Montana Statewide nifH Analysis

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```
## [1] "2019-10-31-09-55-27"
```

Working Directory

```
## [1] "/home/peterslab/Alex Alleman/Statewide Microbiome Analysis/Statewide analysis"
```

```
set.seed(8765)
```

Load packages

```
library(ggplot2)
library(data.table)
library(vegan)
library(dplyr)
library(scales)
library(grid)
library(reshape2)
library(phyloseq)
library(ggpubr)
library(RColorBrewer)
library(ape)
library(grid)
#library(knitr)
library(igraph)
library(Matrix)
library(ggnetwork)
library(intergraph)
library(Hmisc)
library(parallel)
library(ggrepel)
library(tinytex)
```

Colors

```
farm_col<-(c("#8c510a", "#d8b365", "#f6e8c3", "#f5f5f5", "#c7eae5", "#5ab4ac", "#01665e"))
farm_col_dark<-brewer.pal(7, "Dark2")
farm_col_paired<-(c('#fdbf6f','#ff7f00','#b2df8a','#33a02c','#fb9a99','#e31a1c','#cab2d6','#a6cee3','#1
farm_col_paired<-(c('#fdbf6f','#ff7f00','#b2df8a','#33a02c','#fb9a99','#e31a1c','#cab2d6','#a6cee3','#1
```

Load OTU, Taxa, and Meta data

Add OTU table with sample names on top and OTU names as row names

```
OTU_nifH<- read.delim(
  "~/Alex Alleman/Statewide Microbiome Analysis/Statewide analysis/nifH_OTUallspring.csv",
  row.names = 1)
head(OTU_nifH)[,1:10]
```

	JZ017	JZ018	JZ019	JZ020	JZ021	JZ022	JZ023	JZ024	JZ025	JZ026
OTU1	21	32923	48199	22	117	103	147	4054	5597	246
OTU2	35281	5	15	1075	101	1335	26249	376	1705	256
OTU3	11	14	13	49	20	48	9	8	16	9
OTU4	80	4	14	2576	144	136	150	129	29299	77807
OTU5	6	2	7	146	749	526	432	1115	13256	0
OTU6	19317	11274	799	748	1532	348	32	21	28	2517

These taxa were also created by Mr. DNA through a blast program and the NCBI database

```
tax_nifH<- read.delim(
  "~/Alex Alleman/Statewide Microbiome Analysis/Statewide analysis/nifH_OTU_ids_2016_fixed1.txt",
  row.names = 1)
head(tax_nifH)[,1:8]
```

	kingdom	phylum	class	order	family	genus
OTU1	k___bacteria	p___firmicutes	c___bacilli	o___bacillales	f___paenibacillaceae	g___pae
OTU2	k___bacteria	p___firmicutes	c___bacilli	o___bacillales	f___paenibacillaceae	g___pae
OTU3	k___bacteria	p___proteobacteria	c___alphaproteobacteria	o___rhizobiales	f___rhizobiaceae	g___rhiz
OTU4	k___bacteria	p___firmicutes	c___bacilli	o___bacillales	f___paenibacillaceae	g___pae
OTU5	k___bacteria	p___proteobacteria	c___alphaproteobacteria	o___rhizobiales	f___bradyrhizobiaceae	g___bra
OTU6	k___bacteria	p___proteobacteria	c___alphaproteobacteria	o___rhizobiales	f___bradyrhizobiaceae	g___rho

Meta data set has be placed together from all the spring and summer data with excel

```
meta<- read.delim(
  "~/Alex Alleman/Statewide Microbiome Analysis/Statewide analysis/all_metadata_summer.csv",
                  colClasses = c(rep('factor', 7), rep('numeric', 3), rep('factor', 4), 'numeric',
                                 rep('factor', 3), rep('numeric', 28) ) )
head(meta)[,1:5]
```

	Site	ARC	Season	Sample_dates	Pea_variety
JZ032	Kalispell	NWARC	Summer	2016-summer	Delta
JZ031	Kalispell	NWARC	Summer	2016-summer	CDC Saffron
JZ030	Kalispell	NWARC	Summer	2016-summer	AC Earlystar
JZ034	Kalispell	NWARC	Summer	2016-summer	Majoret
JZ033	Kalispell	NWARC	Summer	2016-summer	DS Admiral
JZ035	Kalispell	NWARC	Summer	2016-summer	Navarro

Removed all Havre for analysis because samples were taken in 6" sections instead of 12"

