

# Phyloseq tutorial

*Daniel Vault*

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## 1 Introduction

This document explains the use of the phyloseq R library to analyze metabarcoding data.

### 1.1 Phyloseq R library

- Phyloseq web site : <https://joey711.github.io/phyloseq/index.html>
- See in particular tutorials for
  - importing data: <https://joey711.github.io/phyloseq/import-data.html>
  - heat maps: [https://joey711.github.io/phyloseq/plot\\_heatmap-examples.html](https://joey711.github.io/phyloseq/plot_heatmap-examples.html)

### 1.2 Data

This tutorial uses a reduced metabarcoding dataset obtained by C. Ribeiro and A. Lopes dos Santos. This dataset originates from the CARBOM cruise in 2013 off Brazil and corresponds to the 18S V4 region amplified on flow cytometry sorted samples (see pptx file for details) and sequenced on an Illumina run 2\*250 bp analyzed with mothur.

### 1.3 References for data

- G rikas Ribeiro, C., Lopes dos Santos, A., Marie, D., Helena Pellizari, V., Pereira Brandini, F., and Vault, D. (2016). Pico and nanoplankton abundance and carbon stocks along the Brazilian Bight. PeerJ 4, e2587. doi:10.7717/peerj.2587.

- G rikas Ribeiro, C., Marie, D., Lopes dos Santos, A., Pereira Brandini, F., and Vault, D. (2016). Estimating microbial populations by flow cytometry: Comparison between instruments. *Limnol. Oceanogr. Methods* 14, 750–758. doi:10.1002/lom3.10135.
- G rikas Ribeiro C, Lopes dos Santos A, Marie D, Brandini P, Vault D. (2018). Relationships between photosynthetic eukaryotes and nitrogen-fixing cyanobacteria off Brazil. *ISME J* in press.

## 2 Prerequisites to be installed

- R : <https://pbil.univ-lyon1.fr/CRAN/>
- R studio : <https://www.rstudio.com/products/rstudio/download/#download>
- Download and install the following libraries by running under R studio the following lines

```
install.packages("dplyr")      # To manipulate dataframes
install.packages("readxl")    # To read Excel files into R

install.packages("ggplot2")   # for high quality graphics

source("https://bioconductor.org/biocLite.R")
biocLite("phyloseq")
```

## 3 Script description

- Load the script file : **Phyloseq\_tutorial.R**

### 3.1 Load necessary libraries

```
library("phyloseq")
library("ggplot2")      # graphics
library("readxl")       # necessary to import the data from Excel file
library("dplyr")        # filter and reformat data frames
```

```
##
## Attaching package: 'dplyr'

## The following objects are masked from 'package:stats':
##
##   filter, lag

## The following objects are masked from 'package:base':
##
##   intersect, setdiff, setequal, union
```

### 3.2 Read the data and create phyloseq objects

Three tables are needed :

- \* OTU
- \* Taxonomy
- \* Samples

They are read from a single Excel file where each sheet contains one of the tables

```
otu_mat<- read_excel("CARBOM data.xlsx", sheet = "OTU matrix")
tax_mat<- read_excel("CARBOM data.xlsx", sheet = "Taxonomy table")
samples_df <- read_excel("CARBOM data.xlsx", sheet = "Samples")
```

Phyloseq objects need to have row.names

- define the row names from the otu column

```
row.names(otu_mat) <- otu_mat$otu
```

## Warning: Setting row names on a tibble is deprecated.

- remove the column otu since it is now used as a row name

```
otu_mat <- otu_mat %>% select (-otu)
```

- Idem for the two other matrixes

```
row.names(tax_mat) <- tax_mat$otu
```

## Warning: Setting row names on a tibble is deprecated.

```
tax_mat <- tax_mat %>% select (-otu)
```

```
row.names(samples_df) <- samples_df$sample
```

## Warning: Setting row names on a tibble is deprecated.

```
samples_df <- samples_df %>% select (-sample)
```

Transform into matrixes otu and tax tables (sample table can be left as data frame)

```
otu_mat <- as.matrix(otu_mat)
tax_mat <- as.matrix(tax_mat)
```

Transform to phyloseq objects

```
OTU = otu_table(otu_mat, taxa_are_rows = TRUE)
TAX = tax_table(tax_mat)
samples = sample_data(samples_df)
```

```
carbom <- phyloseq(OTU, TAX, samples)
carbom
```

## phyloseq-class experiment-level object

```
## otu_table() OTU Table: [ 287 taxa and 55 samples ]
## sample_data() Sample Data: [ 55 samples by 27 sample variables ]
## tax_table() Taxonomy Table: [ 287 taxa by 7 taxonomic ranks ]
```

Visualize data

```
sample_names(carbom)
```

```
## [1] "X10n" "X10p" "X11n" "X11p" "X120n" "X120p" "X121n"
## [8] "X121p" "X122n" "X122p" "X125n" "X125p" "X126n" "X126p"
## [15] "X127n" "X13n" "X13p" "X140n" "X140p" "X141n" "X141p"
## [22] "X142n" "X142p" "X155n" "X155p" "X156n" "X156p" "X157n"
## [29] "X157p" "X15n" "X15p" "X165n" "X165p" "X166n" "X166p"
## [36] "X167n" "X167p" "X1n" "X1p" "X2n" "X2p" "X3n"
## [43] "X3p" "X5n" "X5p" "X7n" "X7p" "X9n" "X9p"
## [50] "tri01n" "tri01p" "tri02n" "tri02p" "tri03n" "tri03p"
```

```
rank_names(carbom)
```

```
## [1] "Domain"      "Supergroup" "Division"    "Class"       "Order"
## [6] "Family"      "Genus"
```

```
sample_variables(carbom)
```

```
## [1] "fraction"      "Select_18S_nifH" "total_18S"
## [4] "total_16S"     "total_nifH"      "sample_number"
## [7] "transect"      "station"         "depth"
## [10] "latitude"      "longitude"       "picoeuks"
## [13] "nanoeuks"      "bottom_depth"    "level"
## [16] "transect_distance" "date"           "time"
## [19] "phosphates"    "silicates"       "ammonia"
## [22] "nitrates"      "nitrites"        "temperature"
## [25] "fluorescence"  "salinity"        "sample_label"
```

Keep only samples to be analyzed

```
carbom <- subset_samples(carbom, Select_18S_nifH == "Yes")
carbom
```

```
## phyloseq-class experiment-level object
## otu_table() OTU Table: [ 287 taxa and 54 samples ]
## sample_data() Sample Data: [ 54 samples by 27 sample variables ]
## tax_table() Taxonomy Table: [ 287 taxa by 7 taxonomic ranks ]
```

Keep only photosynthetic taxa

```
carbom <- subset_taxa(carbom, Division %in% c("Chlorophyta", "Dinophyta", "Cryptophyta",
                                              "Haptophyta", "Ochromytha", "Cercosoa"))
carbom <- subset_taxa(carbom, !(Class %in% c("Syndiniales", "Sarcomonadea")))
carbom
```

```
## phyloseq-class experiment-level object
## otu_table() OTU Table: [ 205 taxa and 54 samples ]
## sample_data() Sample Data: [ 54 samples by 27 sample variables ]
## tax_table() Taxonomy Table: [ 205 taxa by 7 taxonomic ranks ]
```

Normalize number of reads in each sample using median sequencing depth.

