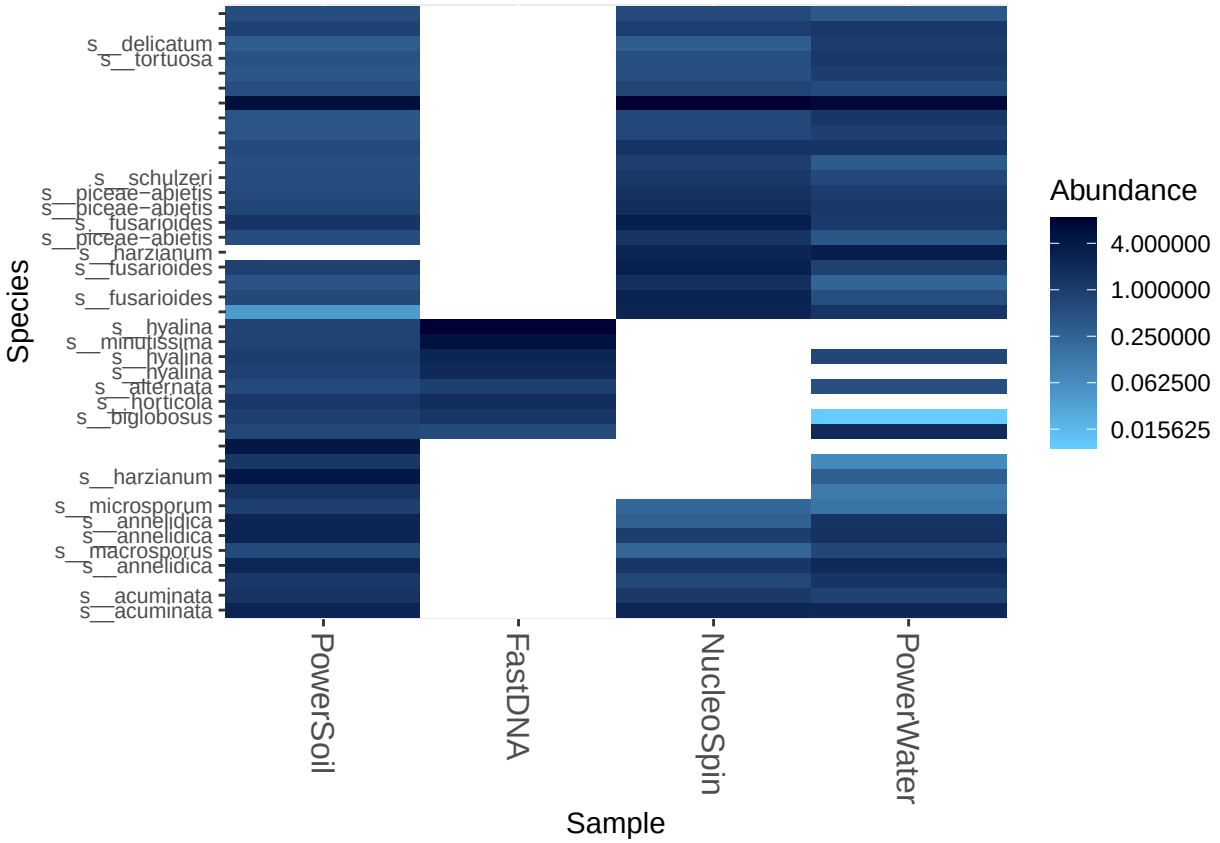
```
  coord_flip() +
  ylab("Percentage of Sequences") + ylim(0, 45) +
  geom_bar(aes(color = Species, fill = Species),
           stat = "identity", position = "stack")
plotSpecies
```