```
meta2 <- meta[-c(19:48),]
sapply(meta2, class)
```

```
##             Site              ARC           Season     Sample_dates
##         "factor"         "factor"         "factor"         "factor"
##      Pea_variety             Plot    season_precip        irrgation
##         "factor"         "factor"        "numeric"        "numeric"
## total_precip_irr     sample_depth             Date          Tillage
##        "numeric"         "factor"         "factor"         "factor"
##        prev_crop      grain_yield        elevation              lat
##         "factor"        "numeric"         "factor"         "factor"
##              lon   Organic_Matter Moisture_Content  Nitrate_Nitrite
##         "factor"        "numeric"        "numeric"        "numeric"
##          Ammonia    Av_Phosphorus     Av_Potassium   Sulfate_Sulfur
##        "numeric"        "numeric"        "numeric"        "numeric"
##               pH            Boron          Arsenic           Barium
##        "numeric"        "numeric"        "numeric"        "numeric"
##          Cadmium          Calcium         Chromium           Cobalt
##        "numeric"        "numeric"        "numeric"        "numeric"
##           Copper             Iron             Lead        Magnesium
##        "numeric"        "numeric"        "numeric"        "numeric"
##        Manganese       Molybdenum           Nickel       Phosphorus
##        "numeric"        "numeric"        "numeric"        "numeric"
##        Potassium           Sodium           Sulfur         Vanadium
##        "numeric"        "numeric"        "numeric"        "numeric"
##             Zinc
##        "numeric"
```

Convert to matrix

```
OTU_nifH_m<-as.matrix(OTU_nifH)
tax_nifH_m<-as.matrix(tax_nifH)
meta_m<-as.matrix(meta2)

class(OTU_nifH_m)
```

```
## [1] "matrix"
```

```
class(tax_nifH_m)
```

```
## [1] "matrix"
```

```
class(meta_m)
```

```
## [1] "matrix"
```

Make phyloseq object

```
OTUnifH = otu_table(OTU_nifH_m, taxa_are_rows = TRUE)
TAXnifH = tax_table(tax_nifH_m)

physeq_nifH = phyloseq(OTUnifH, TAXnifH)
```

Get physeq info

```
physeq_nifH
```

```
## phyloseq-class experiment-level object
## otu_table()   OTU Table:         [ 8821 taxa and 101 samples ]
## tax_table()   Taxonomy Table:    [ 8821 taxa by 8 taxonomic ranks ]
```

Add meta data to phyloseq object

```
meta_phy <- sample_data(meta2)
sample_names(meta_phy)
```

```
##  [1] "JZ032" "JZ031" "JZ030" "JZ034" "JZ033" "JZ035" "JZ040" "JZ046"
##  [9] "JZ042" "JZ044" "JZ038" "JZ036" "JZ041" "JZ047" "JZ043" "JZ045"
## [17] "JZ039" "JZ037" "JZ081" "JZ078" "JZ082" "JZ079" "JZ080" "JZ083"
## [25] "JZ105" "JZ107" "JZ103" "JZ102" "JZ106" "JZ104" "JZ084" "JZ085"
## [33] "JZ086" "JZ087" "JZ088" "JZ089" "JZ090" "JZ091" "JZ092" "JZ093"
## [41] "JZ094" "JZ095" "JZ096" "JZ097" "JZ098" "JZ099" "JZ100" "JZ101"
## [49] "JZ112" "JZ109" "JZ110" "JZ111" "JZ108" "JZ113"
```

```
physeq_nifH<-merge_phyloseq(physeq_nifH, meta_phy)
physeq_nifH
```

```
## phyloseq-class experiment-level object
## otu_table()   OTU Table:         [ 8821 taxa and 54 samples ]
## sample_data() Sample Data:       [ 54 samples by 45 sample variables ]
## tax_table()   Taxonomy Table:    [ 8821 taxa by 8 taxonomic ranks ]
```