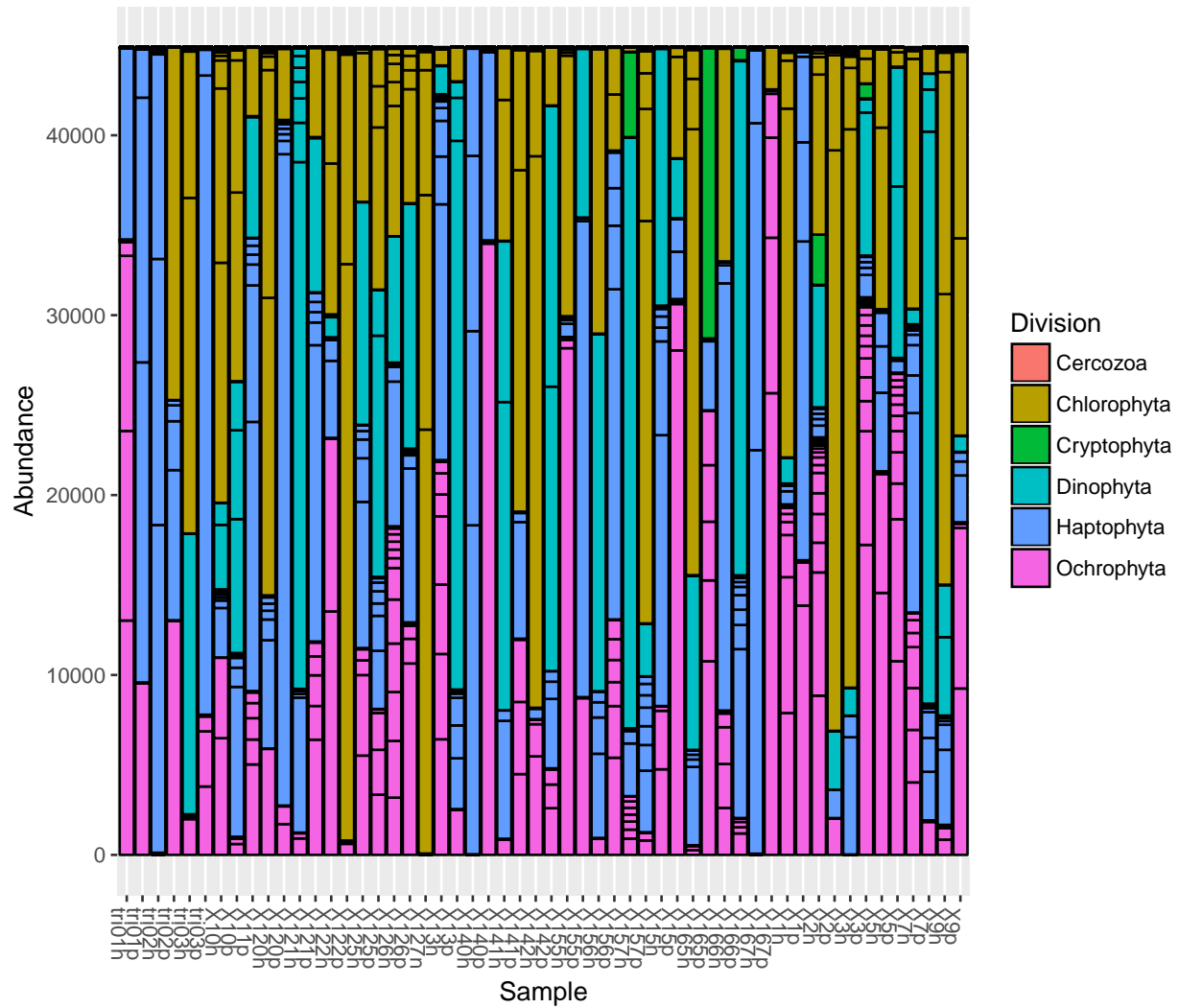
```
total = median(sample_sums(carbom))
standf = function(x, t=total) round(t * (x / sum(x)))
carbom = transform_sample_counts(carbom, standf)
```

The number of reads used for normalization is **44903**.

### 3.3 Bar graphs

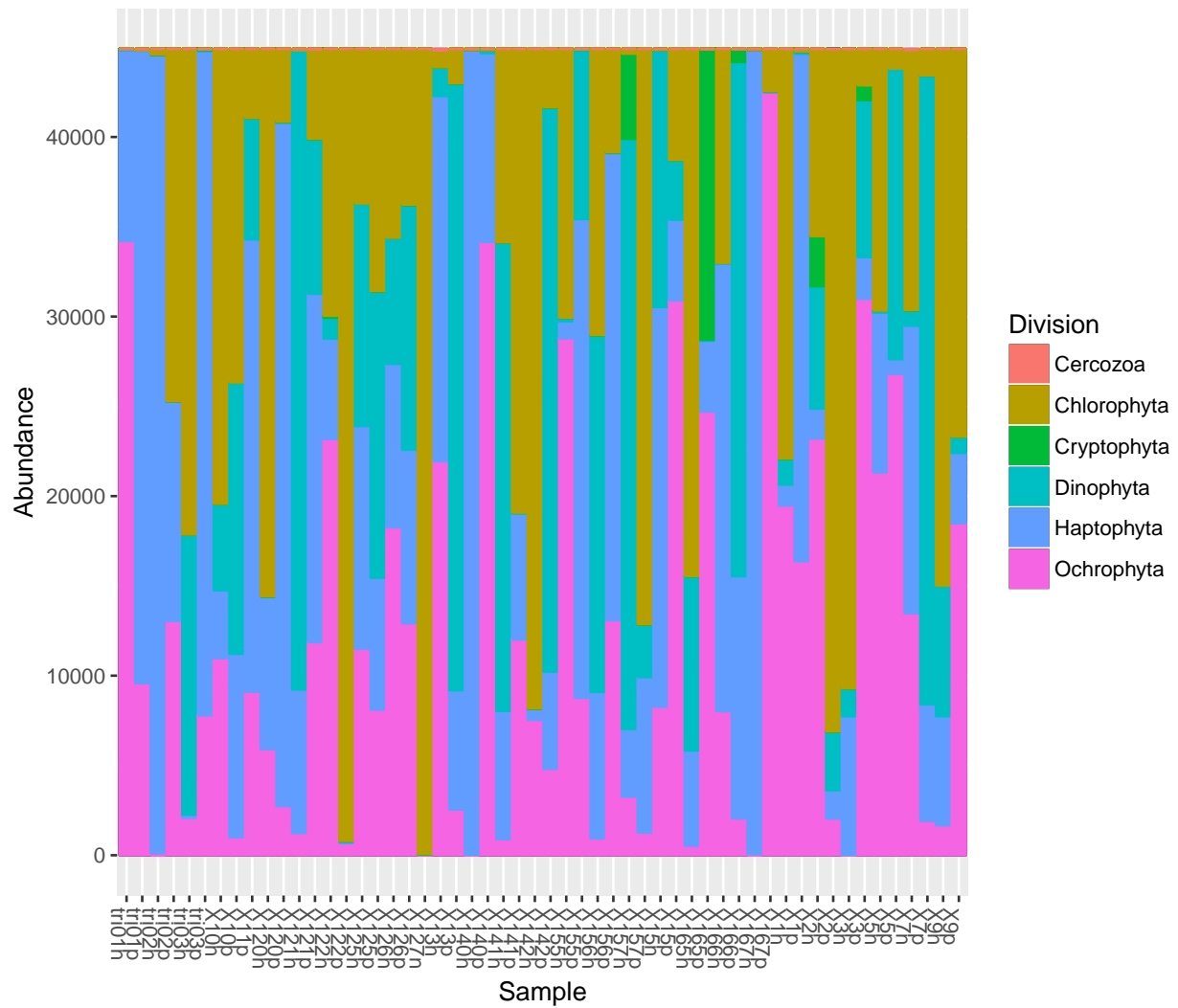
Basic bar graph based on Division

```
plot_bar(carbom, fill = "Division")
```



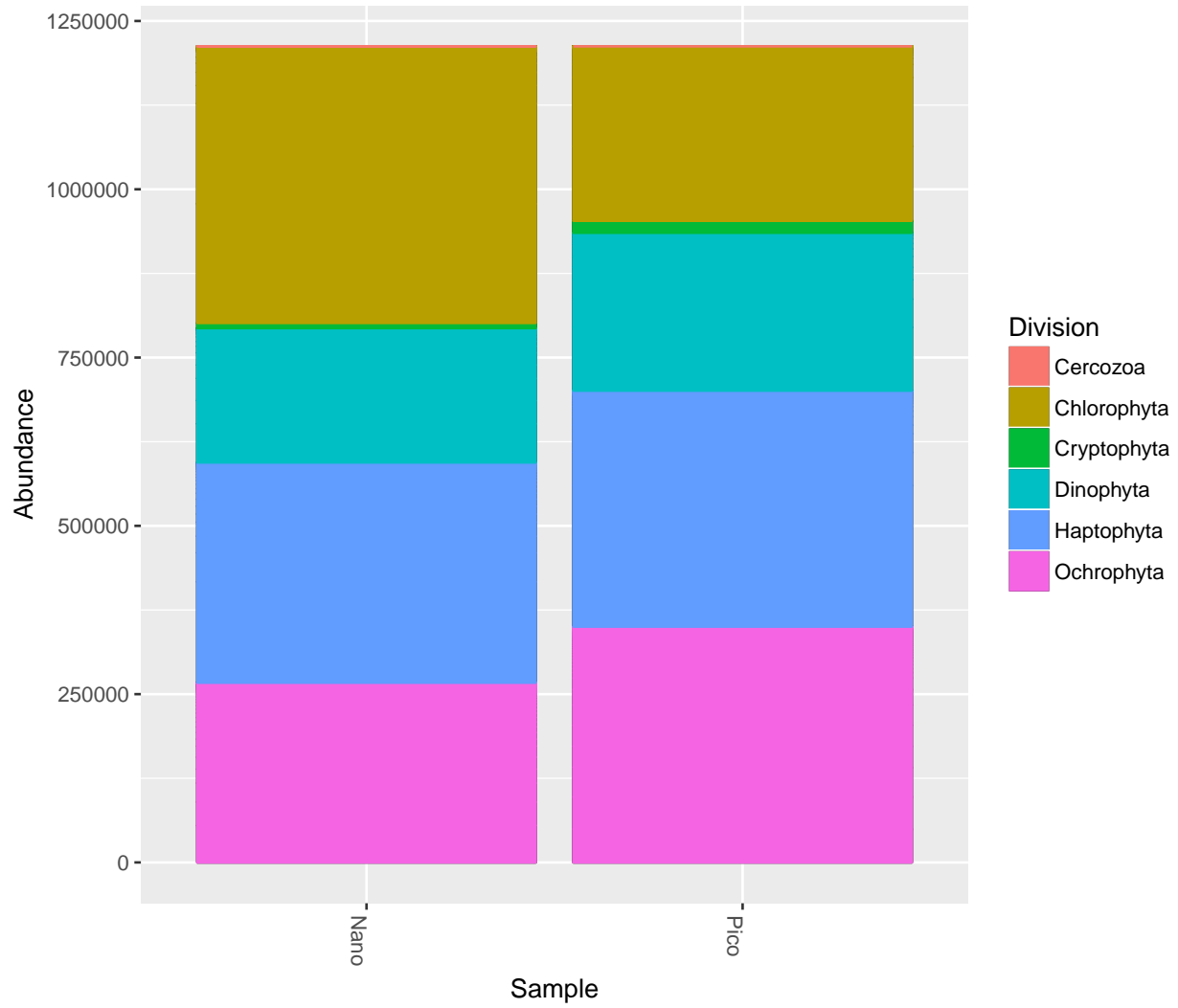
Make the bargraph nicer by removing OTUs boundaries. This is done by adding ggplot2 modifier.

```
plot_bar(carbon, fill = "Division") +  
geom_bar(aes(color=Division, fill=Division), stat="identity", position="stack")
```



