Cryopreservation of a soil microbiome using a Stirling Cycle approach – a genomic (16s data) assessment

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Contents

0.1 Cyropreserve CABI 1

0.1 Cyropreserve CABI

Soil microbiomes are responsive to seasonal and long-term environmental factors, impacting their composition and function. This manuscript explores cryopreservation techniques using a controlled rate cooler and assesses the genomic integrity and bacterial growth of an exemplar soil sample before and after cryopreservation. The study demonstrates that the controlled rate cooler effectively preserves the DNA content of the microbiome. Two cryopreservation methods were compared with control samples, and the results indicate successful cryopreservation using metabarcoding. Enrichment with liquid medium showed similar responses between cryopreserved and non-cryopreserved soil samples, supporting the efficacy of cryopreservation. This study represents the first report of cryopreservation of soil using a Stirling cycle cooling approach, highlighting its potential for future microbiome research.

0.1.1 Load the required packages

```r
# install.packages(c("ggplot2", "ggpubr", "dplyr",
#                    "rstatix", "purrr", "reshape2",
#                    "UpSetR","plyr", "dplyr", "RColorBrewer"))
library("ggplot2")
library("ggpubr")
library("dplyr")
library("rstatix")
library("purrr")
library("reshape2")
library("UpSetR")
library("plyr")
library("dplyr")
library("RColorBrewer")

# if (!require("BiocManager", quietly = TRUE))
#     install.packages("BiocManager")
# BiocManager::install(c("phyloseq", "DESeq2", "microbiome"))
library("phyloseq")
```

```r
library("DESeq2")
library("microbiome")

# if(!requireNamespace("devtools", quietly = TRUE)){install.packages("devtools")}
# devtools::install_github("jbisanz/qiime2R") # current version is 0.99.20
library("qiime2R")

# devtools::install_github("pmartinezarbizu/pairwiseAdonis/pairwiseAdonis")
library("pairwiseAdonis")

# if (!require(devtools)) install.packages("devtools")
# devtools::install_github("yanlinlin82/ggvenn")
library("ggvenn")
```

0.1.2 Qiime2 to Phyloseq

To work with QIIME2 outcomes in the R environment, it is beneficial to convert the data into the phyloseq object structure. This process involves importing and transforming the feature table and sample metadata, allowing for comprehensive analysis and visualisation of microbial community profiles. The phyloseq package in R provides functions to organize and manipulate the data within the phyloseq object, enabling various analyses such as diversity assessments, differential abundance testing, and taxonomic profile visualization. By converting QIIME2 outcomes to phyloseq, researchers can leverage the capabilities of R for advanced statistical analysis, integration with other omics data, and gaining deeper insights into the microbiome datasets.

```r
# Convert qiime2 to phyloseq format
physeq <- qza_to_phyloseq(
  features = "qiime2/430_327_213_table-with-phyla-no-mitochondria-no-chloroplast.qza", # table.qza
  # tree = "inst/artifacts/2020.2_moving-pictures/rooted-tree.qza",
  taxonomy = "qiime2/430_327_213_taxonomy.qza",
  metadata = "16s-meta-data.txt"
)

physeq ## confirm the object
```

```
## phyloseq-class experiment-level object
## otu_table()   OTU Table:         [ 14243 taxa and 29 samples ]
## sample_data() Sample Data:       [ 29 samples by 5 sample variables ]
## tax_table()   Taxonomy Table:    [ 14243 taxa by 7 taxonomic ranks ]
```

0.1.3 Normalise number of reads in each sample by using median sequencing depth

In the process of data normalisation, we employed a technique known as Total Sum Scaling (TSS). This method is particularly effective in scaling the counts in each sample to correspond with the median sequencing depth observed across all samples. Consequently, this approach yields normalised sequence counts.

```r
# Calculate the median sequencing depth
total <- median(sample_sums(physeq))
# Define a scaling function
standf <- function(x, t = total) round(t * (x / sum(x)))
# Normalise the sample counts using the scaling function
```

```r
physeq.norm <- transform_sample_counts(physeq, standf)

# Clean up by removing objects that are no longer needed
rm(total, standf)
```

0.1.4 Sub-grouping

Separate analysis is necessary for the uncultivated and enriched experiments since the data contained in each subset differs and requires distinct examination.

```r
## Subgroup - Uncultivated
physeq.norm.ori <- subset_samples(physeq.norm, Comparison=="Uncultivated")
# Get the column names of the sample_data
colnames(sample_data(physeq.norm.ori))
```

```
## [1] "Label"      "Group"      "Comparison" "Category"   "Raw_Reads"
```

```r
# Find the index of the "Group" column
group_index <- which(colnames(sample_data(physeq.norm.ori)) == "Group")
# Rename the "Group" column to "Uncultivated"
colnames(sample_data(physeq.norm.ori))[group_index] <- "Uncultivated"
# Copy the column information from "Uncultivated" to "Group"
sample_data(physeq.norm.ori)$Group <- sample_data(physeq.norm.ori)$Uncultivated


## Subgroup - Enriched
physeq.norm.rich <- subset_samples(physeq.norm, Comparison=="Enriched")
# Get the column names of the sample_data
colnames(sample_data(physeq.norm.rich))
```

```
## [1] "Label"      "Group"      "Comparison" "Category"   "Raw_Reads"
```

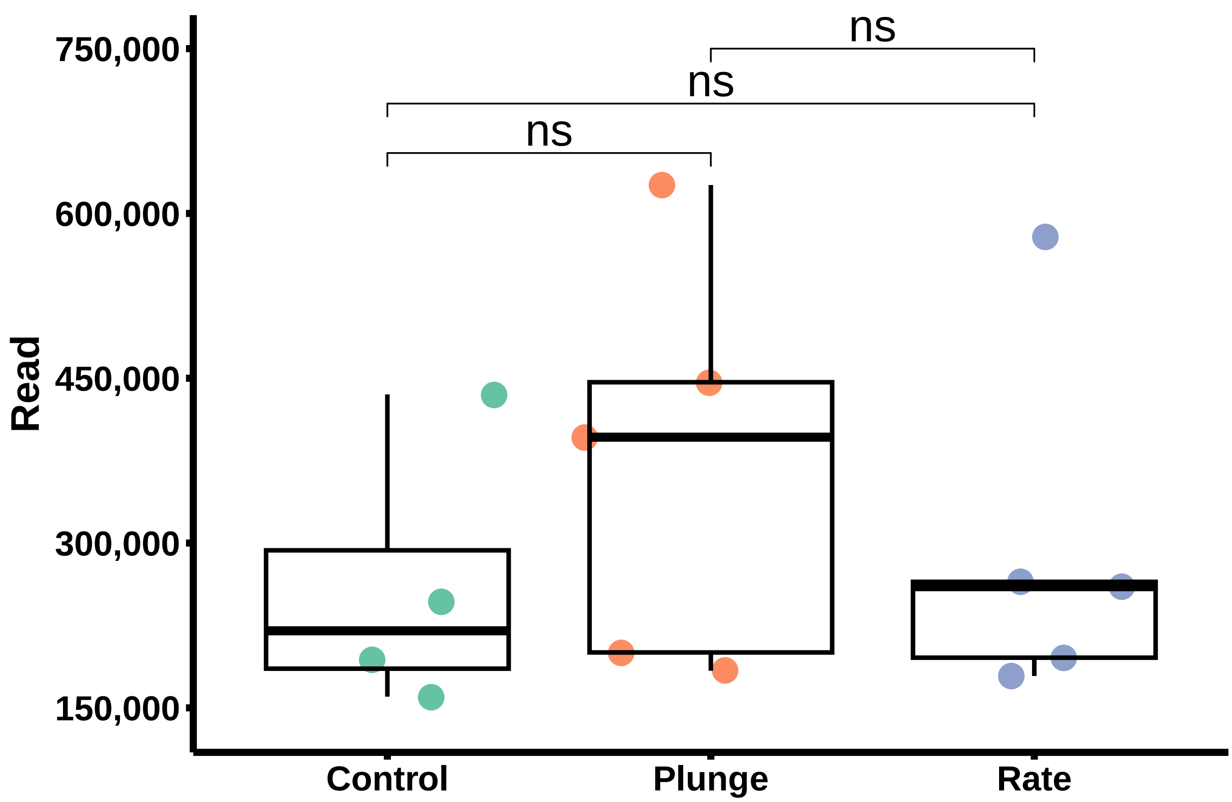
```r
# Find the index of the "Group" column
group_index <- which(colnames(sample_data(physeq.norm.rich)) == "Group")
# Rename the "Group" column to "Uncultivated"
colnames(sample_data(physeq.norm.rich))[group_index] <- "Enriched"
# Copy the column information from "Enriched" to "Group"
sample_data(physeq.norm.rich)$Group <- sample_data(physeq.norm.rich)$Enriched


## Merge the replicate samples for each Group
physeq.norm.group = merge_samples(physeq.norm, "Category") # Sum between replicate samples
sample_data(physeq.norm.group)$Category <- rownames(sample_data(physeq.norm.group))

physeq.norm.ori.group = merge_samples(physeq.norm.ori, "Uncultivated") # Sum between replicate samples
sample_data(physeq.norm.ori.group)$Uncultivated <- rownames(sample_data(physeq.norm.ori.group))

physeq.norm.rich.group = merge_samples(physeq.norm.rich, "Enriched") # Sum between replicate samples
sample_data(physeq.norm.rich.group)$Enriched <- rownames(sample_data(physeq.norm.rich.group))

# Clean up by removing objects that are no longer needed
rm(group_index)
```

0.1.5 Plot the raw reads, Uncultivated

```r
physeq.ori <- subset_samples(physeq, Comparison=="Uncultivated")
# Calculate the total raw reads of for each sample
meta <- data.frame(physeq.ori@sam_data)

# Now you can use 'meta_df' in your functions
stat.test1 <- meta %>%
  t_test(Raw_Reads ~ Group) %>%
  adjust_pvalue(method = "bonferroni") %>%
  add_significance()

print(stat.test1)
```

```
## # A tibble: 3 x 10
##   .y.       group1  group2    n1    n2 statistic    df     p p.adj p.adj.signif
##   <chr>     <chr>   <chr>  <int> <int>     <dbl> <dbl> <dbl> <dbl> <chr>
## 1 Raw_Reads Control Plunge     4     5    -1.09   6.86 0.314 0.942 ns
## 2 Raw_Reads Control Rate       4     5    -0.386  7.00 0.711 1     ns
## 3 Raw_Reads Plunge  Rate       5     5     0.682  7.88 0.515 1     ns
```

```r
# Plot a graph of the abundance of Fusarium for each sample grouped by Group:
Raw_Reads.Ori <- ggplot(subset(meta, Group %in% c("Control","Plunge","Rate")),
                        aes(x = Group, y = Raw_Reads, colour = interaction(Group))) +
  geom_point(alpha = 1, position = "jitter", size = 4) +
  geom_boxplot(alpha = 0, colour = "black", size = 0.8) +
  scale_y_continuous(labels = scales::comma, limits=c(140000, 750000),
                     breaks = c(150000, 300000, 450000, 600000, 750000)) +
    stat_pvalue_manual(stat.test1,
                     y.position = c(655000, 700000, 750000),
                     label = "p.adj.signif",
                     face="bold",
                     size = 6,
                     linetype = 1,
                     tip.length = 0.02,
                     inherit.aes = FALSE) +
  theme_classic() +
  labs(x = "", y = "Read") +
  theme(text = element_text(size=18, colour = "black"),
        axis.ticks = element_line(colour = "black", size = 1.25),
        axis.line = element_line(colour = 'black', size = 1.25),
        axis.text.x = element_text(colour = "black",
                                   angle=0,
                                   size = 13, face="bold"),
        axis.text.y = element_text(angle=0, hjust=0, colour = "black",
                                   size = 13, face="bold"),
        axis.title.y = element_text(color="black", size=15,face="bold"),
        legend.position = "none") +
  scale_color_brewer(palette="Set2")+
  scale_fill_brewer(palette="Set2")

# pdf(file = "Raw_Reads.Ori.pdf", width = 6, height = 5)
Raw_Reads.Ori
```




```
# Close the PDF device and save the plot to a file
# dev.off()


# Clean up by removing objects that are no longer needed
rm(physeq.ori, meta, Raw_Reads.Ori, stat.test1)
```

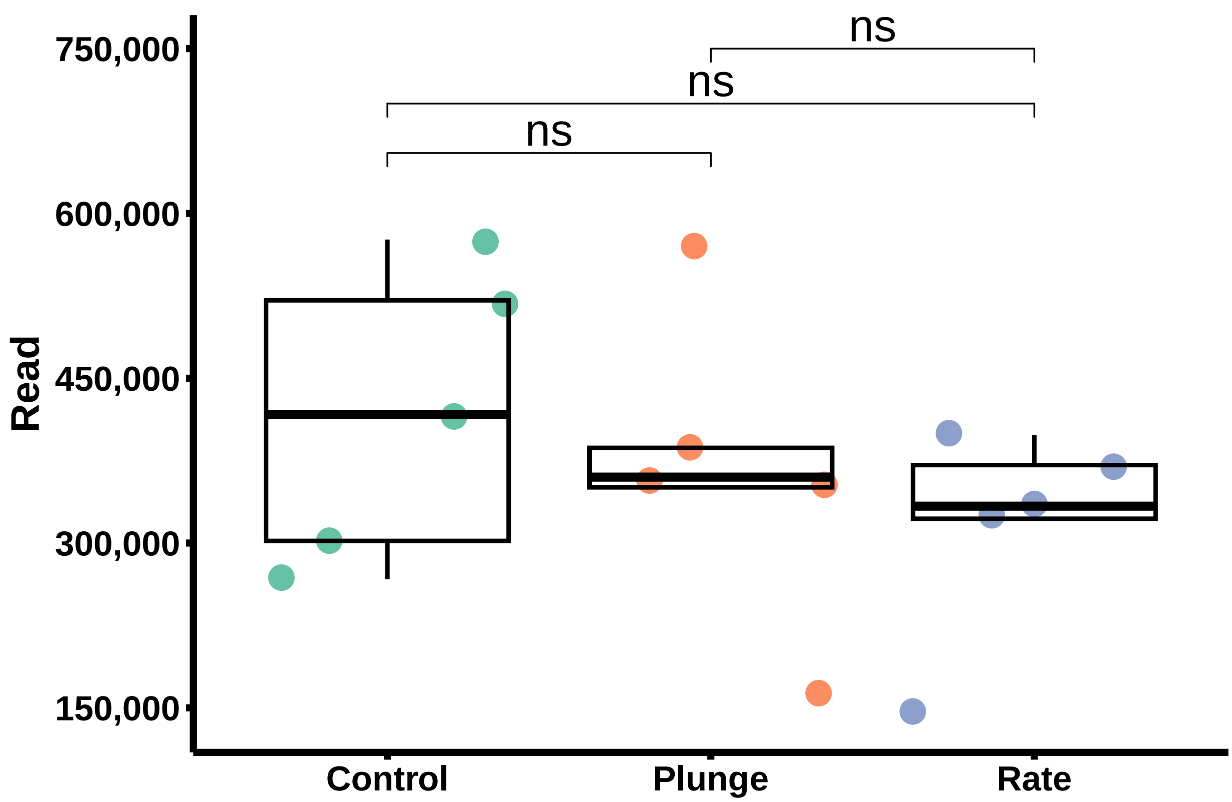
0.1.6 Plot the raw reads, Enriched

```
physeq.rich <- subset_samples(physeq, Comparison=="Enriched")
# Calculate the total raw reads of for each sample
meta <- data.frame(physeq.rich@sam_data)

# Now you can use 'meta_df' in your functions
stat.test1 <- meta %>%
  t_test(Raw_Reads ~ Group) %>%
  adjust_pvalue(method = "bonferroni") %>%
  add_significance()

print(stat.test1)
```

```
## # A tibble: 3 x 10
##   .y.       group1  group2    n1    n2 statistic    df      p p.adj p.adj.signif
```

```
##   <chr>     <chr>   <chr>  <int> <int>     <dbl> <dbl> <dbl> <dbl> <chr>
## 1 Raw_Reads Control Plunge     5     5     0.583  7.97 0.576 1     ns
## 2 Raw_Reads Control Rate       5     5     1.38   7.37 0.209 0.627 ns
## 3 Raw_Reads Plunge  Rate       5     5     0.661  7.12 0.529 1     ns
```

```
# Plot a graph of the abundance of Fusarium for each sample grouped by Group:
Raw_Reads.rich <- ggplot(subset(meta, Group %in% c("Control","Plunge","Rate")),
       aes(x = Group, y = Raw_Reads, colour = interaction(Group))) +
  geom_point(alpha = 1, position = "jitter", size = 4) +
  geom_boxplot(alpha = 0, colour = "black", size = 0.8) +
  scale_y_continuous(labels = scales::comma, limits=c(140000, 750000),
                     breaks = c(150000, 300000, 450000, 600000, 750000)) +
      stat_pvalue_manual(stat.test1,
                     y.position = c(655000, 700000, 750000),
                     label = "p.adj.signif",
                     face="bold",
                     size = 6,
                     linetype = 1,
                     tip.length = 0.02,
                     inherit.aes = FALSE) +
  theme_classic() +
  labs(x = "", y = "Read") +
  theme(text = element_text(size=18, colour = "black"),
        axis.ticks = element_line(colour = "black", size = 1.25),
        axis.line = element_line(colour = 'black', size = 1.25),
        axis.text.x = element_text(colour = "black",
                                   angle=0,
                                   size = 13, face="bold"),
        axis.text.y = element_text(angle=0, hjust=0, colour = "black",
                                   size = 13, face="bold"),
        axis.title.y = element_text(color="black", size=15,face="bold"),
        legend.position = "none") +
  scale_color_brewer(palette="Set2")+
  scale_fill_brewer(palette="Set2")

# pdf(file = "Raw_Reads.rich.pdf", width = 6, height = 5)
Raw_Reads.rich
```




```r
# Close the PDF device and save the plot to a file
# dev.off()


# Clean up by removing objects that are no longer needed
rm(physeq.rich, meta, Raw_Reads.rich)
```

0.1.7 Beta diversity

Beta diversity is a measure used in ecological and microbial community studies to assess the dissimilarity of species or taxa compositions between different samples. It quantifies the variation in community structure and helps researchers understand the diversity and uniqueness of microbial communities. Various metrics, such as Bray-Curtis dissimilarity and Jaccard index, are employed to calculate beta diversity values, which can be visualised using techniques like Principal Coordinate Analysis or Non-Metric Multidimensional Scaling. Beta diversity analysis allows for comparisons of microbial communities across habitats, treatments, or environmental gradients, revealing factors influencing community variation and identifying key drivers of community structure. It provides insights into the functional and ecological significance of different microbial assemblages and their responses to environmental changes, aiding our understanding of microbial community dynamics and their roles in ecology, environmental science, and human health research.