```
physeq_nifH<-rarefy_even_depth(physeq_nifH)
```

```
## You set `rngseed` to FALSE. Make sure you've set & recorded
##  the random seed of your session for reproducibility.
## See `?set.seed`
```

```
## ...
```

```
## 3610OTUs were removed because they are no longer
## present in any sample after random subsampling
```

```
## ...
```

```
physeq_nifH
```

```
## phyloseq-class experiment-level object
## otu_table()   OTU Table:         [ 5211 taxa and 54 samples ]
## sample_data() Sample Data:       [ 54 samples by 45 sample variables ]
## tax_table()   Taxonomy Table:    [ 5211 taxa by 8 taxonomic ranks ]
```

```
OTU_nifH_rare = as(otu_table(physeq_nifH), "matrix")
OTU_nifH_rare = as.data.frame(OTU_nifH_rare)
```

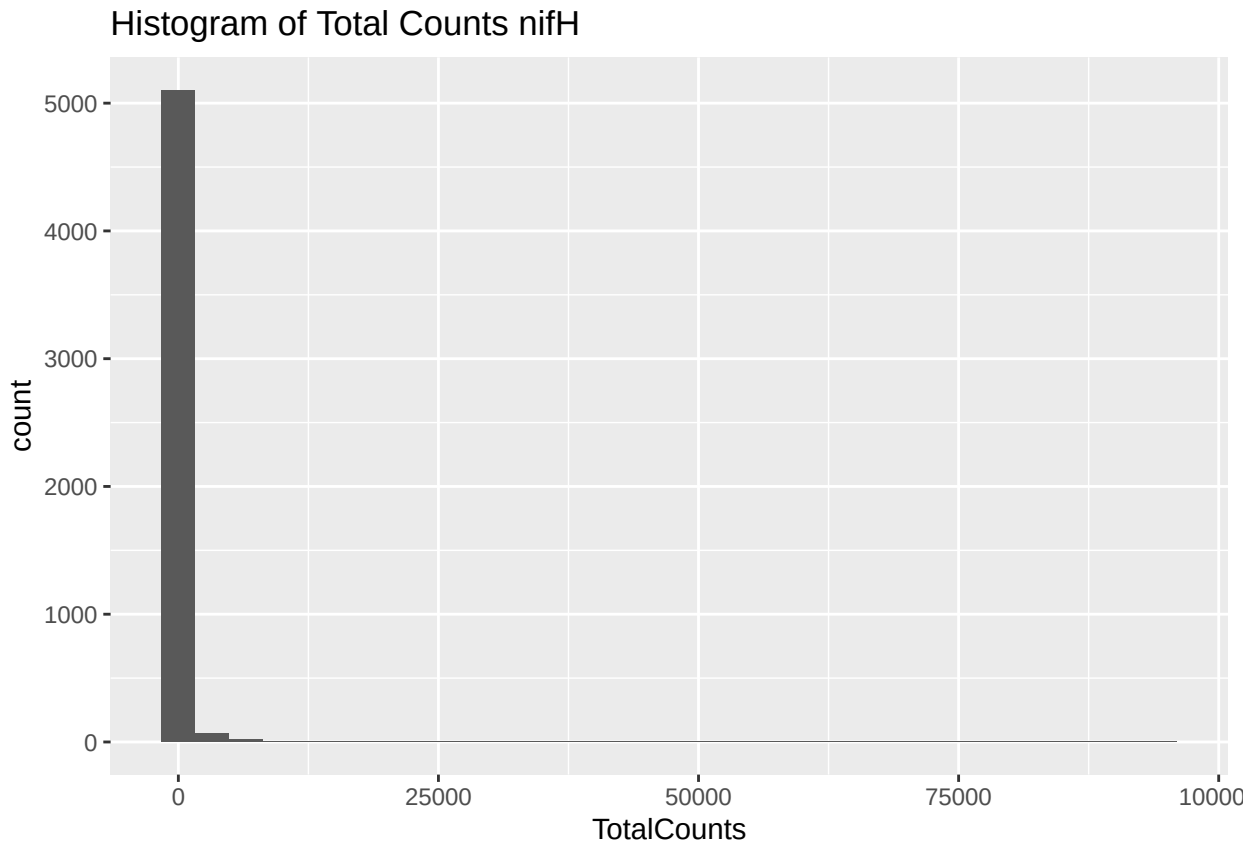
Trim data

Trim data to exclude OTUs that are not in any samples

Source of trim protocol http://evomics.org/wp-content/uploads/2016/01/phyloseq-Lab-01-Answers.html#taxa-total-counts-histogram