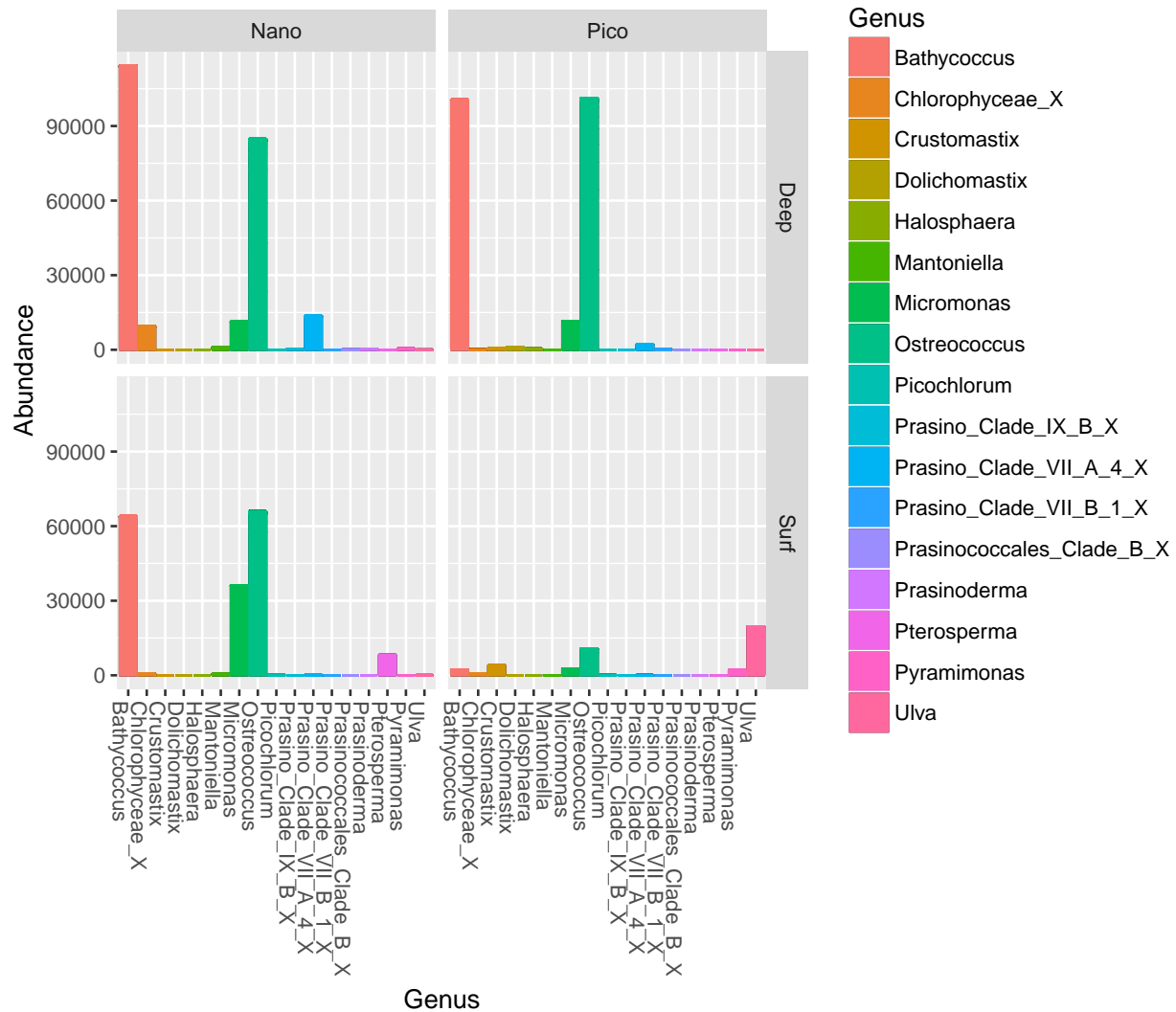
Regroup together Pico vs Nano samples

```
carbom_fraction <- merge_samples(carbom, "fraction")  
plot_bar(carbom_fraction, fill = "Division") +  
geom_bar(aes(color=Division, fill=Division), stat="identity", position="stack")
```



Keep only Chlorophyta and use color according to genus. Do separate panels Pico vs Nano and Surface vs Deep samples.

```
carbom_chloro <- subset_taxa(carbom, Division %in% c("Chlorophyta"))
plot_bar(carbom_chloro, x="Genus", fill = "Genus", facet_grid = level~fraction) +
geom_bar(aes(color=Genus, fill=Genus), stat="identity", position="stack")
```