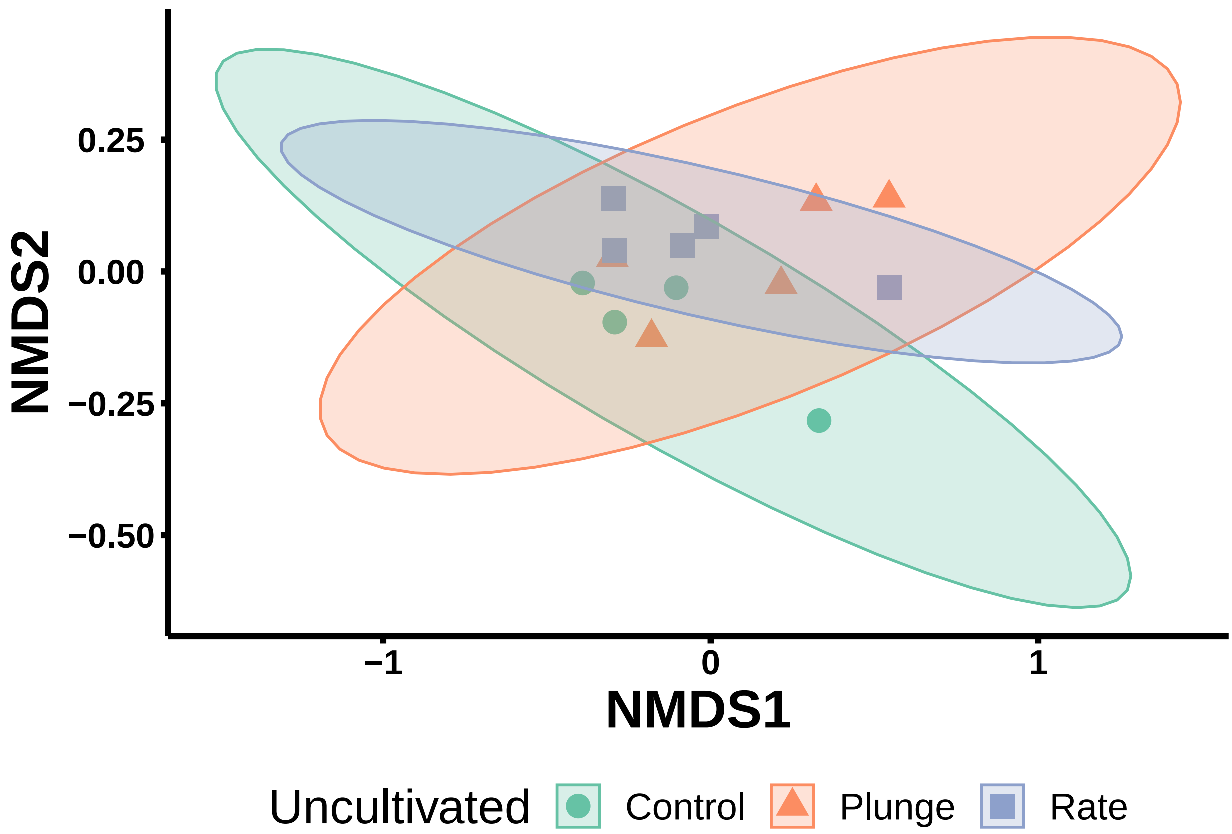
```r
nmds.ori <- ordinate(physeq = physeq.norm.ori, method = "NMDS", distance = "bray")
```

0.1.7.1 Beta diversity - Uncultivated

```
## Square root transformation
## Wisconsin double standardization
## Run 0 stress 0.04166808
## Run 1 stress 0.05749426
## Run 2 stress 0.05088141
## Run 3 stress 0.06272018
## Run 4 stress 0.04166809
## ... Procrustes: rmse 3.805675e-05  max resid 9.083493e-05
## ... Similar to previous best
## Run 5 stress 0.05749429
## Run 6 stress 0.05741062
## Run 7 stress 0.0485738
## Run 8 stress 0.04882746
## Run 9 stress 0.04394384
## Run 10 stress 0.05088148
## Run 11 stress 0.05950353
## Run 12 stress 0.04166809
## ... Procrustes: rmse 1.091577e-05  max resid 2.500461e-05
## ... Similar to previous best
## Run 13 stress 0.05088144
## Run 14 stress 0.05293223
## Run 15 stress 0.04394384
## Run 16 stress 0.04166809
## ... Procrustes: rmse 4.369475e-05  max resid 0.0001019602
## ... Similar to previous best
## Run 17 stress 0.05276266
## Run 18 stress 0.05451054
## Run 19 stress 0.04882751
## Run 20 stress 0.04882746
## *** Best solution repeated 3 times
```

```
Beta.ori <- plot_ordination(
  physeq = physeq.norm.ori,
  ordination = nmds.ori,
  color = "Uncultivated",
  shape = "Uncultivated") +
  theme_classic() +
  geom_point(aes(color = Uncultivated), alpha = 1, size = 4) +
  theme(text = element_text(size=18, colour = "black"),
        axis.ticks = element_line(colour = "black", size = 1.1),
        axis.line = element_line(colour = 'black', size = 1.1),
        axis.text.x = element_text(colour = "black", angle=0,
                                   hjust=0.5, size = 13, face="bold"),
        axis.text.y = element_text(colour = "black", angle=0,
                                   hjust=0.5, size = 13, face="bold"),
        axis.title.y = element_text(color="black", size=20,face="bold"),
        axis.title.x = element_text(color="black", size=20,face="bold"),
        legend.position = "bottom") + # This line moves the legend to the bottom
  stat_ellipse(geom = "polygon", type="norm",
               alpha=0.25, aes(fill = Uncultivated)) + # polygon, path, point
  scale_color_brewer(palette="Set2")+
  scale_fill_brewer(palette="Set2")
```

```r
# pdf(file = "Beta.ori.pdf", width = 6,height = 6.1)
Beta.ori
```




```r
# Close the PDF device and save the plot to a file
# dev.off()

# Clean up by removing objects that are no longer needed
rm(nmds.ori, Beta.ori, nmds.ori, Beta.ori)
```

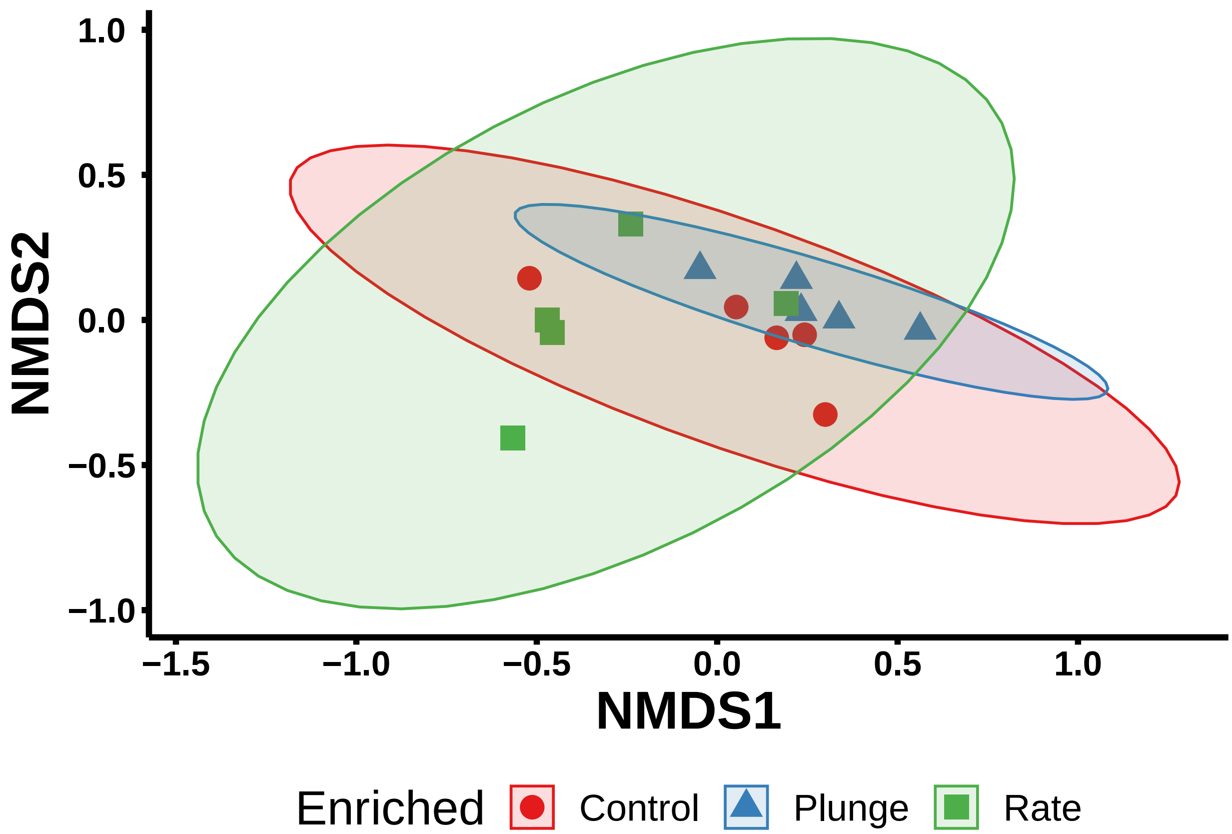
```r
nmds.rich <- ordinate(physeq = physeq.norm.rich, method = "NMDS", distance = "bray")
```

0.1.7.2 Beta diversity - Enriched

```
## Square root transformation
## Wisconsin double standardization
## Run 0 stress 0.08844091
## Run 1 stress 0.08823139
## ... New best solution
## ... Procrustes: rmse 0.04301748  max resid 0.1493551
## Run 2 stress 0.09648967
```

```
## Run 3 stress 0.08815413
## ... New best solution
## ... Procrustes: rmse 0.02369911  max resid 0.05513943
## Run 4 stress 0.09117319
## Run 5 stress 0.0911732
## Run 6 stress 0.08795252
## ... New best solution
## ... Procrustes: rmse 0.01456989  max resid 0.03231104
## Run 7 stress 0.08799876
## ... Procrustes: rmse 0.05831465  max resid 0.1473361
## Run 8 stress 0.09117322
## Run 9 stress 0.08799876
## ... Procrustes: rmse 0.05831727  max resid 0.1473359
## Run 10 stress 0.09575815
## Run 11 stress 0.08878582
## Run 12 stress 0.08835003
## ... Procrustes: rmse 0.02330609  max resid 0.06220255
## Run 13 stress 0.08799878
## ... Procrustes: rmse 0.05833561  max resid 0.1473777
## Run 14 stress 0.09117336
## Run 15 stress 0.09171829
## Run 16 stress 0.08821737
## ... Procrustes: rmse 0.00908115  max resid 0.02505652
## Run 17 stress 0.08799877
## ... Procrustes: rmse 0.05831532  max resid 0.1473623
## Run 18 stress 0.08799876
## ... Procrustes: rmse 0.05831968  max resid 0.1473394
## Run 19 stress 0.09575819
## Run 20 stress 0.09607688
## *** Best solution was not repeated -- monoMDS stopping criteria:
##     20: stress ratio > sratmax
```

```r
Beta.rich <- plot_ordination(
  physeq = physeq.norm.rich,
  ordination = nmds.rich,
  color = "Enriched",
  shape = "Enriched") +
  theme_classic() +
  geom_point(aes(color = Enriched), alpha = 1, size = 4) +
  theme(text = element_text(size=18, colour = "black"),
        axis.ticks = element_line(colour = "black", size = 1.1),
        axis.line = element_line(colour = 'black', size = 1.1),
        axis.text.x = element_text(colour = "black", angle=0, hjust=0.5, size = 13, face="bold"),
        axis.text.y = element_text(colour = "black", angle=0, hjust=0.5, size = 13, face="bold"),
        axis.title.y = element_text(color="black", size=20,face="bold"),
        axis.title.x = element_text(color="black", size=20,face="bold"),
        legend.position = "bottom") + # This line moves the legend to the bottom
  stat_ellipse(geom = "polygon", type="norm", alpha=0.15, aes(fill=Enriched))+
  scale_color_brewer(palette="Set1")+
  scale_fill_brewer(palette="Set1")


# pdf(file = "Beta.rich.pdf", width = 6,height = 6.1)
Beta.rich
```




```
# Close the PDF device and save the plot to a file
# dev.off()

# Clean up by removing objects that are no longer needed
rm(nmds.ori, Beta.ori, nmds.rich, Beta.rich)
```

0.1.8 Alpha diversity

Alpha diversity is a fundamental concept in ecology and refers to the diversity or richness of species within a specific community or habitat. In the context of microbial ecology, alpha diversity represents the diversity of microorganisms within a given sample or microbiome. It provides insights into the variety and evenness of microbial species present in a particular environment. Common measures of alpha diversity include species richness, which counts the number of unique species, and evenness, which assesses the distribution of species abundances. Alpha diversity is crucial for understanding the stability, resilience, and functional potential of microbial communities. It can be influenced by various factors, including environmental conditions, host factors, and perturbations. By comparing alpha diversity across different samples or experimental groups, researchers can gain insights into the impact of factors such as disease, habitat changes, or interventions on microbial community structure.

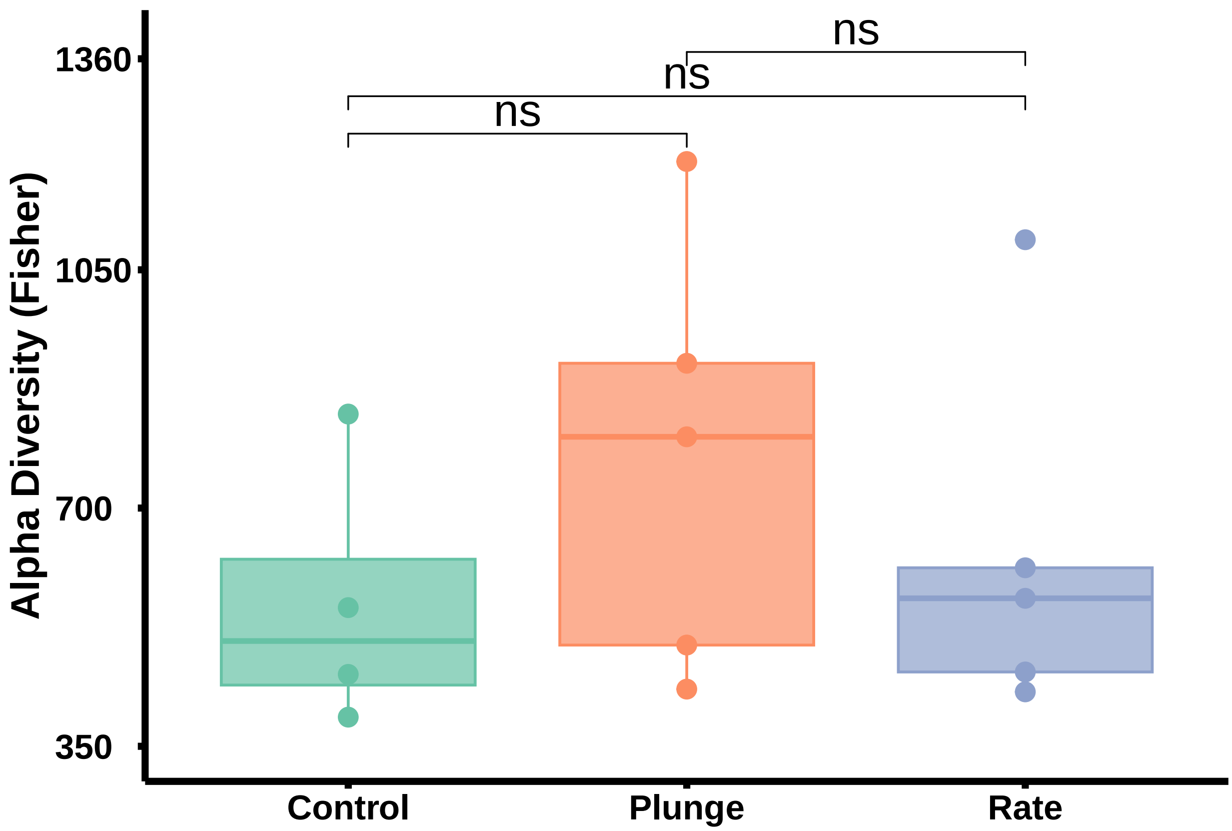
```
tab = cbind(x = sample_data(physeq.norm.ori),
            y = estimate_richness(physeq.norm.ori, measures = 'Fisher'))
```

```
stat.test <- tab %>%
  t_test(Fisher ~ x.Uncultivated) %>%
  adjust_pvalue(method = "bonferroni") %>%
  add_significance()

print(stat.test)
```