Figure 2 Top 17 Fungal Species, attempt 1

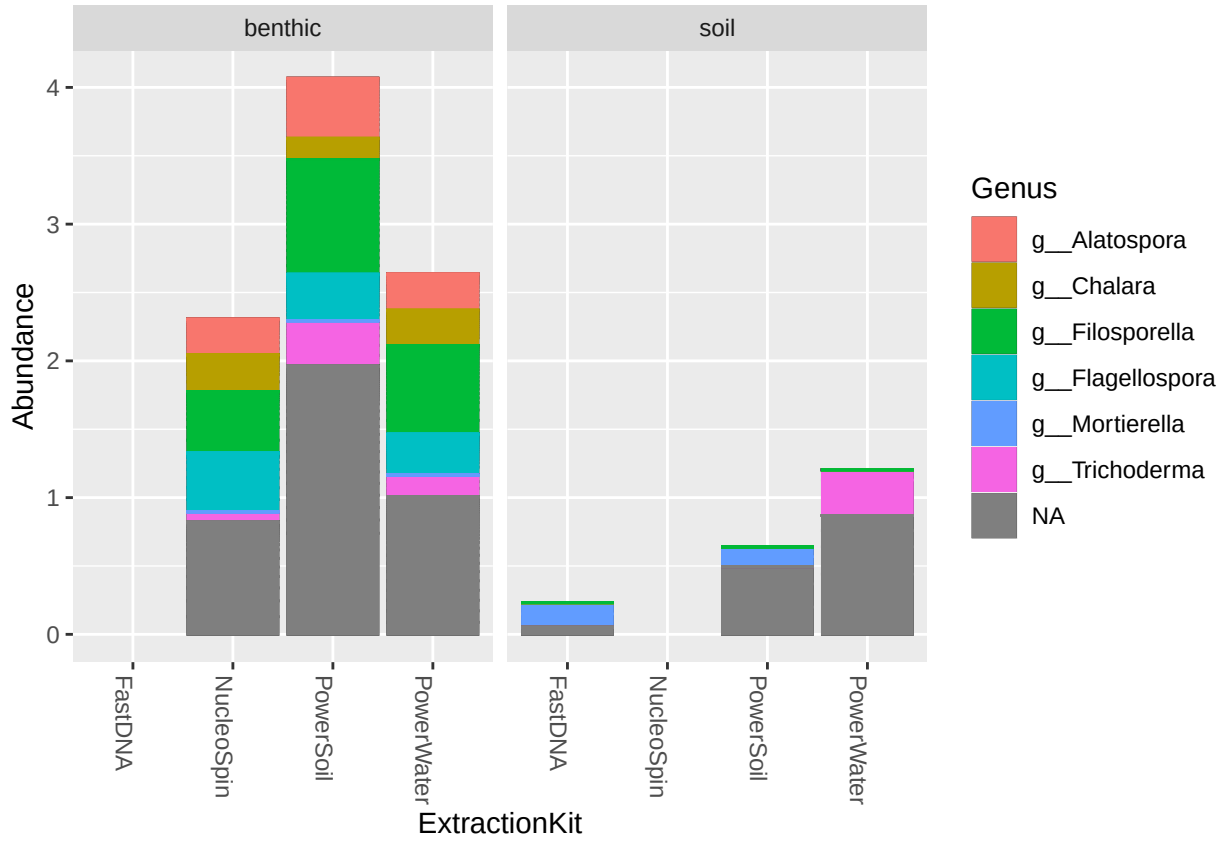


Below I'm testing what a heatmap would look like for taxa abundance across extraction kits:

```
library("phyloseq")
library("ggplot2")
heatPlot <- phyloseq::plot_heatmap(ps2mTop, "PCoA", distance="bray",
                                   sample.label="Sample",
                                   taxa.label="Species",
                                   low="#66CCFF", high="#000033", na.value="white") +
  scale_x_discrete(expand=c(0,0))
heatPlot
```