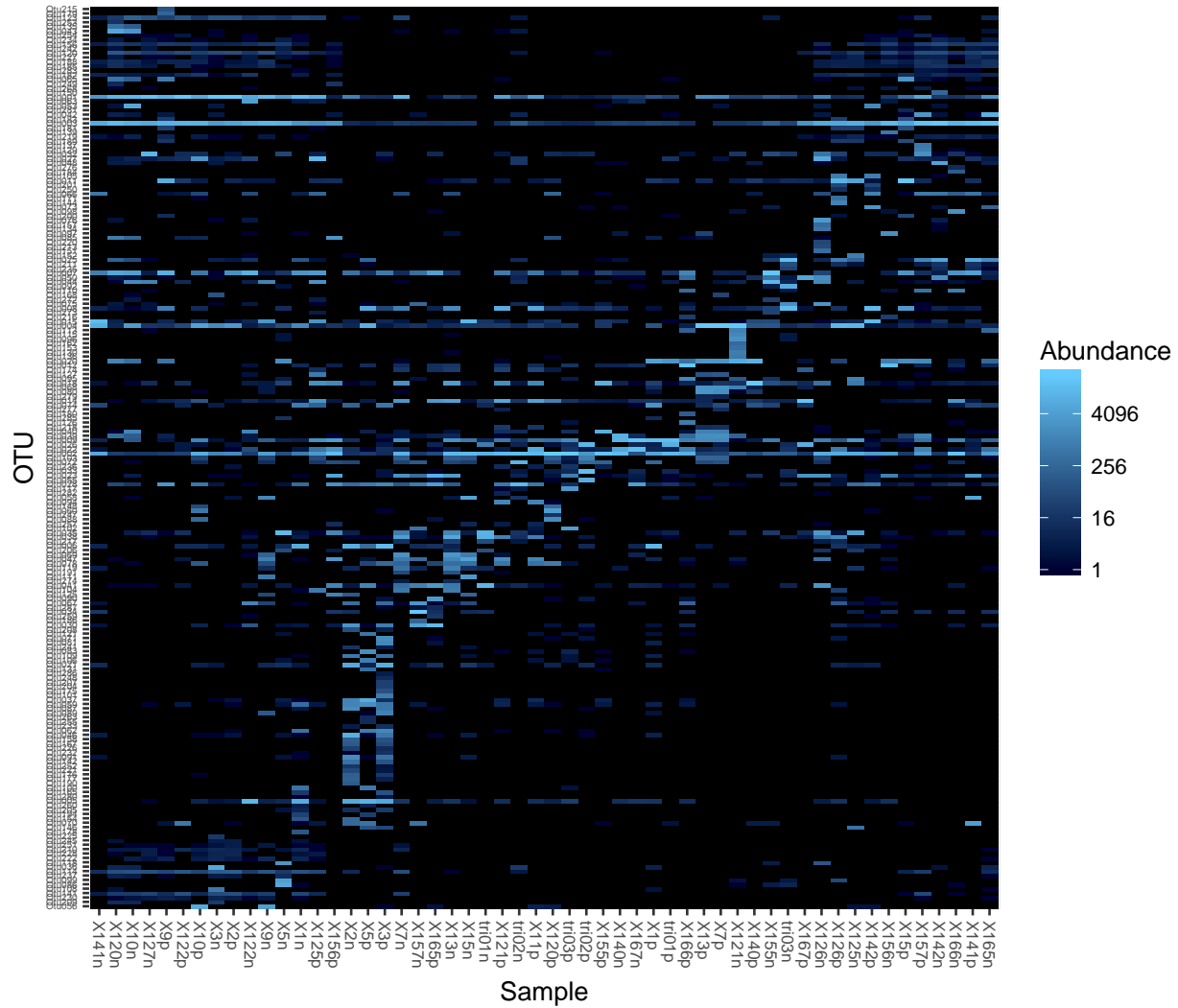


### 3.4 Heatmaps

A basic heatmap using the default parameters.

```
plot_heatmap(carbom, method = "NMDS", distance = "bray")
```

```
## Warning: Transformation introduced infinite values in discrete y-axis
```



It is very very cluttered. It is better to only consider the most abundant OTUs for heatmaps. For example one can only take OTUs that represent at least 20% of reads in at least one sample. Remember we normalized all the samples to median number of reads (total). We are left with only 33 OTUS which makes the reading much more easy.

```
carbom_abund <- filter_taxa(carbom, function(x) sum(x > total*0.20) > 0, TRUE)
carbom_abund
```

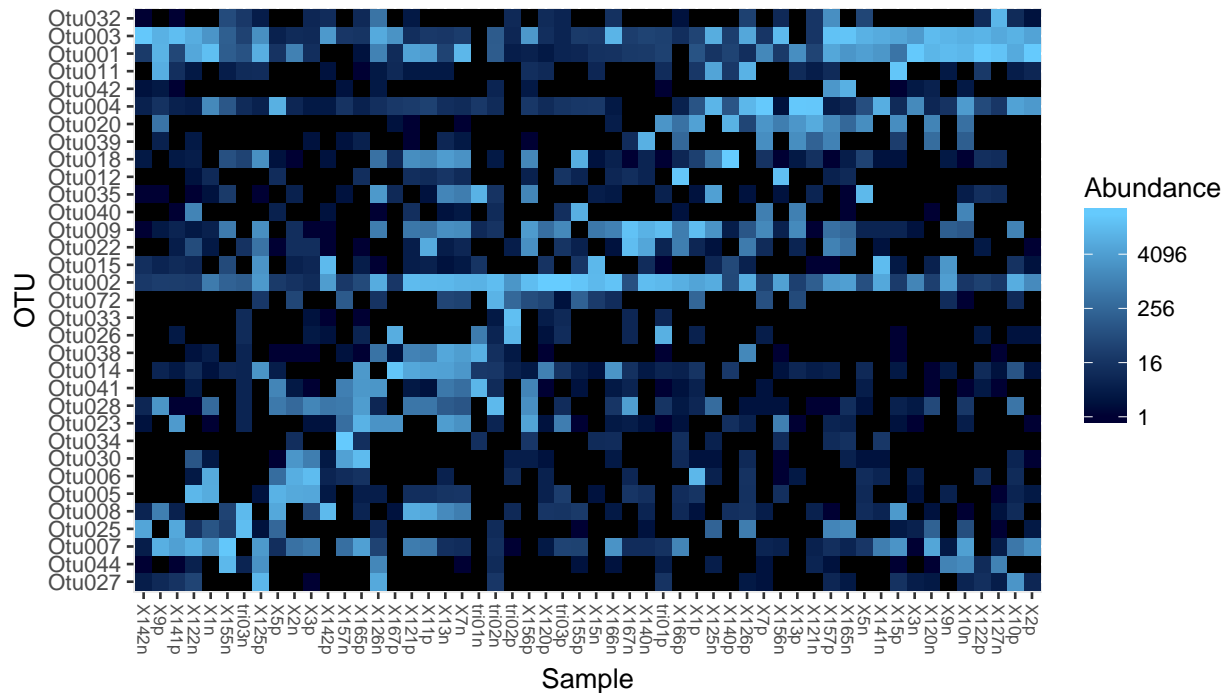
```
## phyloseq-class experiment-level object
## otu_table() OTU Table:      [ 33 taxa and 54 samples ]
## sample_data() Sample Data:  [ 54 samples by 27 sample variables ]
## tax_table() Taxonomy Table: [ 33 taxa by 7 taxonomic ranks ]
```

```
otu_table(carbom_abund)[1:8, 1:5]
```

```
## OTU Table:      [8 taxa and 5 samples]
##               taxa are rows
##      X10n X10p X11p X120n X120p
## Otu001 13339 7346 3804 12662 3
## Otu002 18 8329 14958 30 36206
## Otu003 9692 10488 20 16537 11
## Otu004 3584 4943 33 7 9
## Otu005 0 6 11 0 5
## Otu006 0 9 0 0 5
## Otu007 4473 605 587 5894 3
## Otu008 1 9 6707 2 17
```

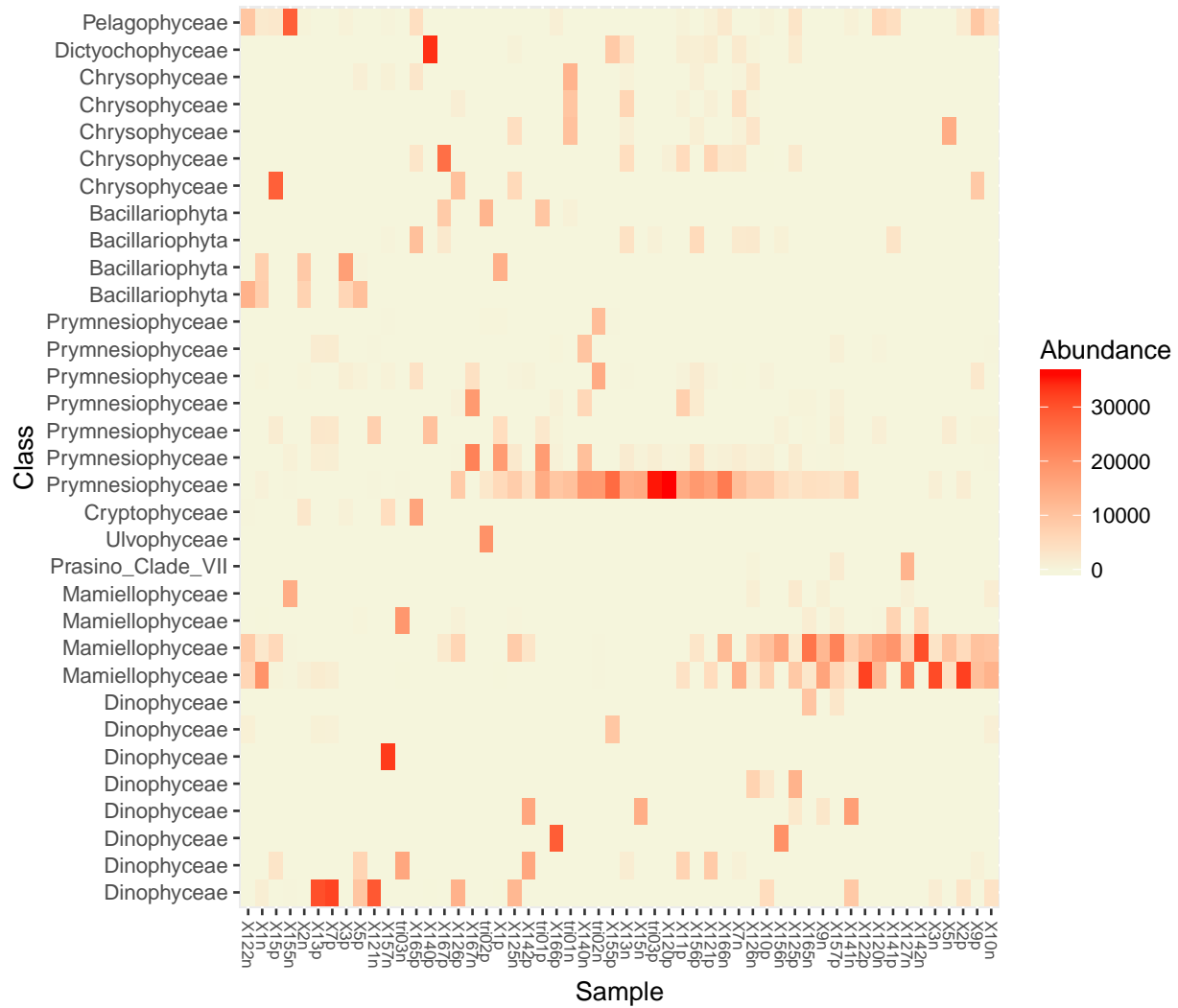
```
plot_heatmap(carbom_abund, method = "NMDS", distance = "bray")
```

```
## Warning: Transformation introduced infinite values in discrete y-axis
```



It is possible to use different distances and different multivariate methods. For example Jaccard distance and MDS and label OTUs with Class, order by Class. We can also change the Palette (the default palette is a bit ugly...).