0.1.8.1 Uncultivated

```
## # A tibble: 3 x 10
##   .y.    group1  group2    n1    n2 statistic    df     p p.adj p.adj.signif
##   <chr>  <chr>   <chr>  <int> <int>     <dbl> <dbl> <dbl> <dbl> <chr>
## 1 Fisher Control Plunge     4     5    -1.23   6.71 0.261 0.783 ns
## 2 Fisher Control Rate       4     5    -0.467  6.98 0.655 1     ns
## 3 Fisher Plunge  Rate       5     5     0.750  7.79 0.475 1     ns
```

```
alpha.ori <- ggplot(data = tab, aes(x = x.Uncultivated,
                                    y = Fisher,
                                    color = x.Uncultivated,
                                    fill = x.Uncultivated)) +
  theme_classic() +
  labs(
    x = element_blank(),
    y = "Alpha Diversity (Fisher)") +
  geom_point(size = 3) +
  geom_boxplot(alpha = 0.7) +
  stat_pvalue_manual(stat.test,
                     y.position = c(1250, 1305, 1370),
                     label = "p.adj.signif",
                     face="bold",
                     size = 6,
                     linetype = 1,
                     tip.length = 0.02,
                     inherit.aes = FALSE) +
  scale_y_continuous(limits=c(350, 1380), breaks = c(350, 700, 1050, 1360)) +
  theme(text = element_text(size=18, colour = "black"),
        axis.ticks = element_line(colour = "black", size = 1.25),
        axis.line = element_line(colour = 'black', size = 1.25),
        axis.text.x = element_text(colour = "black",angle=0,
                                   size = 13, face="bold"),
        axis.text.y = element_text(angle=0, hjust=0, colour = "black",
                                   size = 13, face="bold"),
        axis.title.y = element_text(color="black", size=15,face="bold"),
        legend.position = "none") +
  scale_color_brewer(palette="Set2")+
  scale_fill_brewer(palette="Set2")

# pdf(file = "alpha.ori.pdf", width = 6, height = 5)
alpha.ori
```




```
# Close the PDF device and save the plot to a file
# dev.off()

# Clean up by removing objects that are no longer needed
rm(tab, stat.test, alpha.ori)
```

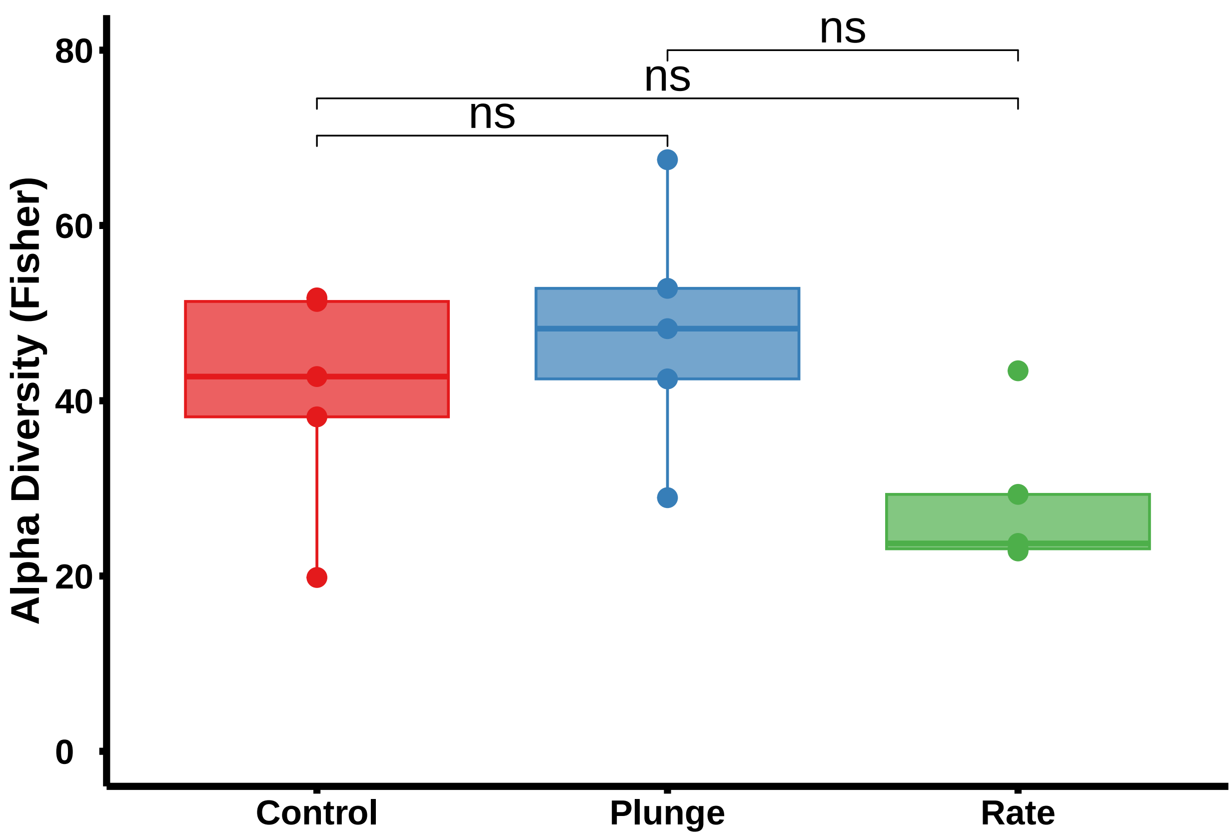
```
tab1 = cbind(x = sample_data(physeq.norm.rich),
             y = estimate_richness(physeq.norm.rich, measures = 'Fisher'))

stat.test1 <- tab1 %>%
  t_test(Fisher ~ x.Enriched) %>%
  adjust_pvalue(method = "bonferroni") %>%
  add_significance()

print(stat.test1)
```

0.1.8.2 Enriched

```
## # A tibble: 3 x 10
##   .y.    group1  group2     n1    n2 statistic    df     p p.adj p.adj.signif
##   <chr>  <chr>   <chr>   <int> <int>     <dbl> <dbl> <dbl> <dbl> <chr>
## 1 Fisher Control Plunge      5     5    -0.841  7.95 0.425 1     ns
```

```
## 2 Fisher Control Rate       5     5     1.75   7.00 0.124 0.372 ns
## 3 Fisher Plunge  Rate       5     5     2.63   6.68 0.036 0.108 ns
```

```r
alpha.rich <- ggplot(data = tab1, aes(x = x.Enriched,
                                     y = Fisher,
                                     color = x.Enriched,
                                     fill = x.Enriched)) +
  theme_classic() +
  labs(
    x = element_blank(),
    y = "Alpha Diversity (Fisher)") +
  geom_point(size = 3) +
  geom_boxplot(alpha = 0.7) +
  stat_pvalue_manual(stat.test1,
                     y.position = c(70.25, 74.5, 80),
                     label = "p.adj.signif",
                     face="bold",
                     size = 6,
                     linetype = 1,
                     tip.length = 0.02,
                     inherit.aes = FALSE) +
  scale_y_continuous(limits=c(0, 80), breaks = c(0, 20, 40, 60, 80)) +
  theme(text = element_text(size=18, colour = "black"),
        axis.ticks = element_line(colour = "black", size = 1.25),
        axis.line = element_line(colour = 'black', size = 1.25),
        axis.text.x = element_text(colour = "black",
                                   angle=0,
                                   size = 13, face="bold"),
        axis.text.y = element_text(angle=0, hjust=0, colour = "black",
                                   size = 13, face="bold"),
        axis.title.y = element_text(color="black", size=15,face="bold"),
        legend.position = "none") +
  scale_color_brewer(palette="Set1")+
  scale_fill_brewer(palette="Set1")

# pdf(file = "alpha.rich.pdf", width = 6, height = 5)
alpha.rich
```




```r
# Close the PDF device and save the plot to a file
# dev.off()

# Clean up by removing objects that are no longer needed
rm(tab1, stat.test1, alpha.rich)
```

0.1.8.3 Determine the count of taxa within each level and group The purpose of this process is to visualise the distribution of the number of matched abundance across different groups and to identify any patterns in the distribution of the processed abundance within individual group. #### Uncultivated

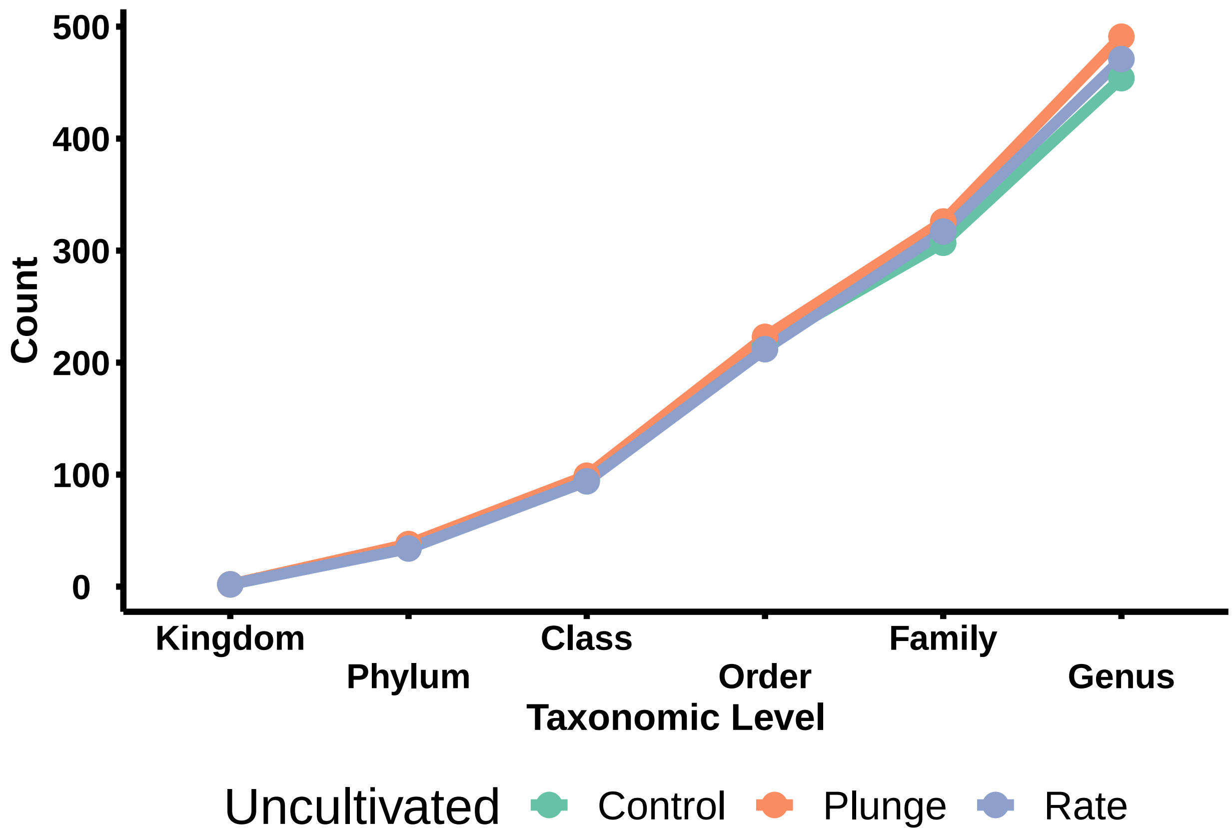
```r
# Create an empty list to store genus-level abundance data for each taxonomic level
gentab_levels <- list()

# Set observation threshold
observationThreshold <- 15

# Define the taxonomic levels
genus_levels <- c("Kingdom", "Phylum", "Class", "Order",
                  "Family", "Genus", "Species")

# loop through all the taxonomic levels
for (level in genus_levels) {

  # create a factor variable for each level
  genfac <- factor(tax_table(physeq.norm.ori.group)[, level])
```

```r
  # calculate the abundance of each level within each sample
  gentab <- apply(otu_table(physeq.norm.ori.group),
                  MARGIN = 1, function(x) {
    tapply(x, INDEX = genfac,
           FUN = sum, na.rm = TRUE,
           simplify = TRUE)
  })

  # calculate the number of samples in which each level is observed above the threshold
  level_counts <- apply(gentab > observationThreshold, 2, sum)

  # create a data frame of level counts with names as row names
  BB <- as.data.frame(level_counts)
  BB$name <- row.names(BB)

  # add the data frame to the gentab_levels list
  gentab_levels[[level]] <- BB
}

# Combine all level counts data frames into one data frame
B2 <- gentab_levels %>% purrr::reduce(dplyr::full_join, by = "name")

# Set row names and column names
rownames(B2) <- B2$name
B2$name <- NULL
colnames(B2)[1:7] <- genus_levels
B2$name <- rownames(B2)
B2$Species <- NULL

print(B2)
```

```
##         Kingdom Phylum Class Order Family Genus    name
## Control       2     35    95   216    307   454 Control
## Plunge        2     38    99   223    326   491  Plunge
## Rate          2     34    94   212    317   471    Rate
```

```r
data_long <- melt(B2, id.vars = "name",
                  variable.name = "Dataset",
                  value.name = "Count")

colnames(data_long) = c("Uncultivated","Taxonomic.Level","Count")


tax.ori <- ggplot(data_long, aes(x = Taxonomic.Level,
                                 y = Count,
                                 color = Uncultivated,
                                 group = Uncultivated)) +
  geom_line(size = 2) +
  geom_point(size = 4) +
  labs(x = "Taxonomic Level", y = "Count", color = "Uncultivated") +
  theme_classic() +
  theme(
```

```
    text = element_text(size = 19, colour = "black"),
    axis.ticks = element_line(colour = "black", size = 1.1),
    axis.line = element_line(colour = 'black', size = 1.1),
    axis.text.x = element_text(colour = "black", angle = 0, hjust = 0.5, size = 13, face = "bold"),
    axis.text.y = element_text(colour = "black", angle = 0, hjust = 0.5, size = 13, face = "bold"),
    axis.title.y = element_text(color = "black", size = 14, face = "bold"),
    axis.title.x = element_text(color = "black", size = 14, face = "bold"),
    legend.position = "bottom") + # This line moves the legend to the bottom
  scale_color_brewer(palette="Set2") +
  scale_x_discrete(guide = guide_axis(n.dodge=2)) +
  scale_y_continuous(breaks=seq(0,600,by=100))


# pdf(file = "tax.ori.pdf", width = 6, height = 6.1)
tax.ori
```



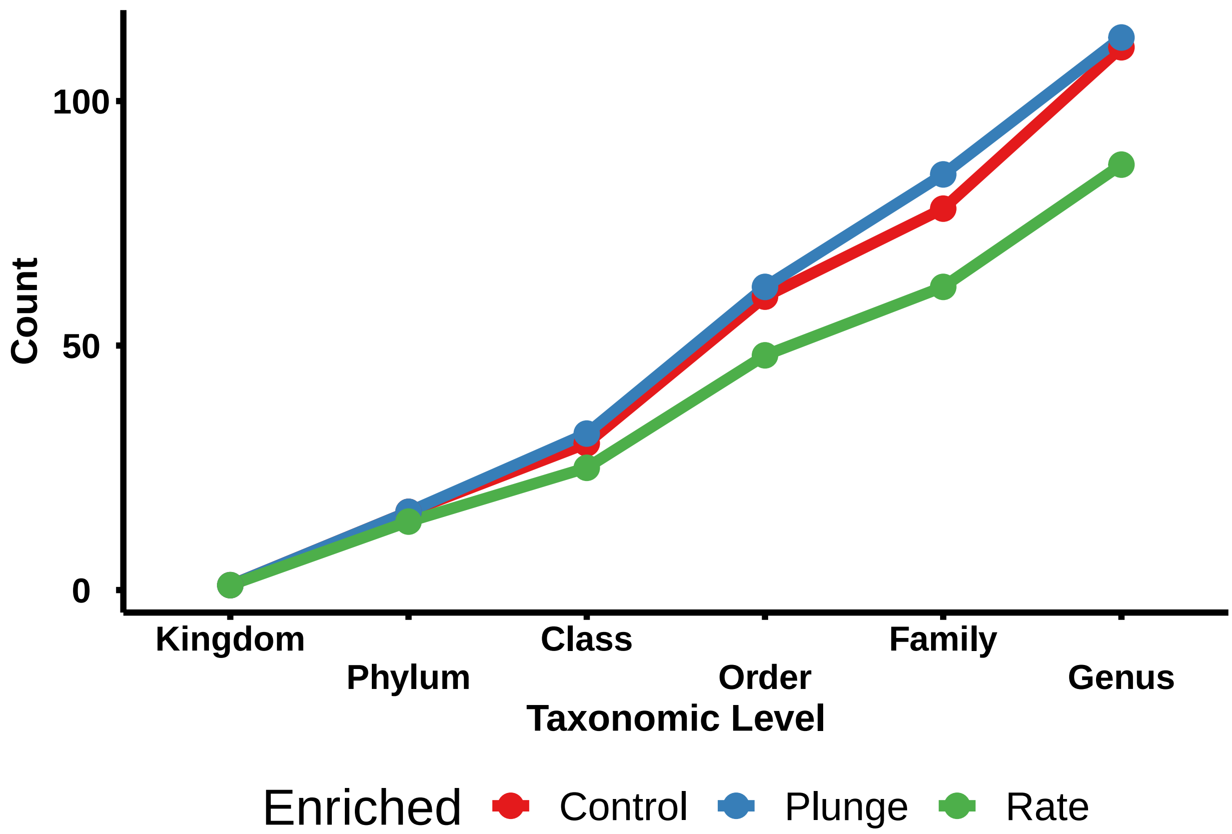

```
# Close the PDF device and save the plot to a file
# dev.off()

# Clean up by removing unnecessary objects
rm(gentab_levels, genus_levels, observationThreshold,
   BB, B2, data_long, gentab, tax.ori, genfac, level, level_counts, alpha.ori, stat.test)
```

```r
# Create an empty list to store genus-level abundance data for each taxonomic level
gentab_levels <- list()

# Set observation threshold
observationThreshold <- 15

# Define the taxonomic levels
genus_levels <- c("Kingdom", "Phylum", "Class", "Order",
                  "Family", "Genus", "Species")

# loop through all the taxonomic levels
for (level in genus_levels) {

  # create a factor variable for each level
  genfac <- factor(tax_table(physeq.norm.rich.group)[, level])

  # calculate the abundance of each genus within each sample
  gentab <- apply(otu_table(physeq.norm.rich.group), MARGIN = 1, function(x) {
    tapply(x, INDEX = genfac, FUN = sum, na.rm = TRUE, simplify = TRUE)
  })

  # calculate the number of samples in which each genus is observed above the threshold
  level_counts <- apply(gentab > observationThreshold, 2, sum)

  # create a data frame of level counts with genus names as row names
  BB <- as.data.frame(level_counts)
  BB$name <- row.names(BB)

  # add the data frame to the gentab_levels list
  gentab_levels[[level]] <- BB
}

# Combine all level counts data frames into one data frame
B2 <- gentab_levels %>% purrr::reduce(dplyr::full_join, by = "name")

# Set row names and column names
rownames(B2) <- B2$name
B2$name <- NULL
colnames(B2)[1:7] <- genus_levels
B2$Species <- NULL
B2$name <- rownames(B2)


print(B2)
```