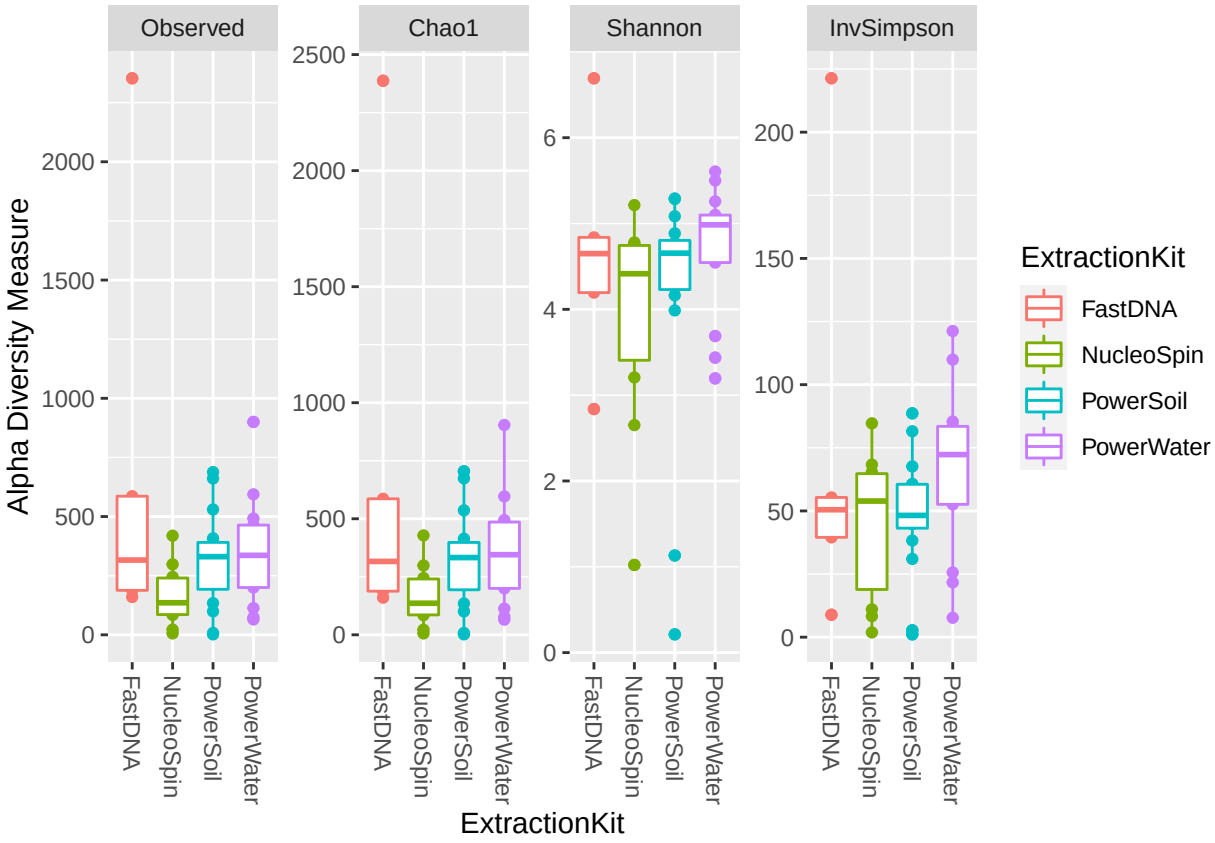

```
library("phyloseq")
library("ggplot2")

top20 <- names(sort(taxa_sums(ps2), decreasing=TRUE))[1:20]
ps.top20 <- phyloseq::transform_sample_counts(ps2, function(OTU) OTU/sum(OTU))
ps.top20 <- phyloseq::prune_taxa(top20, ps.top20)

plotGenus <- phyloseq::plot_bar(ps.top20, x = "ExtractionKit", fill = "Genus",
                                facet_grid = ~sample_Sample)
plotGenus + geom_bar(aes(color = Genus, fill = Genus),
                     stat = "identity",
                     position = "stack")
```



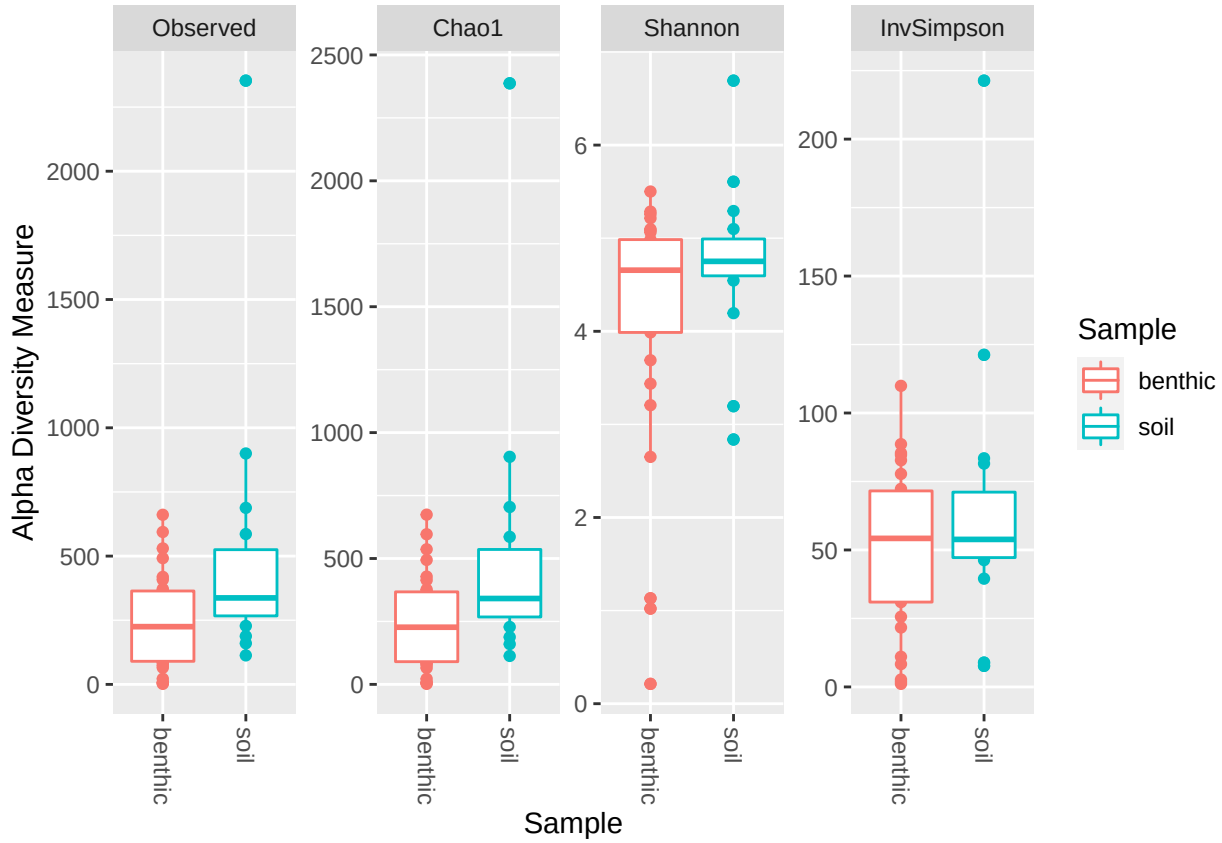
Fo help see:
https://www.gdc-docs.ethz.ch/MDA/handouts/MDA20_PhyloseqFormation_Mahendra_Mariadassou.pdf