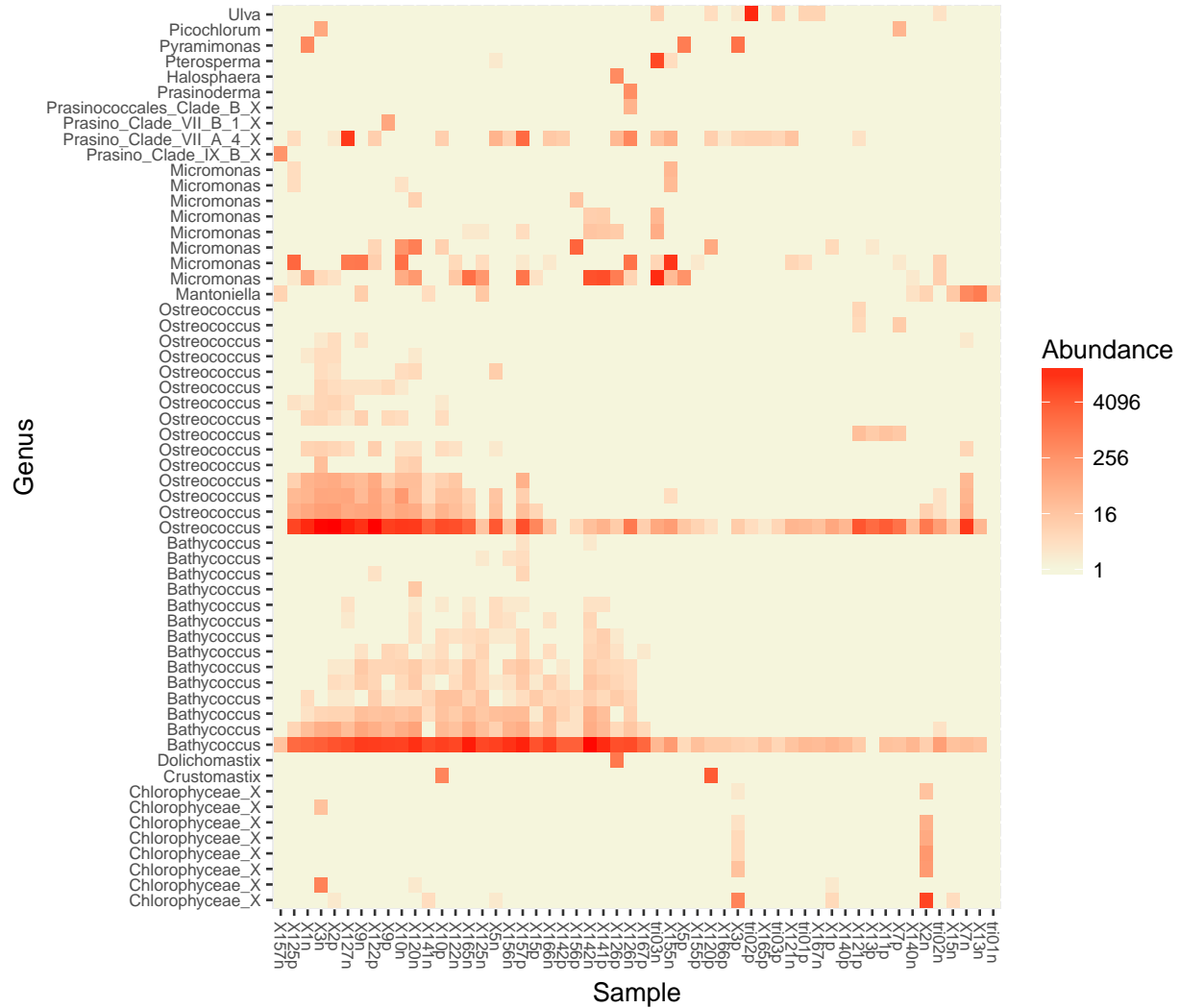
```
plot_heatmap(carbon_abund, method = "MDS", distance = "jaccard",
             taxa.label = "Class", taxa.order = "Class",
             trans=NULL, low="beige", high="red", na.value="beige")
```



Another strategy is to do a heatmap for a specific taxonomy group. For example we can target the Chlorophyta and then label the OTUs using the Genus.

```
plot_heatmap(carbon_chloro, method = "NMDS", distance = "bray",
             taxa.label = "Genus", taxa.order = "Genus",
             low="beige", high="red", na.value="beige")
```

## Warning: Transformation introduced infinite values in discrete y-axis

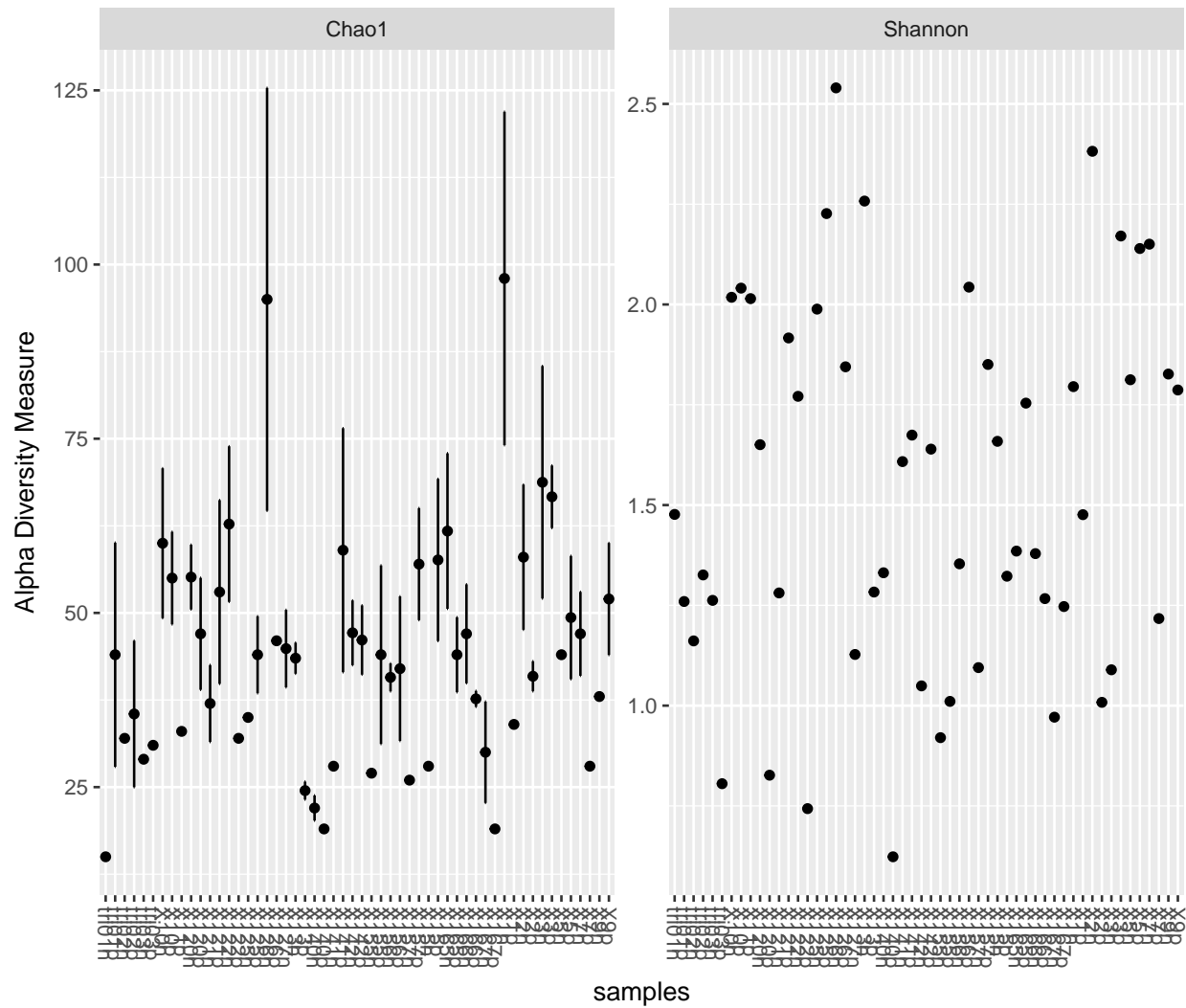


### 3.5 Alpha diversity

Plot Chao1 richness estimator and Shannon diversity estimator.

```
plot_richness(carbom, measures=c("Chao1", "Shannon"))
```