0.1.8.4 Enriched

```
##         Kingdom Phylum Class Order Family Genus    name
## Control       1     16    30    60     78   111 Control
## Plunge        1     16    32    62     85   113  Plunge
## Rate          1     14    25    48     62    87    Rate
```

```r
data_long <- melt(B2, id.vars = "name", variable.name = "Dataset", value.name = "Count")
colnames(data_long) = c("Enriched","Taxonomic.Level","Count")


tax.rich <- ggplot(data_long, aes(x = Taxonomic.Level,
                                  y = Count,
                                  color = Enriched,
                                  group = Enriched)) +
  geom_line(size = 2) +
  geom_point(size = 4) +
  labs(x = "Taxonomic Level", y = "Count", color = "Enriched") +
  theme_classic() +
  theme(
    text = element_text(size = 19, colour = "black"),
    axis.ticks = element_line(colour = "black", size = 1.1),
    axis.line = element_line(colour = 'black', size = 1.1),
    axis.text.x = element_text(colour = "black", angle = 0, hjust = 0.5, size = 13, face = "bold"),
    axis.text.y = element_text(colour = "black", angle = 0, hjust = 0.5, size = 13, face = "bold"),
    axis.title.y = element_text(color = "black", size = 14, face = "bold"),
    axis.title.x = element_text(color = "black", size = 14, face = "bold"),
    legend.position = "bottom") + # This line moves the legend to the bottom
    scale_color_brewer(palette="Set1") +
  scale_x_discrete(guide = guide_axis(n.dodge=2)) +
  scale_y_continuous(breaks=seq(0,200,by=50))

# pdf(file = "tax.rich.pdf", width = 6, height = 6.1)
tax.rich
```




```r
# Close the PDF device and save the plot to a file
# dev.off()

# Clean up by removing unnecessary objects
rm(gentab_levels, genus_levels, observationThreshold,
   BB, B2, data_long, gentab, tax.rich, genfac, level, level_counts, stat.test, alpha.ori)
```

0.1.9 Upset plot using UpsetR

Venn diagrams are commonly used for visualizing sets, but they can become complex with more than five sets. UpSet graphs, on the other hand, offer a more efficient way to display intersections and complements, especially for larger or multiple datasets. They provide a more intuitive and informative data representation. #### Uncultivated

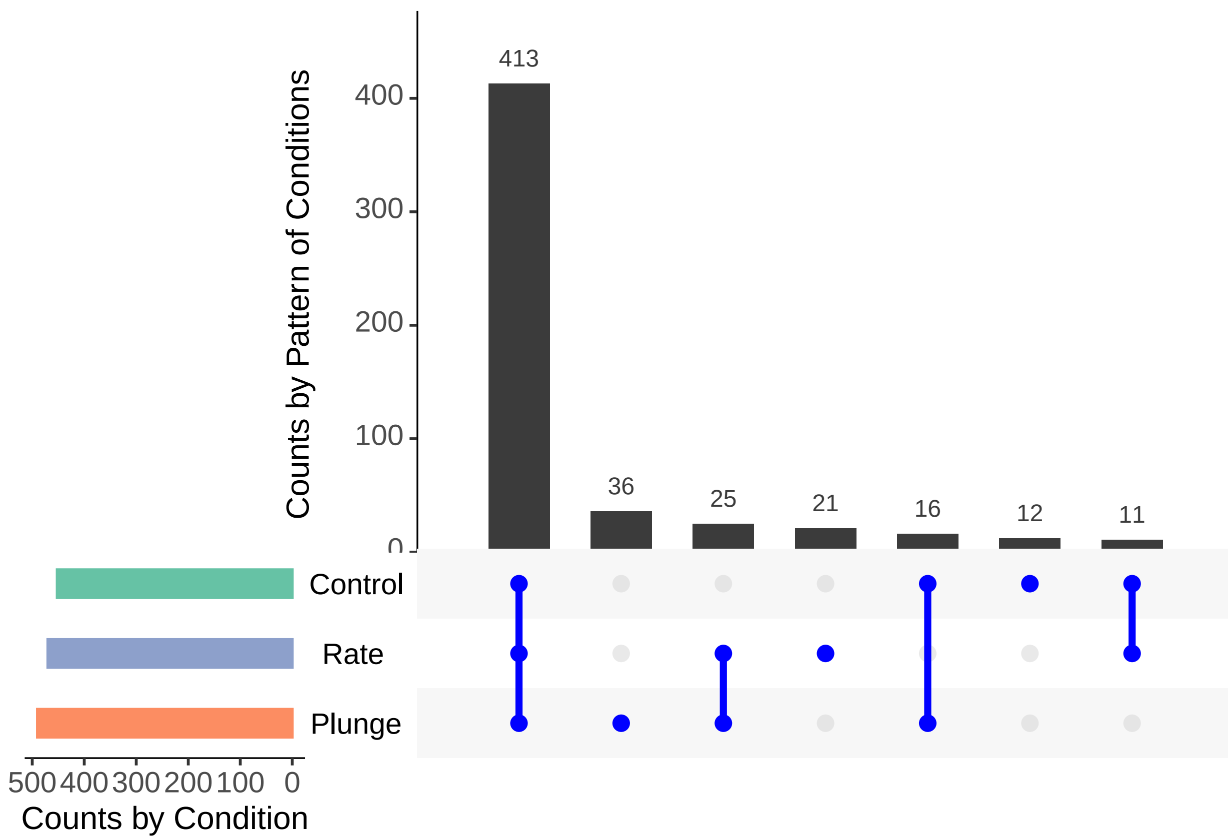
```r
# Aggregate taxa at the genus level
B <- aggregate_taxa(physeq.norm.ori.group, "Genus", verbose = TRUE)
```

```
## [1] "Remove taxonomic information below the target level"
## [1] "Mark the potentially ambiguous taxa"
## [1] "-- split"
## [1] "-- sum"
## [1] "Create phyloseq object"
## [1] "Remove ambiguous levels"
## [1] "-- unique"
```

```
## [1] "-- Rename the lowest level"
## [1] "-- rownames"
## [1] "-- taxa"
## [1] "Convert to taxonomy table"
## [1] "Combine OTU and Taxon matrix into Phyloseq object"
## [1] "Add the metadata as is"
```

```r
# Remove undesired genera
# B2 <- subset_taxa(B, !get("Genus") %in% c("uncultured", "Unknown"))

# Remove unwanted taxon names
taxa_to_remove <- c("uncultured", "Unknown")
B2 <- subset_taxa(B, !get("Genus") %in% taxa_to_remove)

# Extract relevant data from the phyloseq object
sample_data <- sample_data(B2)
otu_table <- otu_table(B2)
abundance <- as.vector(otu_table)

# Create a tibble with the extracted data
D <- tibble(
  Sample = rep(sample_data$Uncultivated, each = nrow(otu_table)),
  ASV = rep(rownames(otu_table), times = ncol(otu_table)),
  Abundance = abundance
) %>%
  group_by(Sample) %>%
  mutate(rank = rank(plyr::desc(Abundance))) %>%
   filter(Abundance > 15) %>%
  ungroup() %>%
  select(Sample, Abundance, ASV)

# Remove the Abundance column
D$Abundance <- NULL

# Rename the second column to "ASV"
names(D)[2] <- "ASV"
names(D)[1] <- "Uncultivated"

# Convert data from long to wide format
E <- dcast(D, ASV ~ Uncultivated)

# Define a binary function
binary_fun <- function(x) {
  x[is.na(x)] <- 0
  ifelse(x > 0, 1, 0)
}


# Apply the binary function to columns 2 to 4
df.uncultivated.family <- apply(E[2:4], 2, binary_fun)
df.uncultivated.family <- as.data.frame(df.uncultivated.family)
rownames(df.uncultivated.family) <- E$ASV

col = c("#FC8D62", "#8DA0CB","#66C2A5")
```

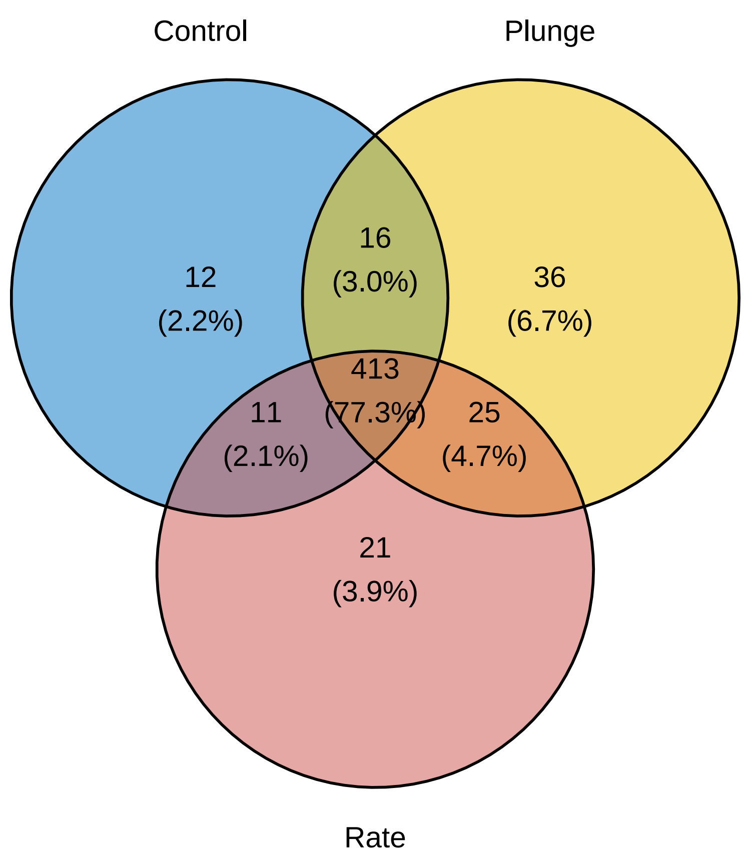
```
# Create an UpSet plot
UpSet.Ori <- upset(df.uncultivated.family,
                    sets = colnames(df.uncultivated.family),
                    sets.bar.color = (col),
                    order.by = "freq",
                    empty.intersections = "on",
                    mainbar.y.label = "Counts by Pattern of Conditions",
                    sets.x.label = "Counts by Condition",
                    matrix.color="blue",
                    mb.ratio = c(0.65, 0.35),
                    point.size= 2.75,
                    line.size = 1.25,
                    text.scale = 1.5
)

# Open a new PDF graphics device
# pdf(file = "UpSet.Ori.pdf", width=6.5,height=4.5)

# Print the UpSet plot
print(UpSet.Ori)
```




```
# Close the PDF device and save the plot to a file
# dev.off()

# Clean up by removing unnecessary objects
```

```r
rm(B, B2, sample_data,
   otu_table, abundance, D, E,
   binary_fun, col, binary_fun, UpSet.Ori)
```

```r
# Extract the rows where the value is 1 for each column
Cont <- rownames(df.uncultivated.family)[df.uncultivated.family$Control == 1]
Plun <- rownames(df.uncultivated.family)[df.uncultivated.family$Plunge == 1]
Rate <- rownames(df.uncultivated.family)[df.uncultivated.family$Rate == 1]

# Create a list with the extracted data
list_data <- list("Control" = Cont, "Plunge" = Plun, "Rate" = Rate)

# Use ggvenn to create the Venn diagram
Venn <- ggvenn(
  list_data,
  fill_color = c("#0073C2FF", "#EFC000FF", "#CD534CFF"),
  stroke_size = 0.5, set_name_size = 4
  )

# Open a new PDF graphics device
# pdf(file = "Fig08C_Venn.pdf", width=5,height=5)

# Print the Venn plot
print(Venn)
```



```r
# Close the PDF device and save the plot to a file
# dev.off()
# Clean up by removing unnecessary objects
rm(df.uncultivated.family, Venn, Cont, Plun, Rate)
```

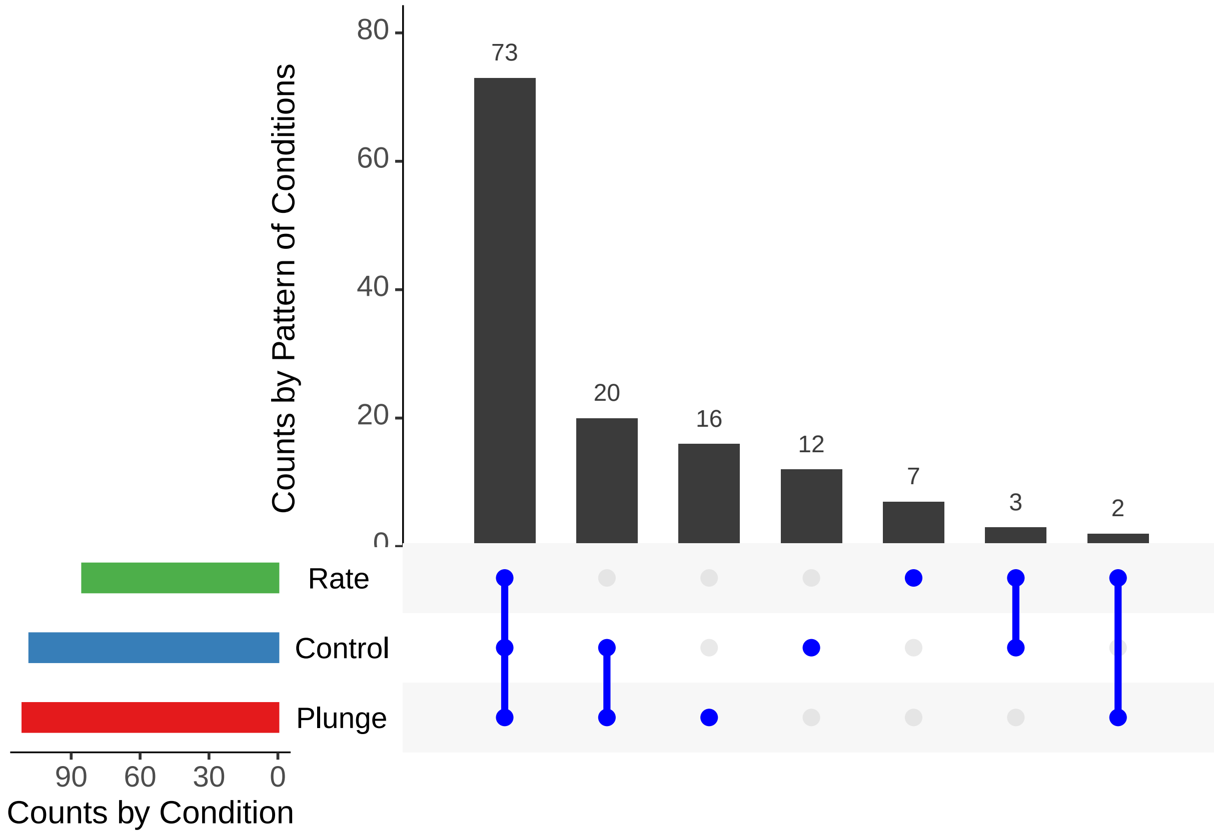
```r
# Aggregate taxa at the genus level
B <- aggregate_taxa(physeq.norm.rich.group, "Genus", verbose = TRUE)
```