```
tdt_nifH = data.table(tax_table(physeq_nifH),
                 TotalCounts = taxa_sums(physeq_nifH),
                 OTU = taxa_names(physeq_nifH))
ggplot(tdt_nifH, aes(TotalCounts)) +
  geom_histogram() +
  ggtitle("Histogram of Total Counts nifH")
```

```
## `stat_bin()` using `bins = 30`. Pick better value with `binwidth`.
```




```
# How many OTUS have low count (Rare)?
tdt_nifH[(TotalCounts <= 0), .N]#zero count
```

```
## [1] 0
```

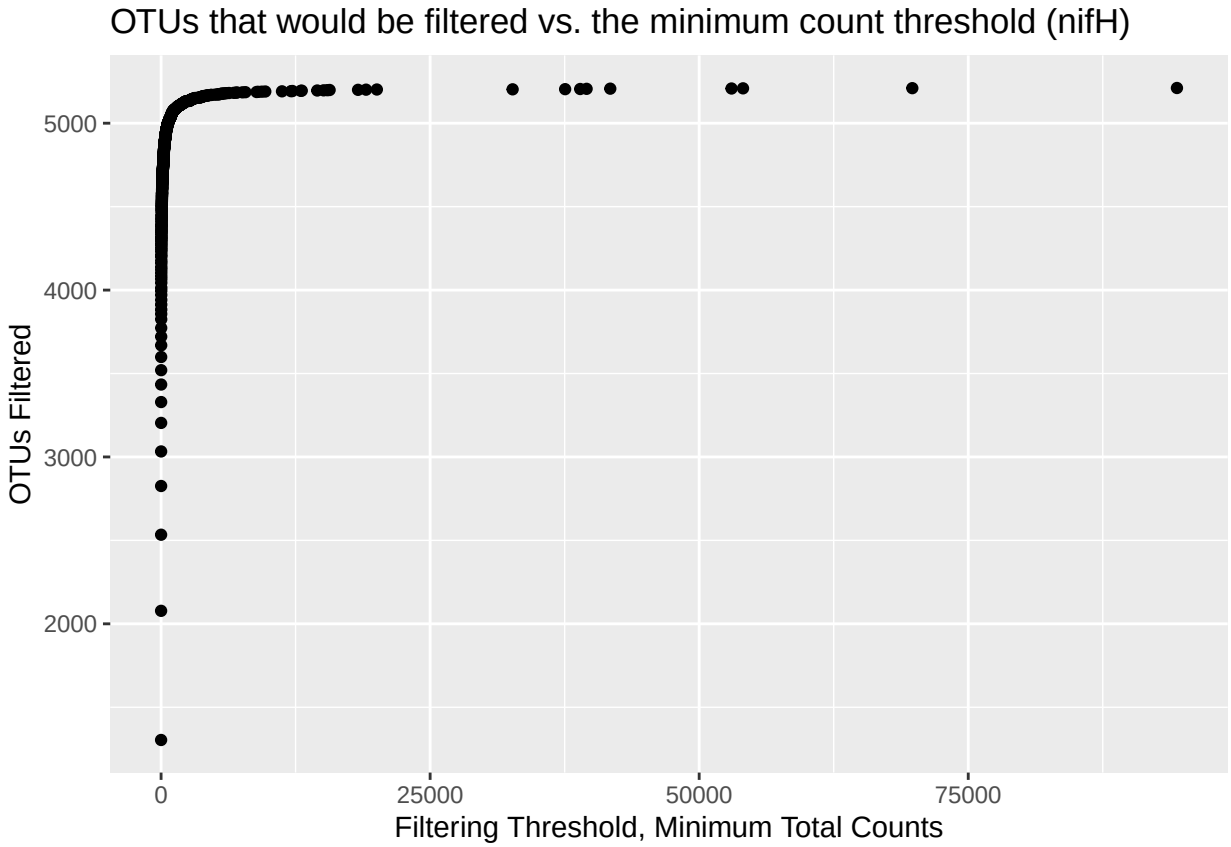
```
tdt_nifH[(TotalCounts <= 1), .N]#single count
```