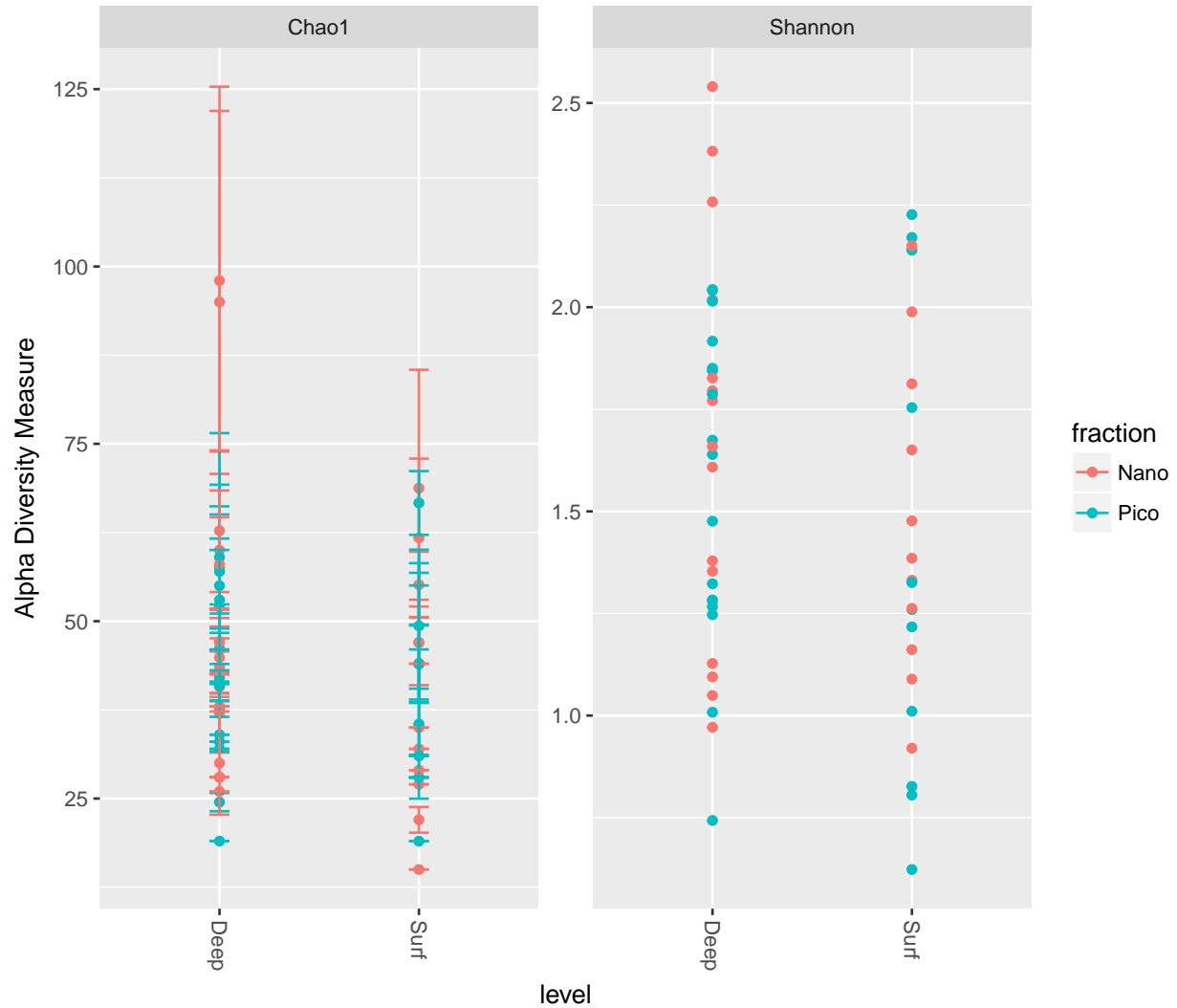
```
## Warning: Removed 54 rows containing missing values (geom_errorbar).
```



Regroup together samples from the same fraction.

```
plot_richness(carbom, measures=c("Chao1", "Shannon"), x="level", color="fraction")
```

## Warning: Removed 54 rows containing missing values (geom\_errorbar).



### 3.6 Ordination

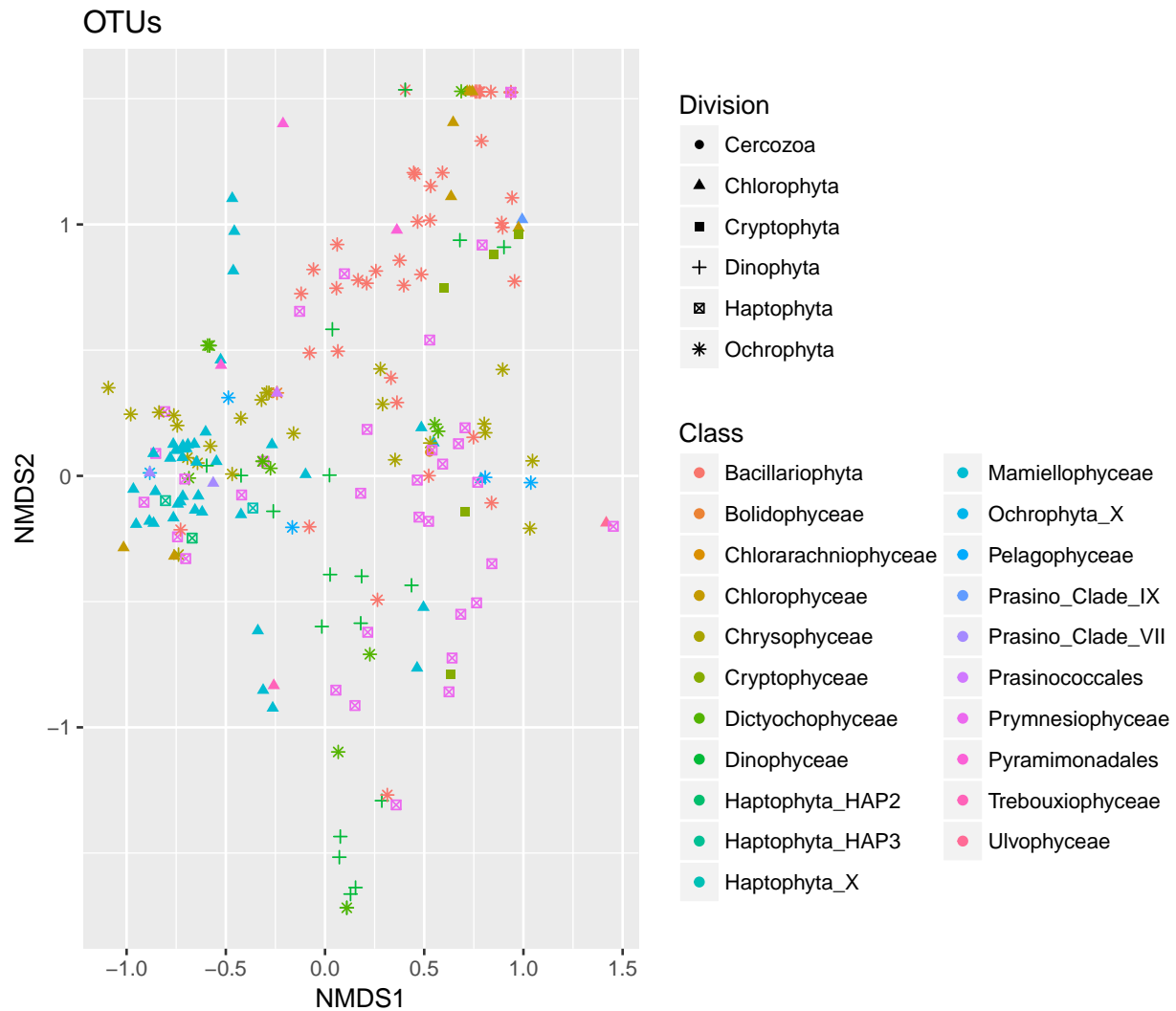
Do multivariate analysis based on Bray-Curtis distance and NMDS ordination.

```
carbom.ord <- ordinate(carbom, "NMDS", "bray")

## Square root transformation
## Wisconsin double standardization
## Run 0 stress 0.2317058
## Run 1 stress 0.2330077
## Run 2 stress 0.2392148
## Run 3 stress 0.2568888
## Run 4 stress 0.2470442
## Run 5 stress 0.2319226
## ... Procrustes: rmse 0.1108061  max resid 0.3714477
## Run 6 stress 0.2521164
## Run 7 stress 0.2294641
## ... New best solution
## ... Procrustes: rmse 0.09893187  max resid 0.3903205
## Run 8 stress 0.241384
## Run 9 stress 0.2419695
## Run 10 stress 0.2411329
## Run 11 stress 0.2496274
## Run 12 stress 0.2545684
## Run 13 stress 0.2487767
## Run 14 stress 0.2484128
## Run 15 stress 0.2457867
## Run 16 stress 0.2337687
## Run 17 stress 0.2506322
## Run 18 stress 0.2472858
## Run 19 stress 0.2439074
## Run 20 stress 0.2467144
## *** No convergence -- monoMDS stopping criteria:
##      20: stress ratio > sratmax
```