0.1.9.1 Enriched

```
## [1] "Remove taxonomic information below the target level"
## [1] "Mark the potentially ambiguous taxa"
## [1] "-- split"
## [1] "-- sum"
## [1] "Create phyloseq object"
## [1] "Remove ambiguous levels"
## [1] "-- unique"
## [1] "-- Rename the lowest level"
## [1] "-- rownames"
## [1] "-- taxa"
## [1] "Convert to taxonomy table"
## [1] "Combine OTU and Taxon matrix into Phyloseq object"
## [1] "Add the metadata as is"
```

```r
# Remove undesired genera
# B2 <- subset_taxa(B, !get("Genus") %in% c("uncultured", "Unknown"))

# Remove unwanted taxon names
taxa_to_remove <- c("uncultured", "Unknown")
B2 <- subset_taxa(B, !get("Genus") %in% taxa_to_remove)

# Extract relevant data from the phyloseq object
sample_data <- sample_data(B2)
otu_table <- otu_table(B2)
abundance <- as.vector(otu_table)

# Create a tibble with the extracted data
D <- tibble(
  Sample = rep(sample_data$Enriched, each = nrow(otu_table)),
  ASV = rep(rownames(otu_table), times = ncol(otu_table)),
  Abundance = abundance
) %>%
  group_by(Sample) %>%
  mutate(rank = rank(plyr::desc(Abundance))) %>%
   filter(Abundance > 15) %>%
  ungroup() %>%
  select(Sample, Abundance, ASV)

# Remove the Abundance column
D$Abundance <- NULL
```

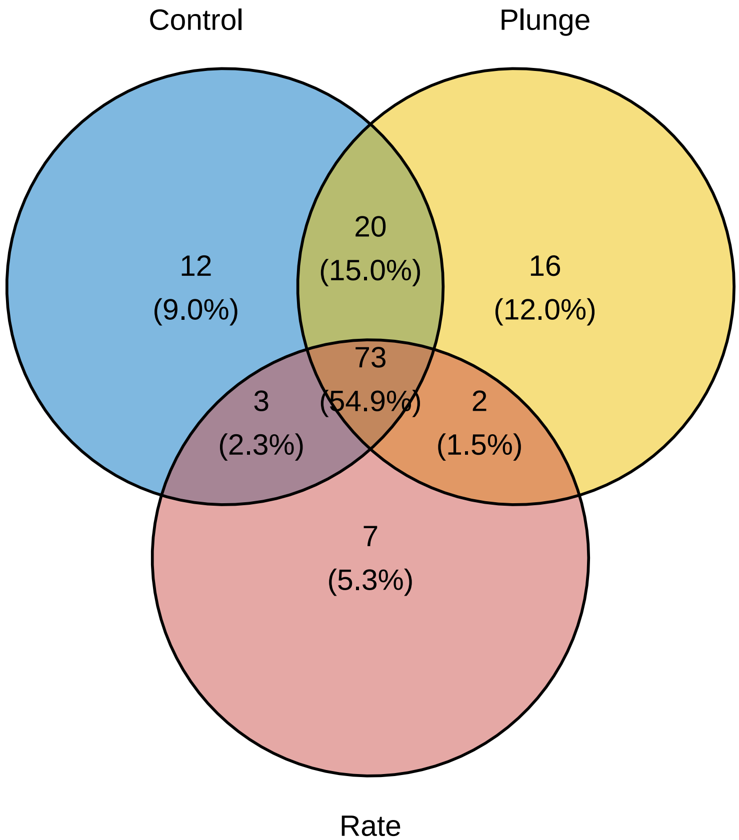
```r
# Rename the second column to "ASV"
names(D)[2] <- "ASV"
names(D)[1] <- "Uncultivated"

# Convert data from long to wide format
E <- dcast(D, ASV ~ Uncultivated)

# Define a binary function
binary_fun <- function(x) {
  x[is.na(x)] <- 0
  ifelse(x > 0, 1, 0)
}

col = brewer.pal(n = 3, name = "Set1")

# Apply the binary function to columns 2 to 4
df.enriched.family <- apply(E[2:4], 2, binary_fun)
df.enriched.family <- as.data.frame(df.enriched.family)

# Create an UpSet plot
upset.Rich <- upset(df.enriched.family,
                    sets = colnames(df.enriched.family),
                    sets.bar.color = (col),
                    order.by = "freq",
                    empty.intersections = "on",
                    mainbar.y.label = "Counts by Pattern of Conditions",
                    sets.x.label = "Counts by Condition",
                    matrix.color="blue",
                    mb.ratio = c(0.65, 0.35),
                    point.size= 2.75,
                    line.size = 1.25,
                    text.scale = 1.5
)


# Open a new PDF graphics device
# pdf(file = "upset.Rich.pdf", width=6.5,height=4.5)

# Print the UpSet plot
print(upset.Rich)
```




```r
# Close the PDF device and save the plot to a file
# dev.off()

# Clean up by removing unnecessary objects
rm(B, B2, sample_data,
   otu_table, abundance, D, E,
   binary_fun, col, binary_fun, upset.Rich)
```

```r
# Extract the rows where the value is 1 for each column
Cont <- rownames(df.enriched.family)[df.enriched.family$Control == 1]
Plun <- rownames(df.enriched.family)[df.enriched.family$Plunge == 1]
Rate <- rownames(df.enriched.family)[df.enriched.family$Rate == 1]

# Create a list with the extracted data
list_data <- list("Control" = Cont, "Plunge" = Plun, "Rate" = Rate)

# Use ggvenn to create the Venn diagram
Venn <- ggvenn(
  list_data,
  fill_color = c("#0073C2FF", "#EFC000FF", "#CD534CFF"),
  stroke_size = 0.5, set_name_size = 4
  )

# Open a new PDF graphics device
# pdf(file = "Fig08C_Venn.pdf", width=5,height=5)
```

```r
# Print the Venn plot
print(Venn)
```




```r
# Close the PDF device and save the plot to a file
# dev.off()

# Clean up by removing unnecessary objects
rm(df.enriched.family, Venn, Cont, Plun, Rate)
```

0.1.10 Pairwise comparison using PERMANOVA

Pairwise PERMANOVA (Permutational Multivariate Analysis of Variance) is a statistical method used in microbial community studies to examine differences between groups or treatments. It assesses the dissimilarity between samples, allowing for the comparison of multivariate data. This approach is useful to focus on specific group comparisons rather than comparing all groups simultaneously. It enables the investigation of the effects of specific treatments on microbial communities, helping to determine if there are significant differences in community composition between selected groups. By considering variation within and between groups, pairwise PERMANOVA offers a robust statistical assessment of dissimilarity, providing insights into community structure differences.

```
##                pairs Df SumsOfSqs  F.Model        R2     p.value p.adjusted sig
## 1 Control vs Plunge  1 0.2135437 2.383923 0.2295783 0.024609754 0.07382926
## 2   Control vs Rate  1 0.1787888 1.287497 0.1386269 0.269437306 0.80831192
## 3    Plunge vs Rate  1 0.3600888 3.368058 0.2962738 0.007749923 0.02324977   .
```

```
##                pairs Df  SumsOfSqs  F.Model        R2    p.value p.adjusted sig
## 1 Control vs Plunge  1 0.05666013 1.131139 0.1391120 0.22237778  0.6671333
## 2   Control vs Rate  1 0.06639183 1.461060 0.1726805 0.06403936  0.1921181
## 3    Plunge vs Rate  1 0.05134752 1.223352 0.1326364 0.12584874  0.3775462
```

0.1.11 Top 10 at family level

Identifying the top 10 bacteria in the top 10 family level and their corresponding percentages provides a snapshot of the microbial community's composition.

```r
##### create a function
standf = function(x) x / sum(x) * 100
##### unwanted taxon names
taxa_to_remove <- c("uncultured", "Unknown")
#####

## Normalised number of reads in percentage
AyBCode.percent = transform_sample_counts(physeq.norm.ori.group, standf)

# Remove unwanted taxon names
AyBCode.percent.B <- subset_taxa(AyBCode.percent, !get("Family") %in% taxa_to_remove)

## Aggregate
AyBCode.percent.B <- aggregate_taxa(AyBCode.percent.B, "Family", verbose = TRUE)
```

0.1.11.1 Uncultivated treatment

```
## [1] "Remove taxonomic information below the target level"
## [1] "Mark the potentially ambiguous taxa"
## [1] "-- split"
## [1] "-- sum"
## [1] "Create phyloseq object"
## [1] "Remove ambiguous levels"
## [1] "-- unique"
## [1] "-- Rename the lowest level"
## [1] "-- rownames"
## [1] "-- taxa"
## [1] "Convert to taxonomy table"
## [1] "Combine OTU and Taxon matrix into Phyloseq object"
## [1] "Add the metadata as is"
```

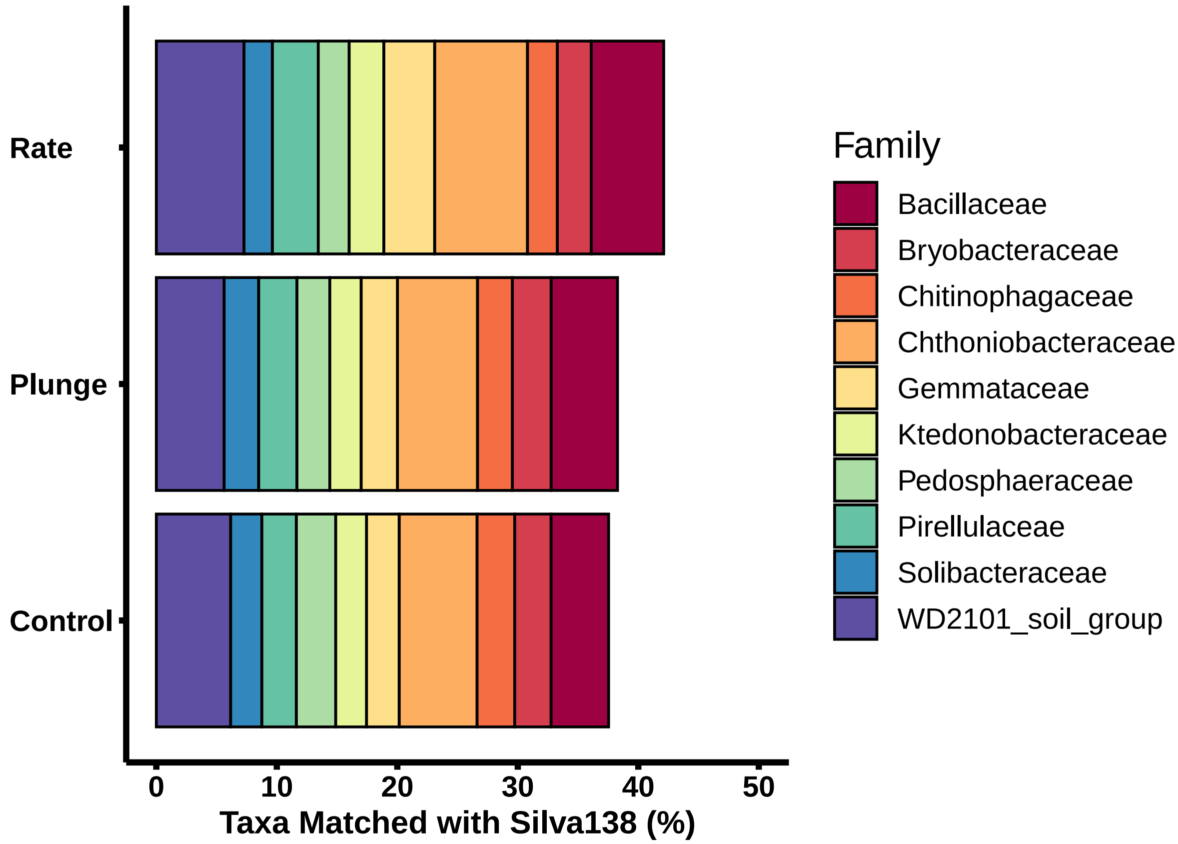
```r
top10otus = names(sort(taxa_sums(AyBCode.percent.B), TRUE)[1:10])
taxtab10 = cbind(tax_table(AyBCode.percent.B), Family = NA)
taxtab10[top10otus, "Family"] <- as(tax_table(AyBCode.percent.B)[top10otus, "Family"],"character")
tax_table(AyBCode.percent.B) <- tax_table(taxtab10)


top10plot = prune_taxa(top10otus, AyBCode.percent.B)
print(top10plot@otu_table)
```

```
## OTU Table:          [10 taxa and 3 samples]
##                      taxa are rows
##                      Control   Plunge     Rate
## Bryobacteraceae     3.014036 3.216168 2.814376
## Solibacteraceae     2.581171 2.859025 2.345029
## Chitinophagaceae    3.121356 2.885015 2.471664
## Ktedonobacteraceae  2.551932 2.597329 2.879709
## Bacillaceae         4.778767 5.512726 6.017349
## WD2101_soil_group   6.168999 5.628070 7.278320
## Gemmataceae         2.709999 3.016132 4.218830
## Pirellulaceae       2.864818 3.183814 3.815266
## Chthoniobacteraceae 6.469898 6.649131 7.710652
## Pedosphaeraceae     3.274158 2.726205 2.557521
```

```
# Calculate the sum of each column
col_sums <- colSums(as.data.frame(top10plot@otu_table))

# Add a new row with the sums
top10plot.df <- rbind('SUM' = col_sums, as.data.frame(top10plot@otu_table))

# Print the dataframe
print(top10plot.df)
```

```
##                       Control    Plunge      Rate
## SUM                 37.535134 38.273615 42.108716
## Bryobacteraceae      3.014036  3.216168  2.814376
## Solibacteraceae      2.581171  2.859025  2.345029
## Chitinophagaceae     3.121356  2.885015  2.471664
## Ktedonobacteraceae   2.551932  2.597329  2.879709
## Bacillaceae          4.778767  5.512726  6.017349
## WD2101_soil_group    6.168999  5.628070  7.278320
## Gemmataceae          2.709999  3.016132  4.218830
## Pirellulaceae        2.864818  3.183814  3.815266
## Chthoniobacteraceae  6.469898  6.649131  7.710652
## Pedosphaeraceae      3.274158  2.726205  2.557521
```

```
top10.ori <- plot_bar(top10plot, fill = "Family") + coord_flip() +
  ylab("Taxa Matched with Silva138 (%)") + ylim(0, 50) +
  theme_classic() +
  theme(text = element_text(size=14, colour = "black"),
        axis.ticks = element_line(colour = "black", size = 1.1),
        axis.line = element_line(colour = 'black', size = 1.1),
        axis.text.x = element_text(colour = "black", angle=0, size = 11, face="bold"),
        axis.text.y = element_text(angle=0, hjust=0, colour = "black", size = 11, face="bold"),
        axis.title.y = element_text(color="black", size=12,face="bold"),
        axis.title.x = element_text(color="black", size=12,face="bold"),
        legend.position = "right") +
  scale_color_brewer(palette="Spectral")+
  scale_fill_brewer(palette="Spectral") +
  xlab("") # This line removes the x-axis label

# pdf(file = "top10.ori.pdf", width = 6.75, height = 5)
top10.ori
```




```
# Close the PDF device and save the plot to a file
# dev.off()

# Clean up by removing objects that are no longer needed
rm(AyBCode.percent, AyBCode.percent.B, taxtab10, tax_table, top10plot, top10.ori)
```

0.1.12 Calculate the statistics in percentange on the top 10 family level

```
## Normalised number of reads in percentage
AyBCode.percent = transform_sample_counts(physeq.norm.ori, standf)

# Subset the phyloseq object for the top 10 OTUs
physeq.top10 <- subset_taxa(AyBCode.percent, Family %in% top10otus)

# Aggregate taxa at the genus level
physeq.top10 <- aggregate_taxa(physeq.top10, "Family", verbose = TRUE)
```

```
## [1] "Remove taxonomic information below the target level"
## [1] "Mark the potentially ambiguous taxa"
## [1] "-- split"
## [1] "-- sum"
## [1] "Create phyloseq object"
## [1] "Remove ambiguous levels"
```

```
## [1] "-- unique"
## [1] "-- Rename the lowest level"
## [1] "-- rownames"
## [1] "-- taxa"
## [1] "Convert to taxonomy table"
## [1] "Combine OTU and Taxon matrix into Phyloseq object"
## [1] "Add the metadata as is"
```

```
# Calculate the total abundance of Fusarium for each sample
meta = AyBCode.percent@sam_data
otudf = as.data.frame(t(as.data.frame(physeq.top10@otu_table)))

# Assuming 'meta' and 'otudf' are your data frames
combined_df <- merge(meta, otudf, by = "row.names", all = TRUE)

# Set row names of the combined data frame
rownames(combined_df) <- combined_df$Row.names

# Remove the 'Row.names' column
combined_df$Row.names <- NULL

# Get the column names from "Bryobacteraceae" onwards
cols <- colnames(combined_df)[which(colnames(combined_df) == "Bryobacteraceae"):ncol(combined_df)]

# Initialize an empty data frame to store the test results
stat.test_df <- data.frame()

# Loop over the columns
for(i in seq_along(cols)){
  # Perform the t-test for each column
  stats <- combined_df %>%
    t_test(reformulate("Group", response=cols[i])) %>%
    adjust_pvalue(method = "bonferroni") %>%
    add_significance()

  # Add a new column to record the run number
  stats$Run <- i

  # Bind the results to the data frame
  stat.test_df <- rbind(stat.test_df, stats)
}

# Print the data frame
print(stat.test_df)
```

```
## # A tibble: 30 x 11
##    .y.        group1 group2    n1    n2 statistic    df     p p.adj p.adj.signif
##    <chr>      <chr>  <chr>  <int> <int>     <dbl> <dbl> <dbl> <dbl> <chr>
##  1 Bryobacte~ Contr~ Plunge     4     5    -0.670  6.01 0.528 1     ns
##  2 Bryobacte~ Contr~ Rate       4     5     0.746  4.46 0.493 1     ns
##  3 Bryobacte~ Plunge Rate       5     5     1.84   6.86 0.11  0.33  ns
##  4 Solibacte~ Contr~ Plunge     4     5    -0.758  6.95 0.473 1     ns
##  5 Solibacte~ Contr~ Rate       4     5     0.928  4.48 0.401 1     ns
##  6 Solibacte~ Plunge Rate       5     5     1.67   5.23 0.154 0.462 ns
```