```
## [1] 1304
```

```
tdt_nifH[(TotalCounts <= 2), .N]#double count
```

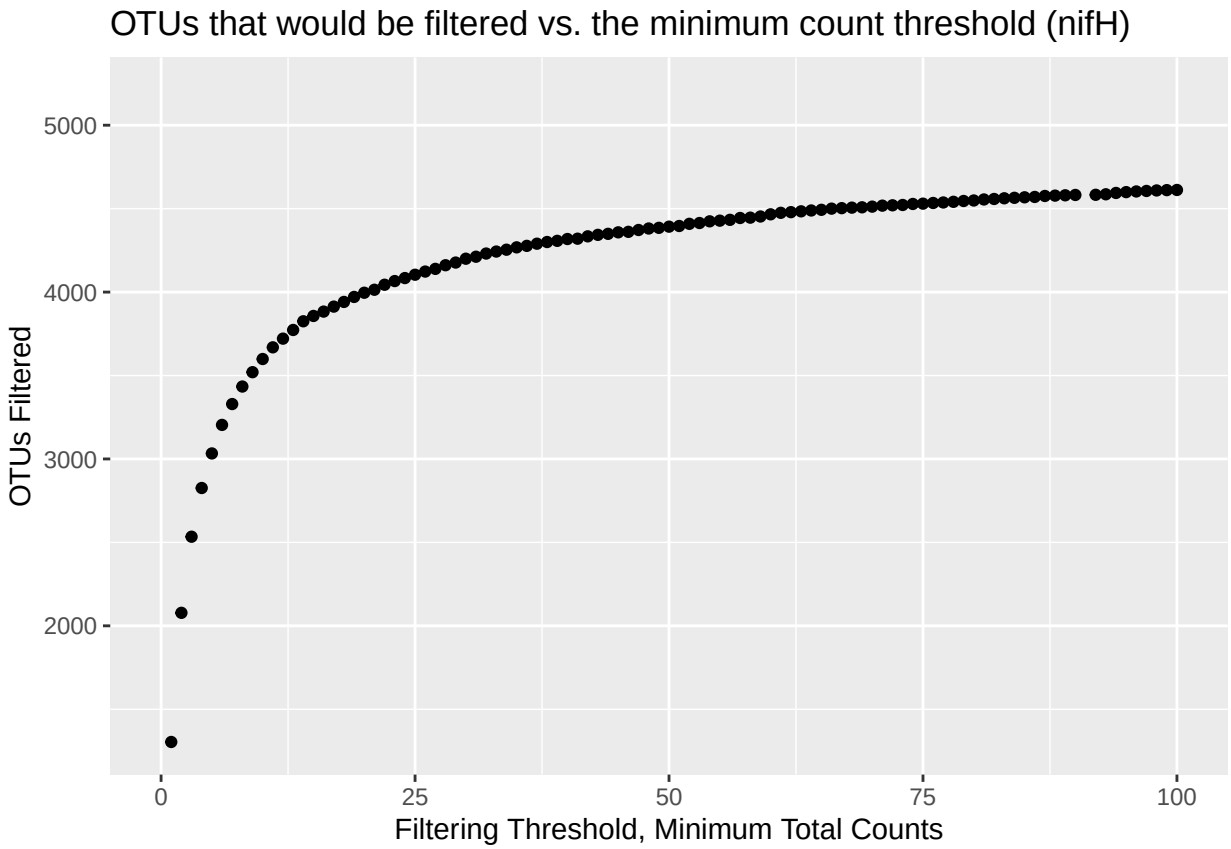
```
## [1] 2078
```

```
# taxa cumulative sum
taxcumsum_nifH = tdt_nifH[, .N, by = TotalCounts]
setkey(taxcumsum_nifH, TotalCounts)
taxcumsum_nifH[, CumSum := cumsum(N)]
# Define the plot
pCumSum_nifH = ggplot(taxcumsum_nifH, aes(TotalCounts, CumSum)) +
  geom_point() +
  xlab("Filtering Threshold, Minimum Total Counts") +
  ylab("OTUs Filtered") +
  ggtitle("OTUs that would be filtered vs. the minimum count threshold (nifH)")
pCumSum_nifH
```



Zoom in