Plot OTUs

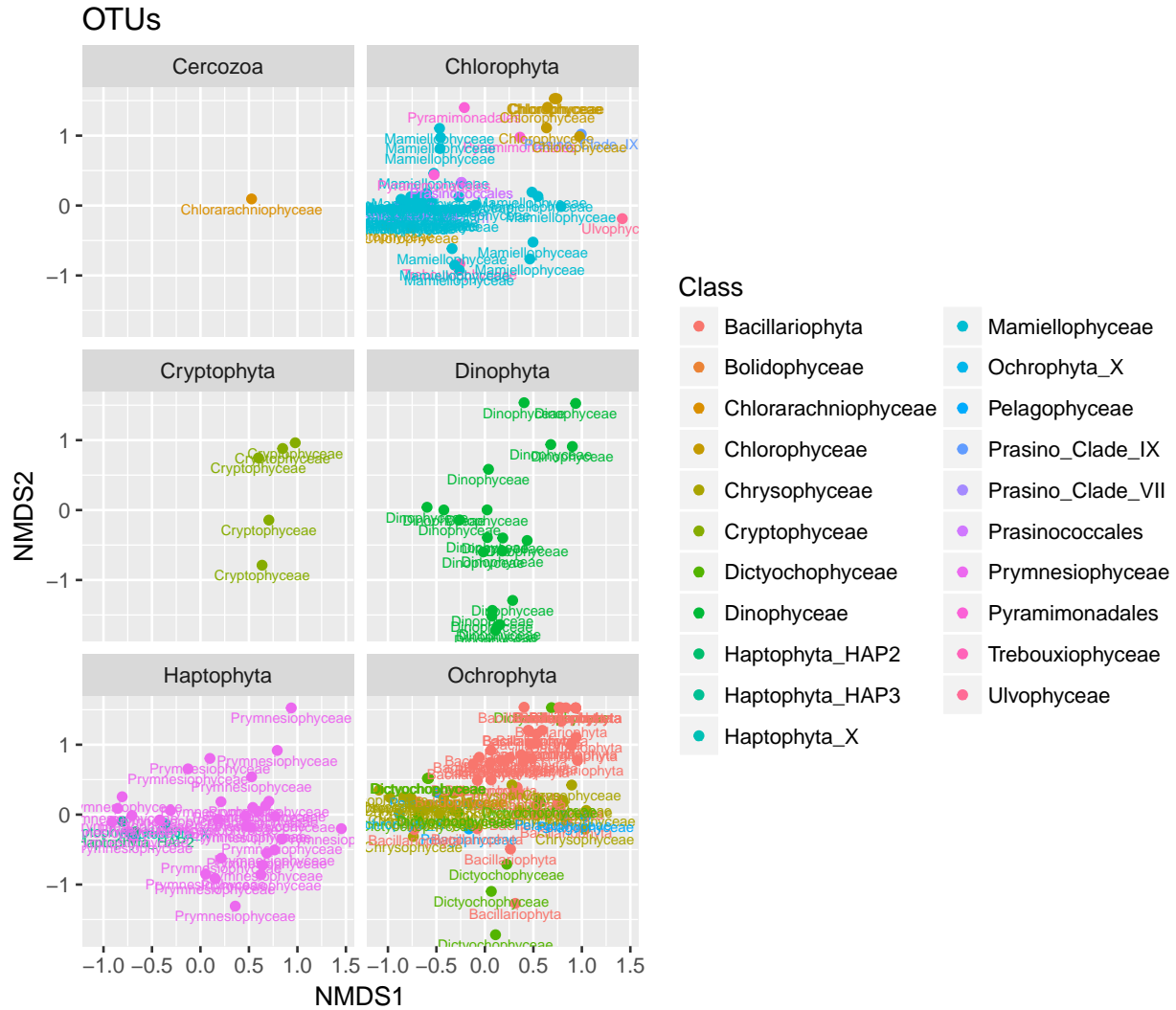
```
plot_ordination(carbom, carbom.ord, type="taxa", color="Class", shape="Division",
               title="OTUs")
```





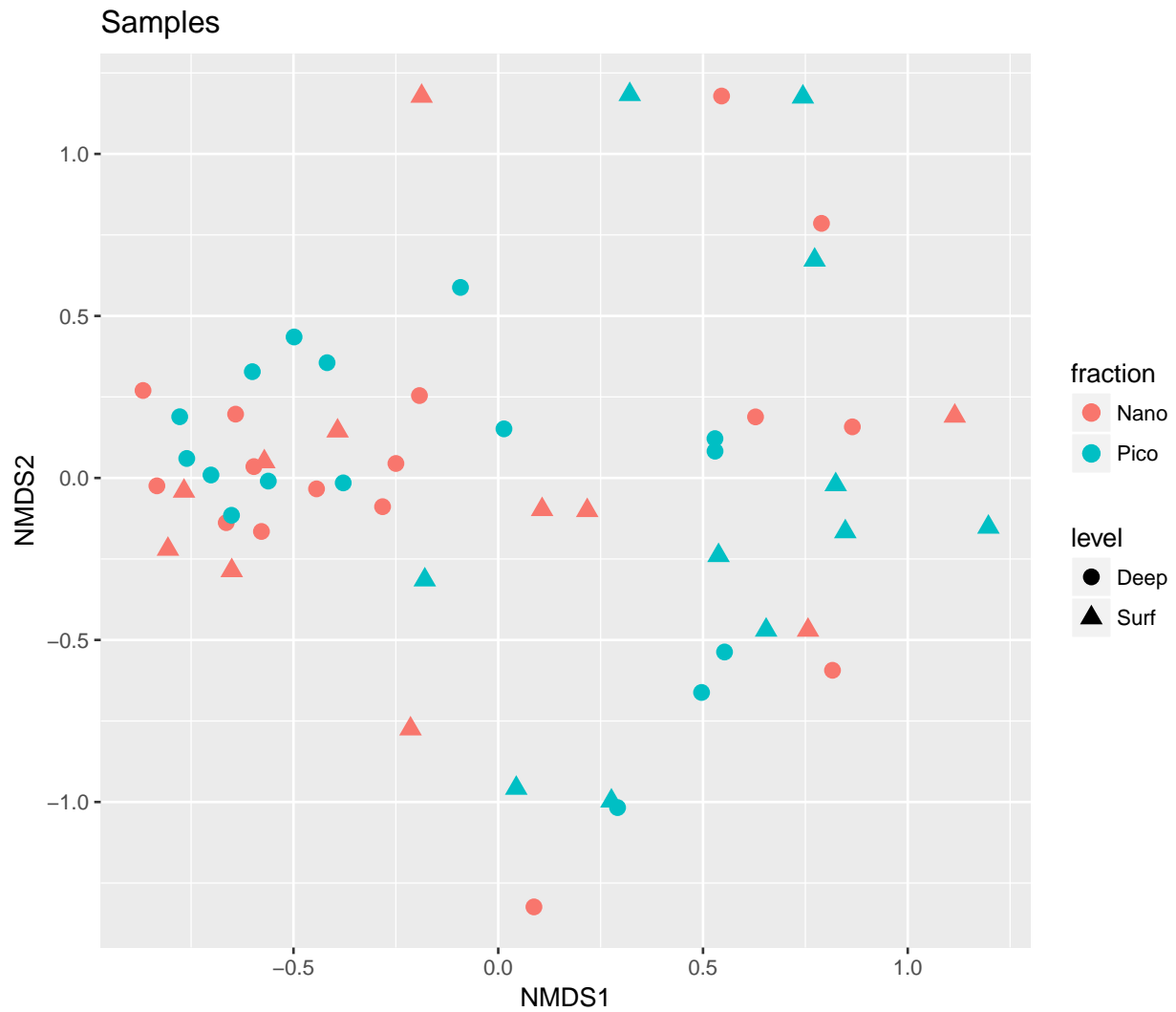
A bit confusing, so make it more easy to visualize by breaking according to taxonomic division.

```
plot_ordination(carbom, carbom.ord, type="taxa", color="Class",
               title="OTUs", label="Class") +
facet_wrap(~Division, 3)
```