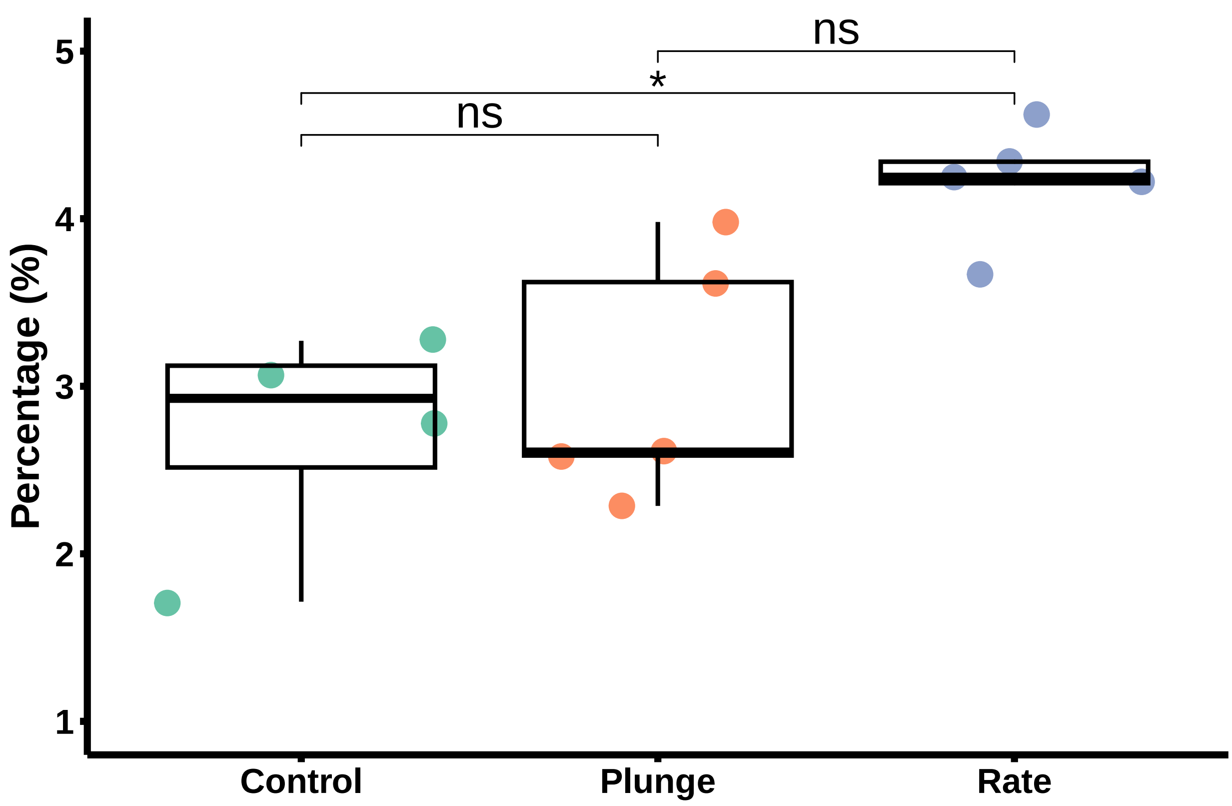
```
##  7 Chitinoph~ Contr~ Plunge     4     5     0.491   4.90 0.644 1     ns
##  8 Chitinoph~ Contr~ Rate       4     5     1.37    4.70 0.232 0.696 ns
##  9 Chitinoph~ Plunge Rate       5     5     1.26    7.97 0.244 0.732 ns
## 10 Ktedonoba~ Contr~ Plunge     4     5    -0.105   6.53 0.92  1     ns
## # i 20 more rows
## # i 1 more variable: Run <int>
```

```
# Clean up by removing objects that are no longer needed
rm(physeq.top10, meta, otudf, combined_df, cols, col, stats, calc_stats, top10otus, stats_list)
```

0.1.13 Plot the graph for Gemmataceae , Uncultivated

```
physeq.a.genus <- subset_taxa(AyBCode.percent, Family == "Gemmataceae")

# Calculate the total abundance of Fusarium for each sample
meta <- data.frame(AyBCode.percent@sam_data)
otudf = as.data.frame(t(as.data.frame(physeq.a.genus@otu_table)))
meta$Bacillaceae = rowSums(otudf)

# Now you can use 'meta_df' in your functions
stat.test1 <- meta %>%
  t_test(Bacillaceae ~ Group) %>%
  adjust_pvalue(method = "bonferroni") %>%
  add_significance()

# Plot a graph of the abundance of Fusarium for each sample grouped by Group:
Gemmataceae.Ori <- ggplot(subset(meta, Group %in% c("Control","Plunge","Rate")),
       aes(x = Group, y = Bacillaceae, colour = interaction(Group))) +
  geom_point(alpha = 1, position = "jitter", size = 4) +
  geom_boxplot(alpha = 0, colour = "black", size = 0.8)+
  theme_classic() +
  labs(x = "", y = "Percentage (%)") +
    stat_pvalue_manual(stat.test1,
                     y.position = c(4.5, 4.75, 5),
                     label = "p.adj.signif",
                     face="bold",
                     size = 6,
                     linetype = 1,
                     tip.length = 0.02,
                     inherit.aes = FALSE) +
  scale_y_continuous(limits=c(1, 5), breaks = c(1, 2, 3, 4, 5)) +
  theme(text = element_text(size=18, colour = "black"),
        axis.ticks = element_line(colour = "black", size = 1.25),
        axis.line = element_line(colour = 'black', size = 1.25),
        axis.text.x = element_text(colour = "black",
                                   angle=0,
                                   size = 13, face="bold"),
        axis.text.y = element_text(angle=0, hjust=0, colour = "black",
                                   size = 13, face="bold"),
        axis.title.y = element_text(color="black", size=15,face="bold"),
        legend.position = "none") +
  scale_color_brewer(palette="Set2")+
```

```
  scale_fill_brewer(palette="Set2")

# pdf(file = "Gemmataceae.ori.pdf", width = 6, height = 5)
Gemmataceae.Ori
```

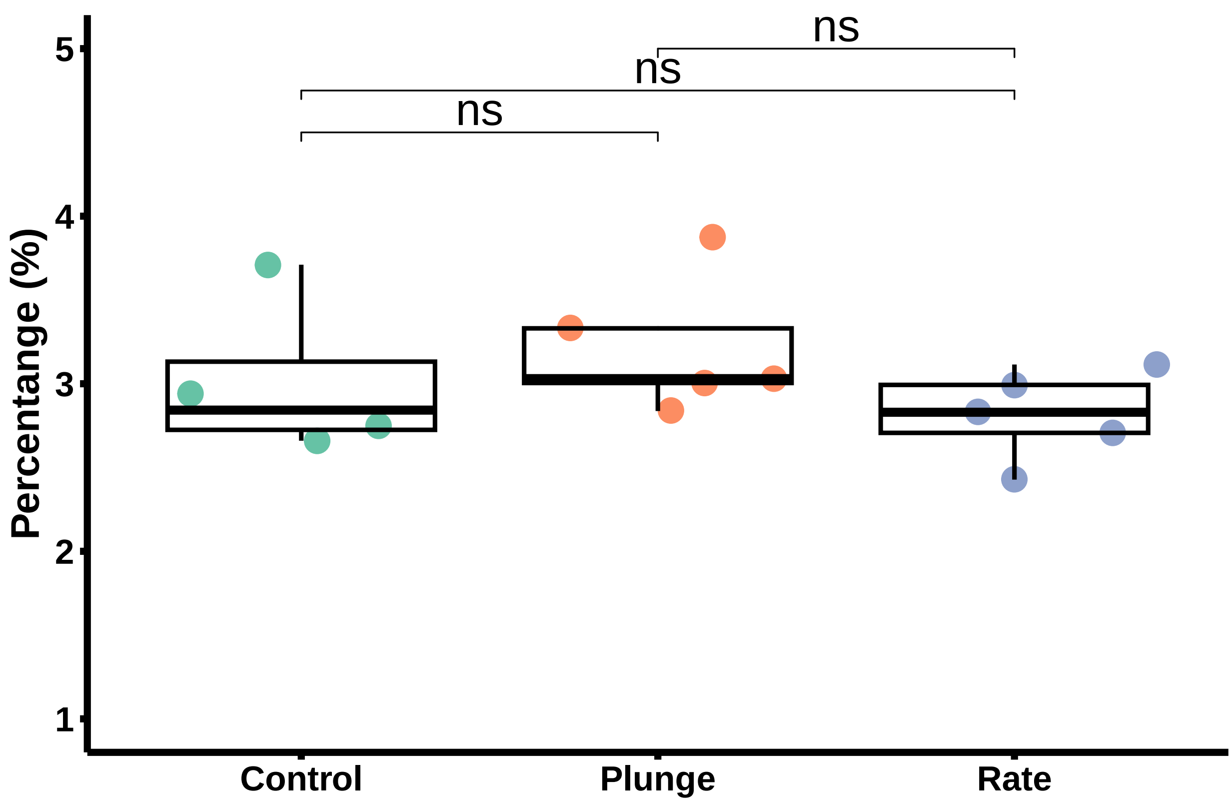



```
# Close the PDF device and save the plot to a file
# dev.off()


# Clean up by removing objects that are no longer needed
rm(physeq.a.genus, meta, otudf, Bacillaceae.Ori)
```

0.1.14 Plot the graph for Bryobacteraceae, Uncultivated

```
physeq.a.genus <- subset_taxa(AyBCode.percent, Family == "Bryobacteraceae")

# Calculate the total abundance of Fusarium for each sample
meta = data.frame(AyBCode.percent@sam_data)
otudf = as.data.frame(t(as.data.frame(physeq.a.genus@otu_table)))
meta$Bryobacteraceae = rowSums(otudf)

stat.test1 <- meta %>%
  t_test(Bryobacteraceae ~ Group) %>%
```

```r
  adjust_pvalue(method = "bonferroni") %>%
  add_significance()

# Plot a graph of the abundance of Fusarium for each sample grouped by Group:
Bryobacteraceae.Ori <- ggplot(subset(meta, Group %in% c("Control","Plunge","Rate")),
             aes(x = Group, y = Bryobacteraceae, colour = interaction(Group))) +
  geom_point(alpha = 1, position = "jitter", size = 4) +
  geom_boxplot(alpha = 0, colour = "black", size = 0.8)+
  theme_classic() +
  labs(x = "", y = "Percentange (%)") +
      stat_pvalue_manual(stat.test1,
                     y.position = c(4.5, 4.75, 5),
                     label = "p.adj.signif",
                     face="bold",
                     size = 6,
                     linetype = 1,
                     tip.length = 0.02,
                     inherit.aes = FALSE) +
  scale_y_continuous(limits=c(1, 5), breaks = c(1, 2, 3, 4 , 5)) +
  theme(text = element_text(size=18, colour = "black"),
        axis.ticks = element_line(colour = "black", size = 1.25),
        axis.line = element_line(colour = 'black', size = 1.25),
        axis.text.x = element_text(colour = "black",
                                   angle=0,
                                   size = 13, face="bold"),
        axis.text.y = element_text(angle=0, hjust=0, colour = "black",
                                   size = 13, face="bold"),
        axis.title.y = element_text(color="black", size=15,face="bold"),
        legend.position = "none") +
  scale_color_brewer(palette="Set2")+
  scale_fill_brewer(palette="Set2")


# pdf(file = "Bryobacteraceae.ori.pdf", width = 6, height = 5)
Bryobacteraceae.Ori
```




```r
# Close the PDF device and save the plot to a file
# dev.off()

# Clean up by removing objects that are no longer needed
rm(meta, otudf, Bryobacteraceae.Ori)
```

```r
## Normalised number of reads in percentage
AyBCode.percent = transform_sample_counts(physeq.norm.rich.group, standf)

# Remove unwanted taxon names
AyBCode.percent.B <- subset_taxa(AyBCode.percent, !get("Family") %in% taxa_to_remove)
AyBCode.percent.B <- aggregate_taxa(AyBCode.percent.B, "Family", verbose = TRUE)
```

0.1.14.1 Enriched treatment

```
## [1] "Remove taxonomic information below the target level"
## [1] "Mark the potentially ambiguous taxa"
## [1] "-- split"
## [1] "-- sum"
## [1] "Create phyloseq object"
## [1] "Remove ambiguous levels"
## [1] "-- unique"
```

```
## [1] "-- Rename the lowest level"
## [1] "-- rownames"
## [1] "-- taxa"
## [1] "Convert to taxonomy table"
## [1] "Combine OTU and Taxon matrix into Phyloseq object"
## [1] "Add the metadata as is"
```

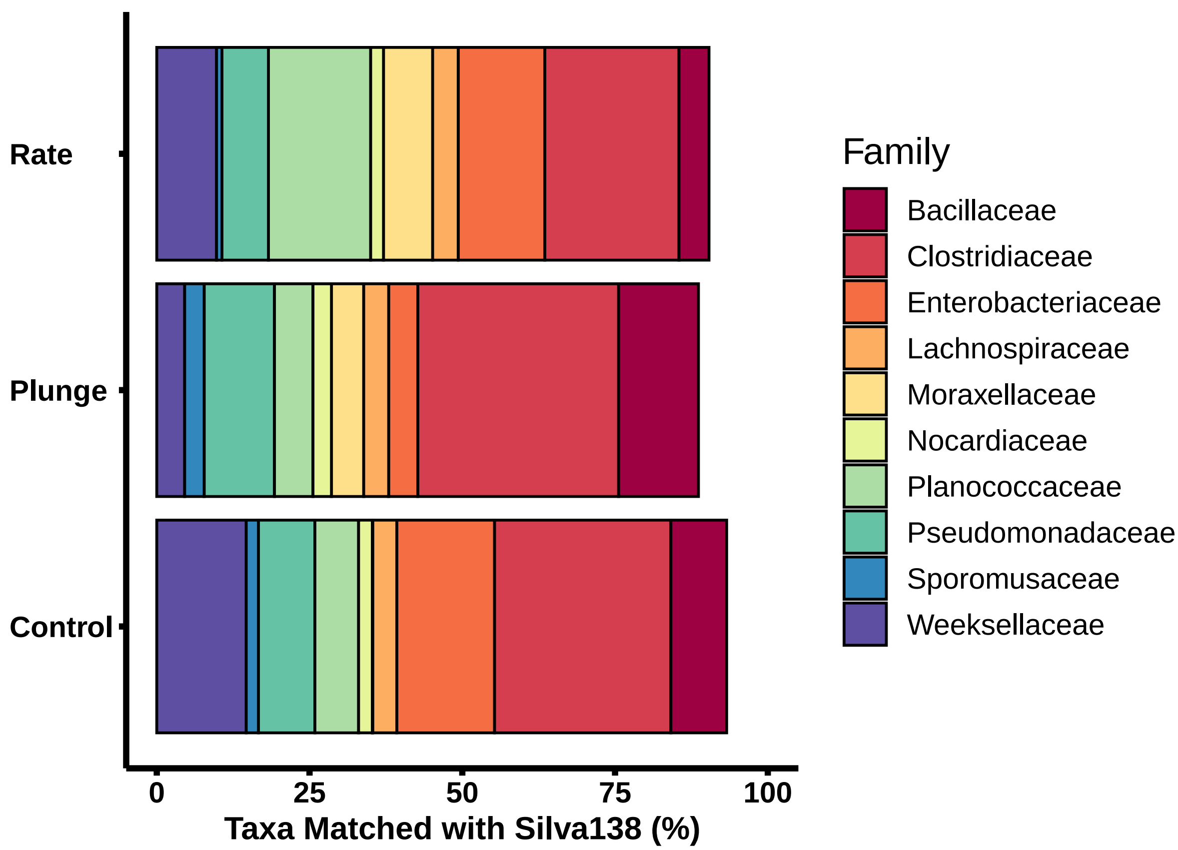
```
top10otus = names(sort(taxa_sums(AyBCode.percent.B), TRUE)[1:10])
taxtab10 = cbind(tax_table(AyBCode.percent.B), Family = NA)
taxtab10[top10otus, "Family"] <- as(tax_table(AyBCode.percent.B)[top10otus, "Family"],"character")
tax_table(AyBCode.percent.B) <- tax_table(taxtab10)



top10plot = prune_taxa(top10otus, AyBCode.percent.B)
print(top10plot@otu_table)
```

```
## OTU Table:          [10 taxa and 3 samples]
##                      taxa are rows
##                         Control    Plunge       Rate
## Nocardiaceae         2.28226721  3.024618  2.0966366
## Weeksellaceae       14.63879639  4.567538  9.7892338
## Bacillaceae          9.12871030 13.069239  4.9176978
## Planococcaceae       7.14868977  6.302729 16.7317711
## Clostridiaceae      28.86527524 32.869044 21.9672225
## Lachnospiraceae      3.95878617  4.076597  4.1803658
## Sporomusaceae        2.00377360  3.183544  0.8770719
## Enterobacteriaceae 15.99012231  4.766890 14.1617756
## Moraxellaceae        0.00779818  5.274863  8.0465632
## Pseudomonadaceae     9.23582322 11.511888  7.6069965
```

```
# Calculate the sum of each column
col_sums <- colSums(as.data.frame(top10plot@otu_table))

# Add a new row with the sums
top10plot.df <- rbind('SUM' = col_sums, as.data.frame(top10plot@otu_table))

# Print the dataframe
print(top10plot.df)
```

```
##                         Control    Plunge       Rate
## SUM                 93.26004240 88.646949 90.3753348
## Nocardiaceae         2.28226721  3.024618  2.0966366
## Weeksellaceae       14.63879639  4.567538  9.7892338
## Bacillaceae          9.12871030 13.069239  4.9176978
## Planococcaceae       7.14868977  6.302729 16.7317711
## Clostridiaceae      28.86527524 32.869044 21.9672225
## Lachnospiraceae      3.95878617  4.076597  4.1803658
## Sporomusaceae        2.00377360  3.183544  0.8770719
## Enterobacteriaceae 15.99012231  4.766890 14.1617756
## Moraxellaceae        0.00779818  5.274863  8.0465632
## Pseudomonadaceae     9.23582322 11.511888  7.6069965
```

```r
top10.rich <- plot_bar(top10plot, fill = "Family") + coord_flip() +
  ylab("Taxa Matched with Silva138 (%)") + ylim(0, 100) +
  theme_classic() +
  theme(text = element_text(size=14, colour = "black"),
        axis.ticks = element_line(colour = "black", size = 1.1),
        axis.line = element_line(colour = 'black', size = 1.1),
        axis.text.x = element_text(colour = "black", angle=0, size = 11, face="bold"),
        axis.text.y = element_text(angle=0, hjust=0, colour = "black", size = 11, face="bold"),
        axis.title.y = element_text(color="black", size=12,face="bold"),
        axis.title.x = element_text(color="black", size=12,face="bold"),
        legend.position = "right") +
  scale_color_brewer(palette="Spectral")+
  scale_fill_brewer(palette="Spectral") +
  xlab("") # This line removes the x-axis label

# pdf(file = "top10.rich.pdf", width = 6.75, height = 5)
top10.rich
```




```r
# Close the PDF device and save the plot to a file
# dev.off()

# Clean up by removing objects that are no longer needed
rm(physeq.ori, physeq.rich, AyBCode,
   AyBCode.percent.B, taxtab10, top10plot, top10.ori, top10.rich)
```