```
pCumSum_nifH + xlim(0, 100)
```



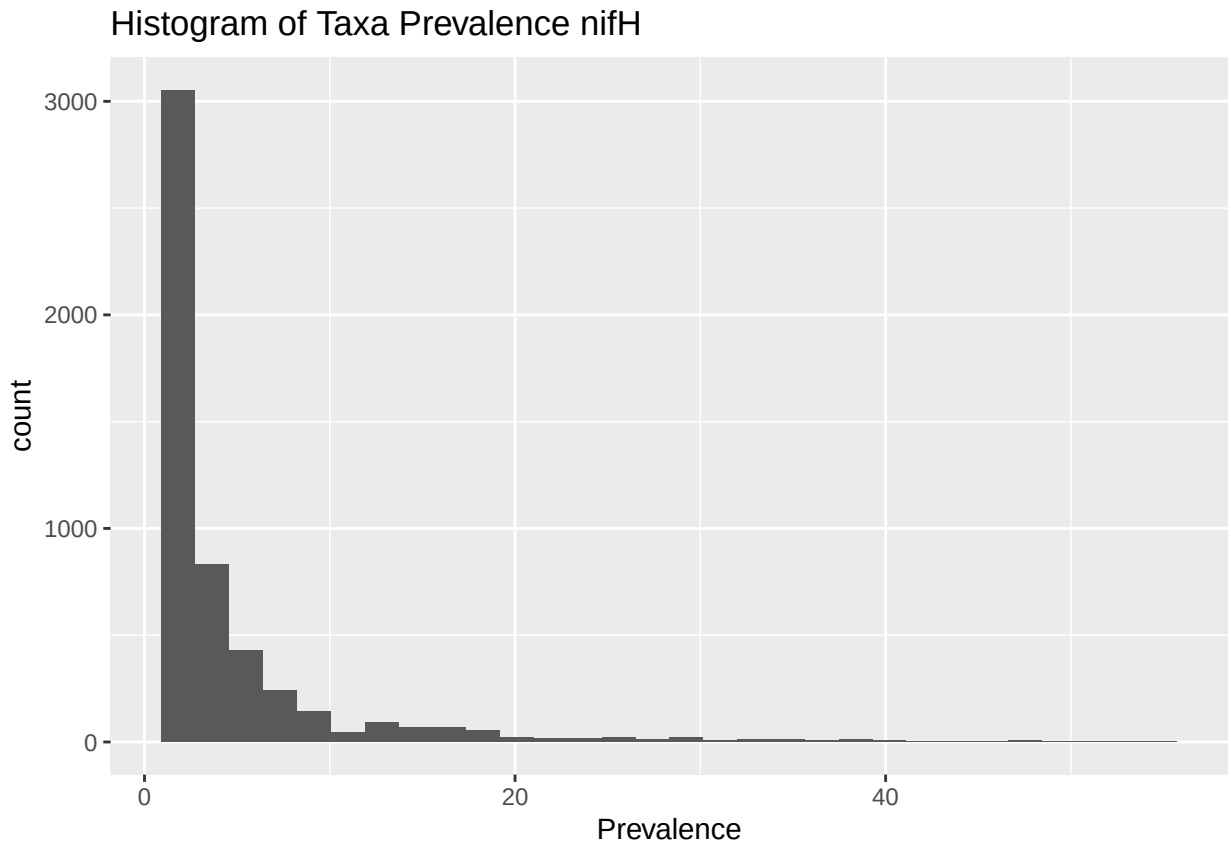
So if we filter ~9 Total count so remove every OTU with less than 9 counts we would remove 5000 OTUs. If we remove OTUS with less than 3 counts per OTU we only remove ~2900 OTUS.

We will now to the same with OTU prevlenace (How many samples is the OTU in)

```
mdt_nifH = fast_melt(physeq_nifH)
prevdtnifH = mdt_nifH[, list(Prevalence = sum(count > 0),
                      TotalCounts = sum(count)),
              by = TaxaID]
```