Not yet working:
Transform data to proportions as appropriate for Bray-Curtis distances:

```
library("phyloseq")
library("ggplot2")
count_to_prop <- function(x) x/sum(x)
# psProp <- phyloseq::transformSampleCounts(ps2, function(otu) otu/sum(otu))
psProp <- phyloseq::transformSampleCounts(ps2, count_to_prop)
# ordNmdsBray1 <- phyloseq::ordinate(psProp, method="NMDS", distance="bray")
# sample_sums(psProp)[1:5]

p <- plot_richness(ps, color = "ExtractionKit", x = "ExtractionKit",
                   measures = c("Observed", "Chao1", "Shannon", "InvSimpson"))
p <- p + geom_boxplot()
plot(p)
```




```
p <- plot_richness(ps, color = "Sample", x = "Sample",
                   measures = c("Observed", "Chao1", "Shannon", "InvSimpson"))
p <- p + geom_boxplot()
#plot(p)
p
```



Statistical analysis Post hoc comparisons between the four tested methods were made using the Tukey HSD test. OTUs or OTUs pooled at phylum, class, order, family or genera level with different abundances were identified using a generalized linear model where the counts follow an overdispersed Poisson distribution (Kristiansson, Hugenholtz and Dalevi 2009; Jonsson et al.2016). The p-values were corrected for multiple testing using the false discovery rate (FDR) method. The OTU abundance was used for principal component analysis (PCA). Shared OTUs between DNA extraction methods were graphically visualised in Venn diagrams using the corresponding OTU tables exported from QIIME. The hypergeometric distribution was used to test the distribution of gram negatives and gram positives among the taxa identified with the respective four DNA extraction methods. Pearson correlations were used to test for correlations between descriptors of DNA quantity and quality (Table 1), and descriptors of taxonomic diversity (Table 2). The statistical significance for all the analyses was set to $P < 0.05$ or FDR< 0.05. All statistical analyses were carried out using the R v.3.2.0 software (R Core Team 2013).

Table 2. Detected 16S rRNA richness and biodiversity from marine periphyton biofilm DNA extracted with the four studied methods.

	FastDNA	Soil	PowerPlant	PowerBiofilm	PlantDNAzol
n	3	2	3	3	P-values
No. of OTUs	666 ± 42	704 ± 58	809 ± 11	791 ± 7	$P < 0.05$
No. of phyla	17 ± 1	17 ± 1	17 ± 1	18 ± 0	ns
No. of classes	39 ± 1	40 ± 1	40 ± 2	41 ± 1	ns
No. of orders	68 ± 3	70 ± 3	71 ± 2	72 ± 1	ns
No. of families	91 ± 2	95 ± 6	104 ± 3	106 ± 2	$P < 0.05$
No. of genera	141 ± 4	145 ± 12	159 ± 4	162 ± 3	$P < 0.05$

Each value represents the arithmetic mean ± standard error of the mean. n: number of replicates. Statistical significance between extraction methods is denoted as $P < 0.05$ (ANOVA).

ns: indicates no statistically significant differences between extraction methods.