0.1.15 Calculate the statistics in percentange on the top 10 family level

```r
## Normalised number of reads in percentage
AyBCode.percent = transform_sample_counts(physeq.norm.rich, standf)

# Subset the phyloseq object for the top 10 OTUs
physeq.top10 <- subset_taxa(AyBCode.percent, Family %in% top10otus)

# Aggregate taxa at the genus level
physeq.top10 <- aggregate_taxa(physeq.top10, "Family", verbose = TRUE)
```

```
## [1] "Remove taxonomic information below the target level"
## [1] "Mark the potentially ambiguous taxa"
## [1] "-- split"
## [1] "-- sum"
## [1] "Create phyloseq object"
## [1] "Remove ambiguous levels"
## [1] "-- unique"
## [1] "-- Rename the lowest level"
## [1] "-- rownames"
## [1] "-- taxa"
## [1] "Convert to taxonomy table"
## [1] "Combine OTU and Taxon matrix into Phyloseq object"
## [1] "Add the metadata as is"
```

```r
# Calculate the total abundance of Fusarium for each sample
meta = AyBCode.percent@sam_data
otudf = as.data.frame(t(as.data.frame(physeq.top10@otu_table)))

# Assuming 'meta' and 'otudf' are your data frames
combined_df <- merge(meta, otudf, by = "row.names", all = TRUE)

# Set row names of the combined data frame
rownames(combined_df) <- combined_df$Row.names

# Remove the 'Row.names' column
combined_df$Row.names <- NULL

# Get the column names from "Nocardiaceae" onwards
cols <- colnames(combined_df)[which(colnames(combined_df) == "Nocardiaceae"):ncol(combined_df)]

# Initialize an empty data frame to store the test results
stat.test_df <- data.frame()

# Loop over the columns
for(i in seq_along(cols)){
  # Perform the t-test for each column
  stats <- combined_df %>%
    t_test(reformulate("Group", response=cols[i])) %>%
    adjust_pvalue(method = "bonferroni") %>%
    add_significance()
```

```r
  # Add a new column to record the run number
  stats$Run <- i

  # Bind the results to the data frame
  stat.test_df <- rbind(stat.test_df, stats)
}

# Print the data frame
print(stat.test_df)
```

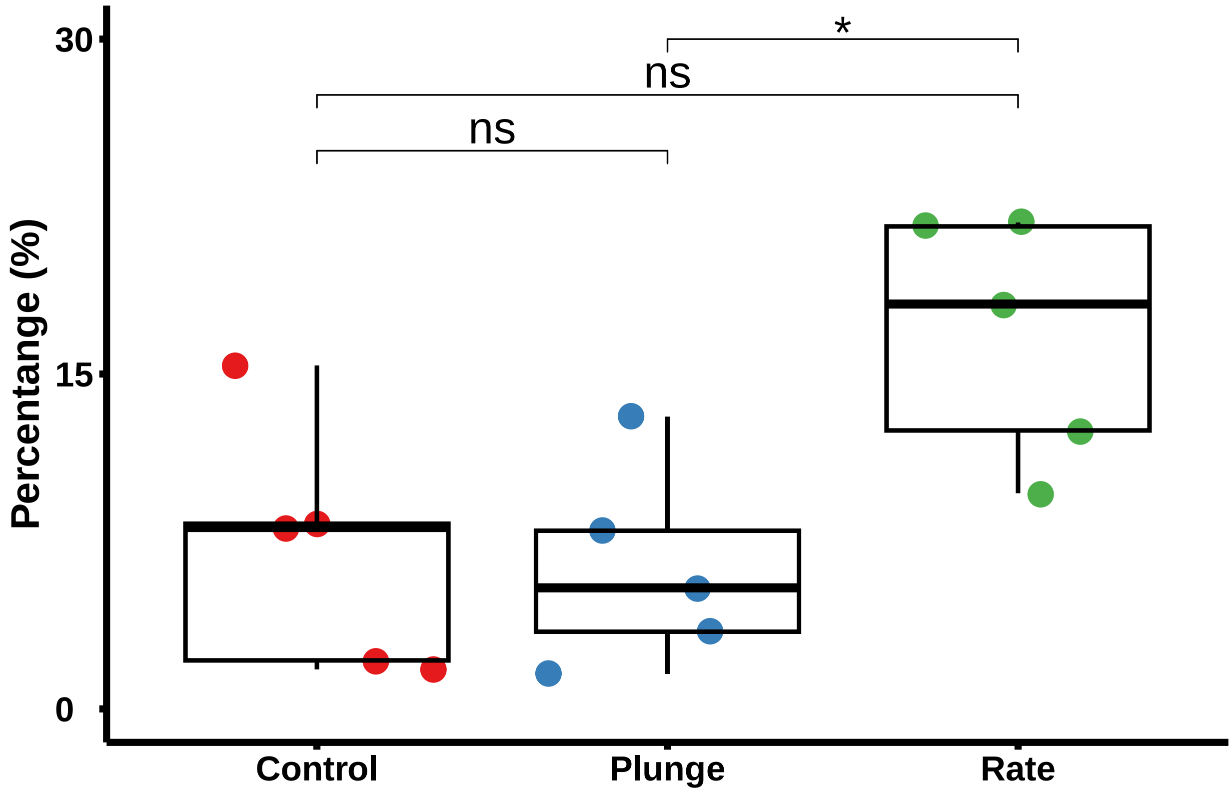
```
## # A tibble: 30 x 11
##    .y.        group1 group2    n1    n2 statistic    df     p p.adj p.adj.signif
##    <chr>      <chr>  <chr>  <int> <int>     <dbl> <dbl> <dbl> <dbl> <chr>
##  1 Nocardiac~ Contr~ Plunge     5     5    -0.871  7.42 0.411 1     ns
##  2 Nocardiac~ Contr~ Rate       5     5     0.298  7.12 0.774 1     ns
##  3 Nocardiac~ Plunge Rate       5     5     1.21   6.02 0.272 0.816 ns
##  4 Weeksella~ Contr~ Plunge     5     5     1.70   6.09 0.139 0.417 ns
##  5 Weeksella~ Contr~ Rate       5     5     0.699  7.86 0.505 1     ns
##  6 Weeksella~ Plunge Rate       5     5    -0.977  6.60 0.363 1     ns
##  7 Bacillace~ Contr~ Plunge     5     5    -2.21   6.56 0.066 0.198 ns
##  8 Bacillace~ Contr~ Rate       5     5     1.55   7.06 0.164 0.492 ns
##  9 Bacillace~ Plunge Rate       5     5     3.36   5.31 0.018 0.054 ns
## 10 Planococc~ Contr~ Plunge     5     5     0.265  7.65 0.798 1     ns
## # i 20 more rows
## # i 1 more variable: Run <int>
```

```r
# Clean up by removing objects that are no longer needed
rm(physeq.top10, meta, otudf, combined_df, cols, col, stats, calc_stats, top10otus)
```

0.1.16 Plot the graph for Clostridiaceae, Enriched

```r
## Normalised number of reads in percentage
AyBCode.percent = transform_sample_counts(physeq.norm.rich, standf)

physeq.a.genus <- subset_taxa(AyBCode.percent, Family == "Planococcaceae")

# Calculate the total abundance of Planococcaceae for each sample
meta = data.frame(AyBCode.percent@sam_data)
otudf = as.data.frame(t(as.data.frame(physeq.a.genus@otu_table)))
meta$Planococcaceae = rowSums(otudf)

stat.test1 <- meta %>%
  t_test(Planococcaceae ~ Group) %>%
  adjust_pvalue(method = "bonferroni") %>%
  add_significance()

# Plot a graph of the abundance of Planococcaceae for each sample grouped by Group:
Planococcaceae.Rich <- ggplot(subset(meta, Group %in% c("Control","Plunge","Rate")),
                              aes(x = Group, y = Planococcaceae, colour = interaction(Group))) +
  geom_point(alpha = 1, position = "jitter", size = 4) +
  geom_boxplot(alpha = 0, colour = "black", size = 0.8)+
```

```
  theme_classic() +
  labs(x = "", y = "Percentange (%)") +
      stat_pvalue_manual(stat.test1,
                         y.position = c(25, 27.5, 30),
                         label = "p.adj.signif",
                         face="bold",
                         size = 6,
                         linetype = 1,
                         tip.length = 0.02,
                         inherit.aes = FALSE) +
  scale_y_continuous(limits=c(0, 30), breaks = c(0, 15, 30)) +
  theme(text = element_text(size=18, colour = "black"),
        axis.ticks = element_line(colour = "black", size = 1.25),
        axis.line = element_line(colour = 'black', size = 1.25),
        axis.text.x = element_text(colour = "black",
                                   angle=0,
                                   size = 13, face="bold"),
        axis.text.y = element_text(angle=0, hjust=0, colour = "black",
                                   size = 13, face="bold"),
        axis.title.y = element_text(color="black", size=15,face="bold"),
        legend.position = "none") +
  scale_color_brewer(palette="Set1")+
  scale_fill_brewer(palette="Set1")

# pdf(file = "Planococcaceae.Rich.pdf", width = 6, height = 5)
Planococcaceae.Rich
```

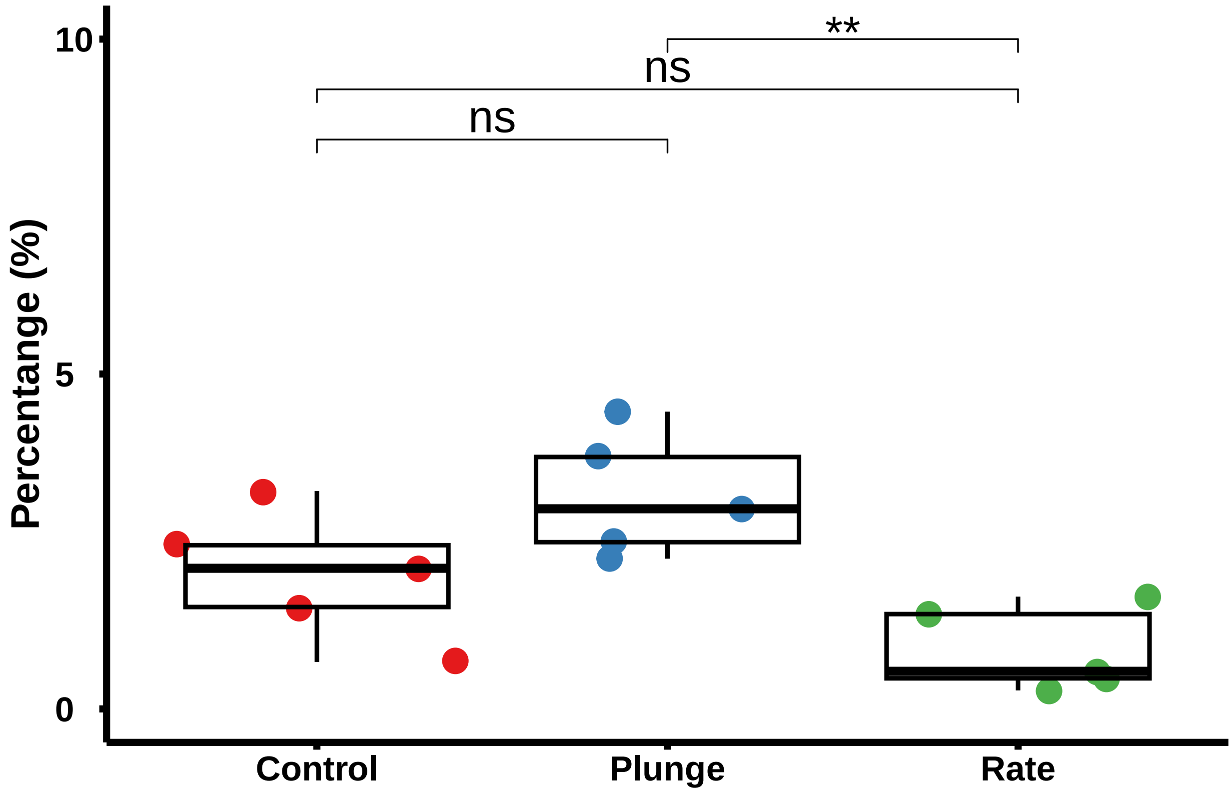



```
# Close the PDF device and save the plot to a file
# dev.off()

# Clean up by removing objects that are no longer needed
rm(physeq.a.genus, meta, otudf, Clostridiaceae.Rich)
```

0.1.17 Plot the graph for Moraxellaceae, Enriched

```
## Normalised number of reads in percentage
AyBCode.percent = transform_sample_counts(physeq.norm.rich, standf)
physeq.a.genus <- subset_taxa(AyBCode.percent, Family == "Sporomusaceae")

# Calculate the total abundance of Sporomusaceae for each sample
meta = data.frame(AyBCode.percent@sam_data)
otudf = as.data.frame(t(as.data.frame(physeq.a.genus@otu_table)))
meta$Sporomusaceae = rowSums(otudf)

stat.test1 <- meta %>%
  t_test(Sporomusaceae ~ Group) %>%
  adjust_pvalue(method = "bonferroni") %>%
  add_significance()

# Plot a graph of the abundance of Fusarium for each sample grouped by Group:
Sporomusaceae.Rich <- ggplot(subset(meta, Group %in% c("Control","Plunge","Rate")),
```

```
                              aes(x = Group, y = Sporomusaceae, colour = interaction(Group))) +
  geom_point(alpha = 1, position = "jitter", size = 4) +
  geom_boxplot(alpha = 0, colour = "black", size = 0.8)+
  theme_classic() +
  labs(x = "", y = "Percentange (%)") +
  stat_pvalue_manual(stat.test1,
                     y.position = c(8.5, 9.25, 10),
                     label = "p.adj.signif",
                     face="bold",
                     size = 6,
                     linetype = 1,
                     tip.length = 0.02,
                     inherit.aes = FALSE) +
  scale_y_continuous(limits=c(0, 10), breaks = c(0, 5, 10)) +
  theme(text = element_text(size=18, colour = "black"),
        axis.ticks = element_line(colour = "black", size = 1.25),
        axis.line = element_line(colour = 'black', size = 1.25),
        axis.text.x = element_text(colour = "black",
                                   angle=0,
                                   size = 13, face="bold"),
        axis.text.y = element_text(angle=0, hjust=0, colour = "black",
                                   size = 13, face="bold"),
        axis.title.y = element_text(color="black", size=15,face="bold"),
        legend.position = "none") +
  scale_color_brewer(palette="Set1")+
  scale_fill_brewer(palette="Set1")

# pdf(file = "Sporomusaceae.Rich.pdf", width = 6, height = 5)
Sporomusaceae.Rich
```