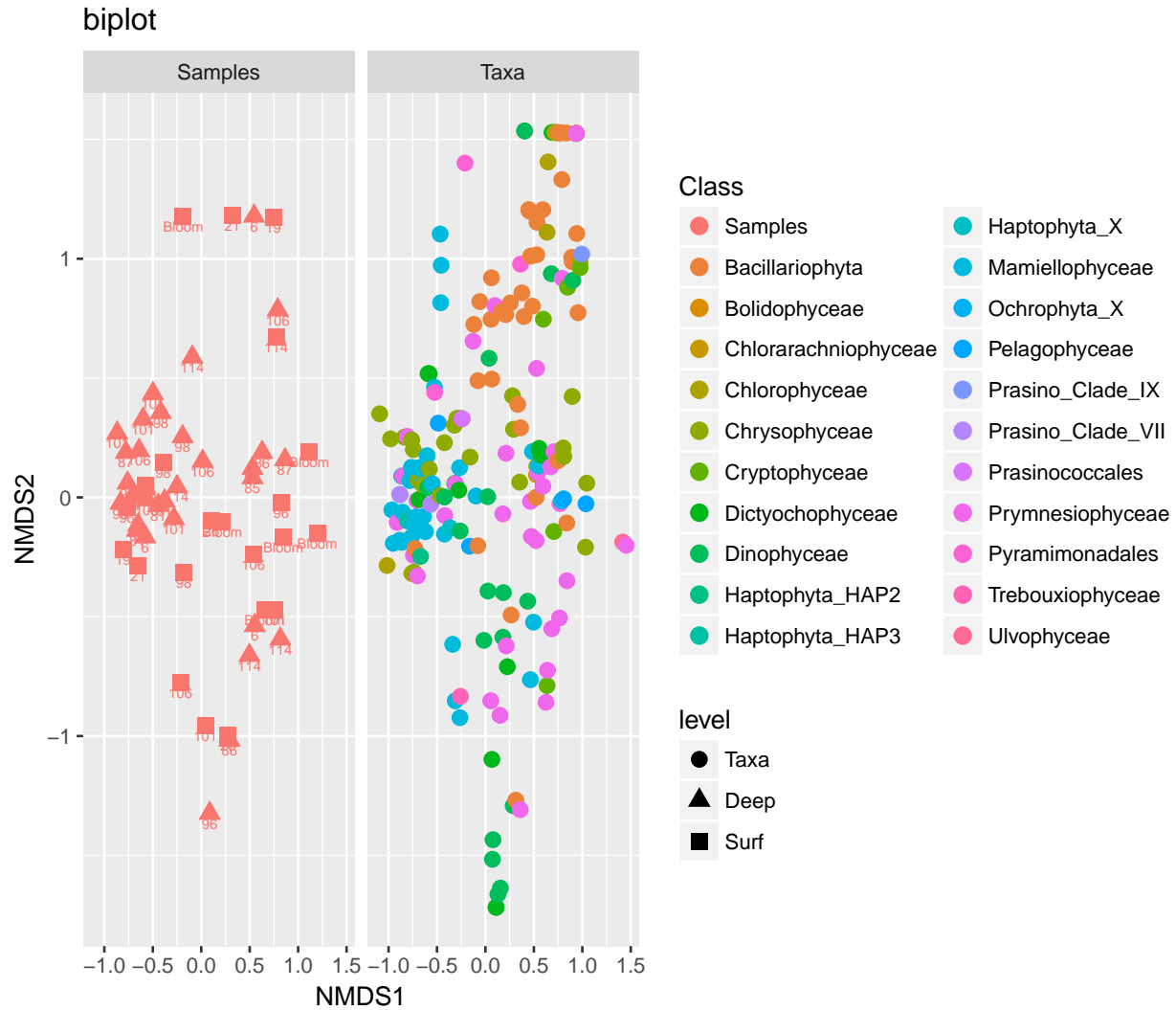
Now display **samples** and enlarge the points to make it more easy to read.

```
plot_ordination(carbom, carbom.ord, type="samples", color="fraction",  
               shape="level", title="Samples") + geom_point(size=3)
```



Display both samples and OTUs but in 2 different panels.

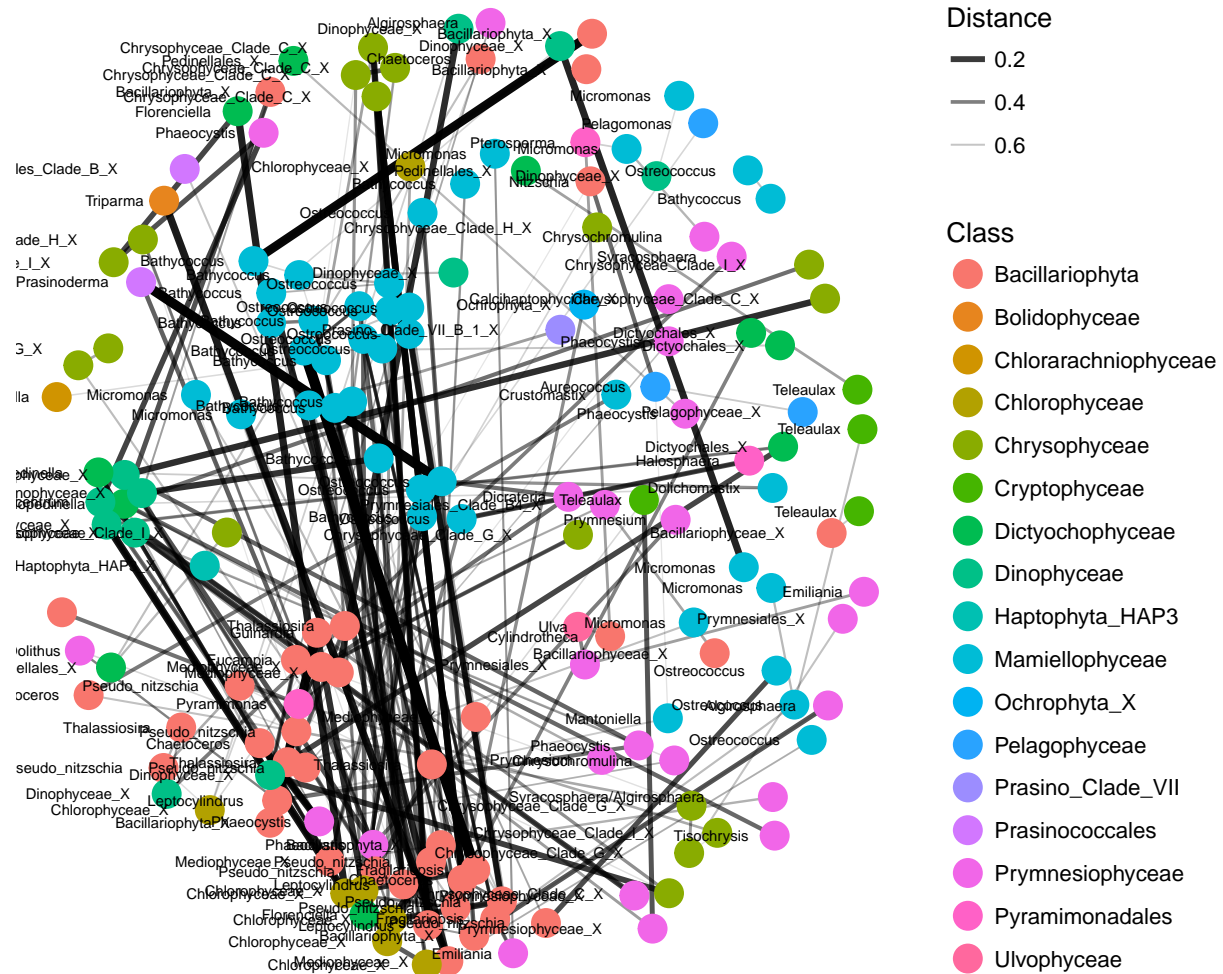
```
plot_ordination(carbom, carbom.ord, type="split", color="Class",
                 shape="level", title="biplot", label = "station") +
geom_point(size=3)
```



## 3.7 Network analysis

Simple network analysis

```
plot_net(carbom, distance = "bray", type = "taxa",
         maxdist = 0.7, color="Class", point_label="Genus")
```



This is quite confusing. Let us make it more simple by using only major OTUs

```
plot_net(carbon_abund, distance = "bray", type = "taxa",
         maxdist = 0.8, color="Class", point_label="Genus")
```