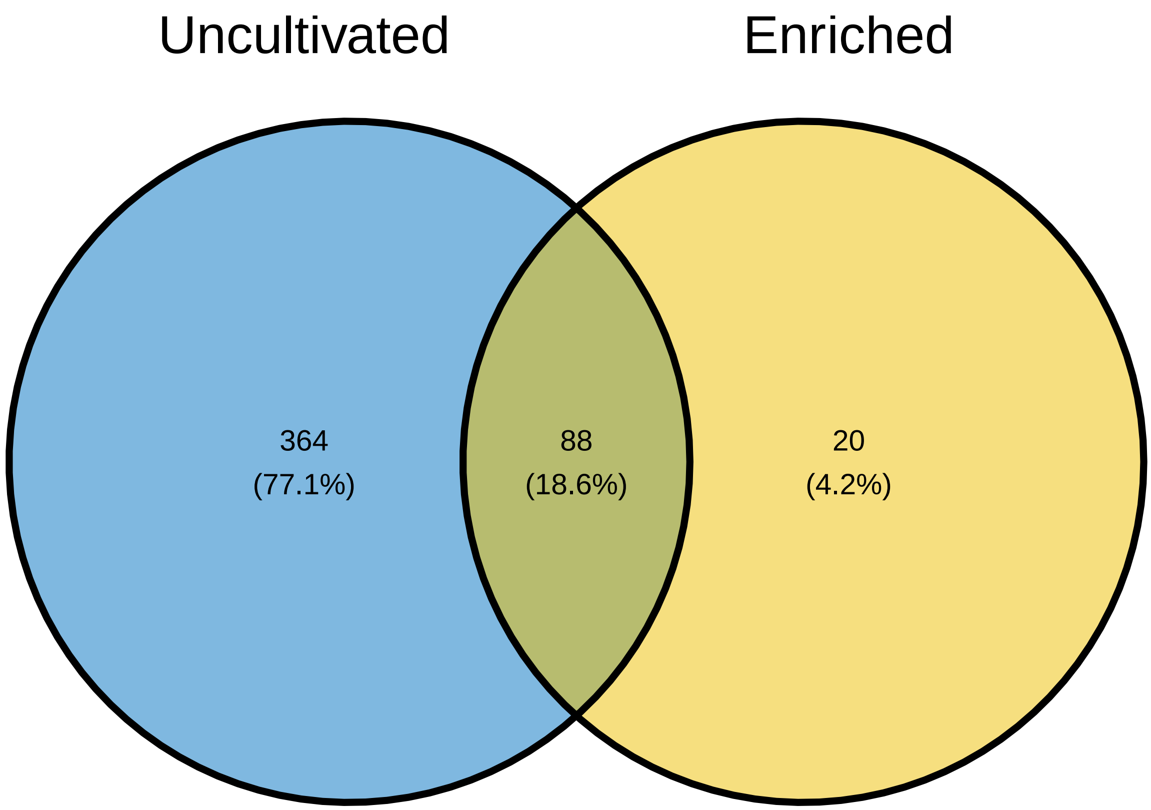

```
# Close the PDF device and save the plot to a file
# dev.off()

# Clean up by removing objects that are no longer needed
rm(physeq.a.genus, meta, otudf, AyBCode.percent,
   stat.test1, Sporomusaceae.Rich, stats_list)
```

```
# Aggregate taxa at the genus level
B <- aggregate_taxa(physeq.norm.group, "Genus", verbose = TRUE)
```

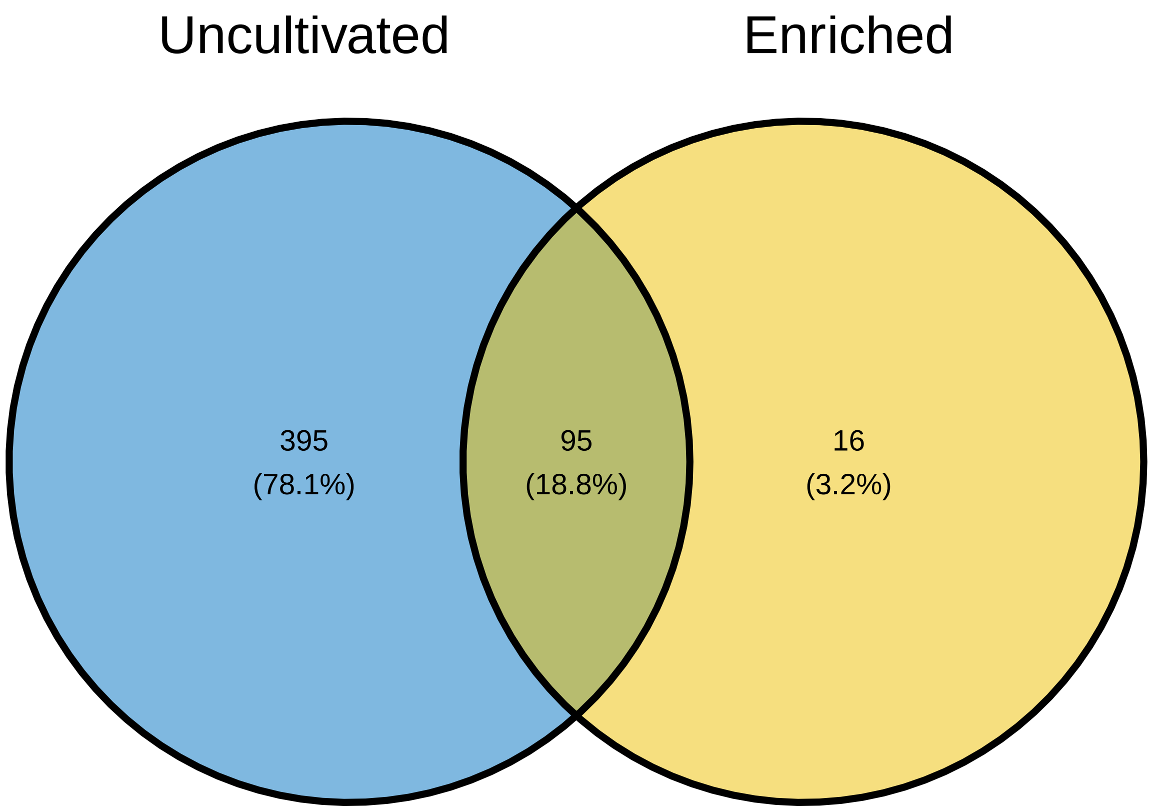
```
## [1] "Remove taxonomic information below the target level"
## [1] "Mark the potentially ambiguous taxa"
## [1] "-- split"
## [1] "-- sum"
## [1] "Create phyloseq object"
## [1] "Remove ambiguous levels"
## [1] "-- unique"
## [1] "-- Rename the lowest level"
## [1] "-- rownames"
## [1] "-- taxa"
## [1] "Convert to taxonomy table"
## [1] "Combine OTU and Taxon matrix into Phyloseq object"
## [1] "Add the metadata as is"
```

```r
# Remove undesired genera
# B2 <- subset_taxa(B, !get("Genus") %in% c("uncultured", "Unknown"))

# Remove unwanted taxon names
taxa_to_remove <- c("uncultured", "Unknown")
B2 <- subset_taxa(B, !get("Genus") %in% taxa_to_remove)

# Extract relevant data from the phyloseq object
sample_data <- sample_data(B2)
otu_table <- otu_table(B2)
abundance <- as.vector(otu_table)



# Create a tibble with the extracted data
D <- tibble(
  Sample = rep(sample_data$Category, each = nrow(otu_table)),
  ASV = rep(rownames(otu_table), times = ncol(otu_table)),
  Abundance = abundance
) %>%
  group_by(Sample) %>%
  mutate(rank = rank(plyr::desc(Abundance))) %>%
   filter(Abundance > 15) %>%
  ungroup() %>%
  select(Sample, Abundance, ASV)

# Remove the Abundance column
D$Abundance <- NULL

# Rename the second column to "ASV"
names(D)[2] <- "ASV"
names(D)[1] <- "Category"

# Convert data from long to wide format
E <- dcast(D, ASV ~ Category)

# Define a binary function
binary_fun <- function(x) {
  x[is.na(x)] <- 0
  ifelse(x > 0, 1, 0)
}

# Apply the binary function to columns 2 to 4
df.Category.family <- apply(E[2:7], 2, binary_fun)
df.Category.family <- as.data.frame(df.Category.family)
rownames(df.Category.family) <- E$ASV


# Clean up by removing unnecessary objects
rm(B,B2, sample_data,
   otu_table, abundance, D, E,
   binary_fun, col, binary_fun, UpSet.Ori, otudf)
```

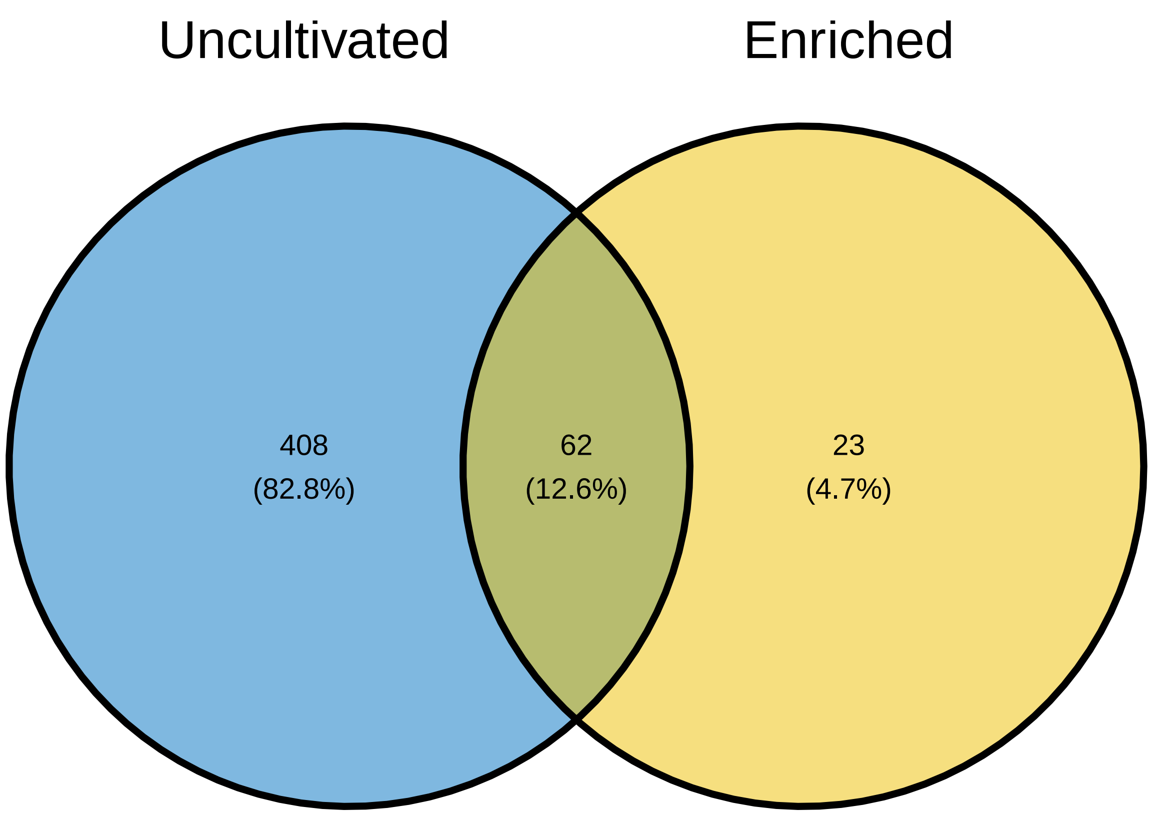
```r
# Extract the rows where the value is 1 for each column
Uncultivated.Control <- rownames(df.Category.family)[df.Category.family$Uncultivated.Control == 1]
Enriched.Control <- rownames(df.Category.family)[df.Category.family$Enriched.Control == 1]

Uncultivated.Plunge <- rownames(df.Category.family)[df.Category.family$Uncultivated.Plunge == 1]
Enriched.Plunge <- rownames(df.Category.family)[df.Category.family$Enriched.Plunge == 1]

Uncultivated.Rate <- rownames(df.Category.family)[df.Category.family$Uncultivated.Rate == 1]
Enriched.Rate <- rownames(df.Category.family)[df.Category.family$Enriched.Rate == 1]

# Create a list with the extracted data
list_data.1 <- list("Uncultivated" = Uncultivated.Control, "Enriched" = Enriched.Control)
list_data.2 <- list("Uncultivated" = Uncultivated.Plunge, "Enriched" = Enriched.Plunge)
list_data.3 <- list("Uncultivated" = Uncultivated.Rate, "Enriched" = Enriched.Rate)

# Use ggvenn to create the Venn diagram
Venn.1 <- ggvenn(
  list_data.1,
  fill_color = c("#0073C2FF", "#EFC000FF"),
  stroke_size = 1.25, set_name_size = 7
)

# Open a new PDF graphics device
# pdf(file = "Venn_control.pdf", width=5,height=5)

# Print the Venn plot
print(Venn.1)
```




```r
# Close the PDF device and save the plot to a file
# dev.off()


# Use ggvenn to create the Venn diagram
Venn.2 <- ggvenn(
  list_data.2,
  fill_color = c("#0073C2FF", "#EFC000FF"),
  stroke_size = 1.25, set_name_size = 7
)

# Open a new PDF graphics device
# pdf(file = "Venn_Plunge.pdf", width=5,height=5)

# Print the Venn plot
print(Venn.2)
```




```r
# Close the PDF device and save the plot to a file
# dev.off()

# Use ggvenn to create the Venn diagram
Venn.3 <- ggvenn(
  list_data.3,
  fill_color = c("#0073C2FF", "#EFC000FF"),
  stroke_size = 1.25, set_name_size = 7
)

# Open a new PDF graphics device
# pdf(file = "Venn_Rate.pdf", width=5,height=5)

# Print the Venn plot
print(Venn.3)
```




```r
# Close the PDF device and save the plot to a file
# dev.off()
```

```r
sessionInfo()
```

```
## R version 4.3.2 (2023-10-31 ucrt)
## Platform: x86_64-w64-mingw32/x64 (64-bit)
## Running under: Windows 11 x64 (build 22631)
##
## Matrix products: default
##
##
## locale:
## [1] LC_COLLATE=English_United Kingdom.utf8
## [2] LC_CTYPE=English_United Kingdom.utf8
## [3] LC_MONETARY=English_United Kingdom.utf8
## [4] LC_NUMERIC=C
## [5] LC_TIME=English_United Kingdom.utf8
##
## time zone: Europe/London
## tzcode source: internal
##
## attached base packages:
## [1] grid      stats4    stats     graphics  grDevices utils     datasets
## [8] methods   base
```

```
##
## other attached packages:
##  [1] ggvenn_0.1.10               pairwiseAdonis_0.4.1
##  [3] cluster_2.1.4               vegan_2.6-4
##  [5] lattice_0.21-9              permute_0.9-7
##  [7] qiime2R_0.99.6              microbiome_1.22.0
##  [9] DESeq2_1.40.2               SummarizedExperiment_1.30.2
## [11] Biobase_2.60.0              MatrixGenerics_1.12.3
## [13] matrixStats_1.0.0           GenomicRanges_1.52.1
## [15] GenomeInfoDb_1.36.4         IRanges_2.34.1
## [17] S4Vectors_0.38.2            BiocGenerics_0.46.0
## [19] phyloseq_1.44.0             RColorBrewer_1.1-3
## [21] plyr_1.8.9                  UpSetR_1.4.0
## [23] reshape2_1.4.4              purrr_1.0.2
## [25] rstatix_0.7.2               dplyr_1.1.3
## [27] ggpubr_0.6.0                ggplot2_3.4.4
##
## loaded via a namespace (and not attached):
##  [1] bitops_1.0-7            gridExtra_2.3           rlang_1.1.1
##  [4] magrittr_2.0.3          ade4_1.7-22             compiler_4.3.2
##  [7] mgcv_1.9-0              vctrs_0.6.3             stringr_1.5.0
## [10] pkgconfig_2.0.3         crayon_1.5.2            fastmap_1.1.1
## [13] backports_1.4.1         XVector_0.40.0          labeling_0.4.3
## [16] utf8_1.2.3              rmarkdown_2.25          xfun_0.40
## [19] zlibbioc_1.46.0         jsonlite_1.8.7          biomformat_1.28.0
## [22] rhdf5filters_1.12.1     DelayedArray_0.26.7     Rhdf5lib_1.22.1
## [25] BiocParallel_1.34.2     broom_1.0.5             parallel_4.3.2
## [28] R6_2.5.1                stringi_1.7.12          zCompositions_1.4.1
## [31] rpart_4.1.21            car_3.1-2               Rcpp_1.0.11
## [34] iterators_1.0.14        knitr_1.44              base64enc_0.1-3
## [37] nnet_7.3-19             Matrix_1.6-1.1          splines_4.3.2
## [40] igraph_1.5.1            tidyselect_1.2.0        rstudioapi_0.15.0
## [43] abind_1.4-5             yaml_2.3.7              codetools_0.2-19
## [46] tibble_3.2.1            withr_2.5.2             evaluate_0.22
## [49] Rtsne_0.16              foreign_0.8-85          survival_3.5-7
## [52] Biostrings_2.68.1       pillar_1.9.0            carData_3.0-5
## [55] DT_0.30                 checkmate_2.2.0         foreach_1.5.2
## [58] NADA_1.6-1.1            generics_0.1.3          RCurl_1.98-1.12
## [61] truncnorm_1.0-9         munsell_0.5.0           scales_1.3.0
## [64] glue_1.6.2              Hmisc_5.1-1             tools_4.3.2
## [67] data.table_1.14.8       locfit_1.5-9.8          ggsignif_0.6.4
## [70] rhdf5_2.44.0            tidyr_1.3.0             ape_5.7-1
## [73] colorspace_2.1-0        nlme_3.1-163            GenomeInfoDbData_1.2.10
## [76] htmlTable_2.4.1         Formula_1.2-5           cli_3.6.1
## [79] fansi_1.0.4             S4Arrays_1.0.6          gtable_0.3.4
## [82] digest_0.6.33           farver_2.1.1            htmlwidgets_1.6.2
## [85] htmltools_0.5.6         multtest_2.56.0         lifecycle_1.0.4
## [88] MASS_7.3-60
```