```
ggplot(prevdtnifH, aes(Prevalence)) +
  geom_histogram() +
  ggtitle("Histogram of Taxa Prevalence nifH")
```

```
## `stat_bin()` using `bins = 30`. Pick better value with `binwidth`.
```




```
# How many OTUS have low prevelance (Rare)?
prevdtnifH[(Prevalence <= 0), .N]#zero
```

```
## [1] 0
```

```
prevdtnifH[(Prevalence <= 1), .N]#single
```

```
## [1] 2144
```

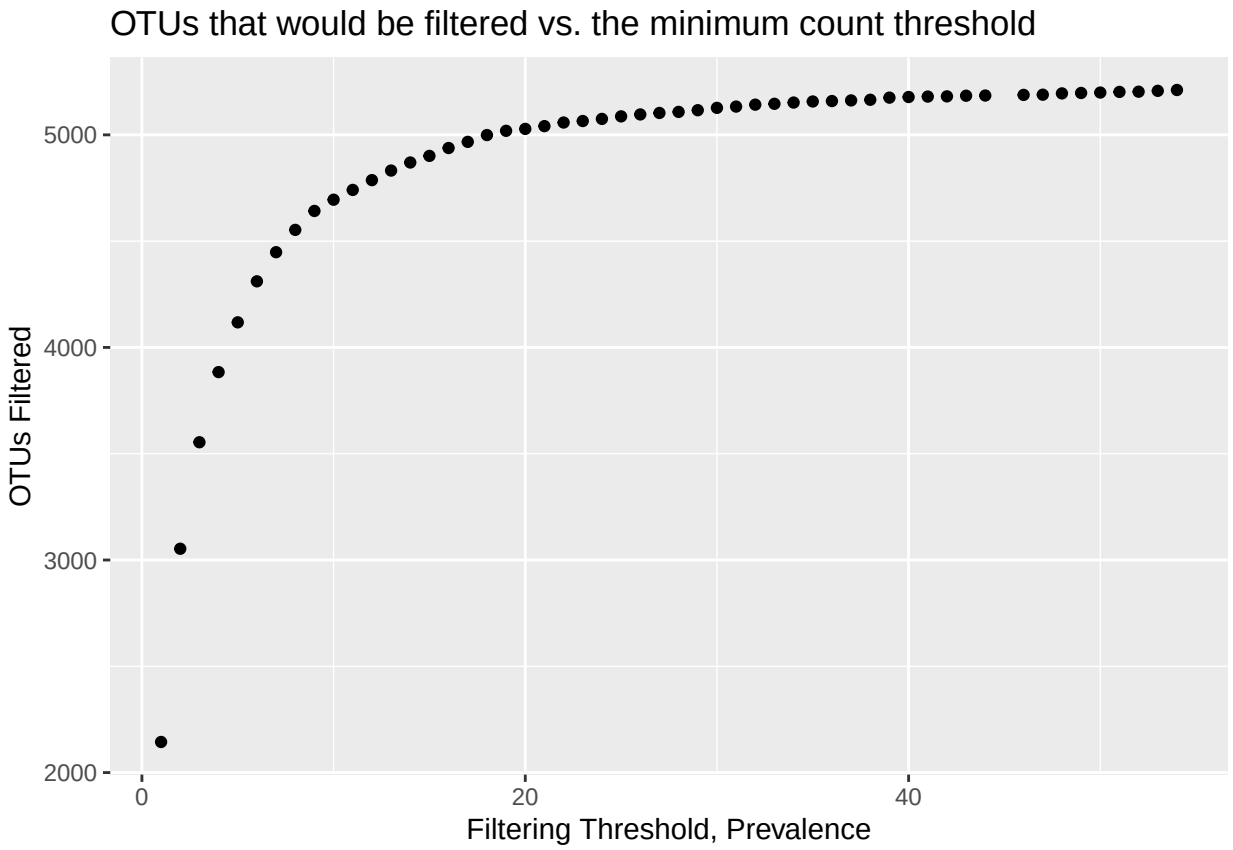
```
prevdtnifH[(Prevalence <= 2), .N]#double
```

```
## [1] 3053
```

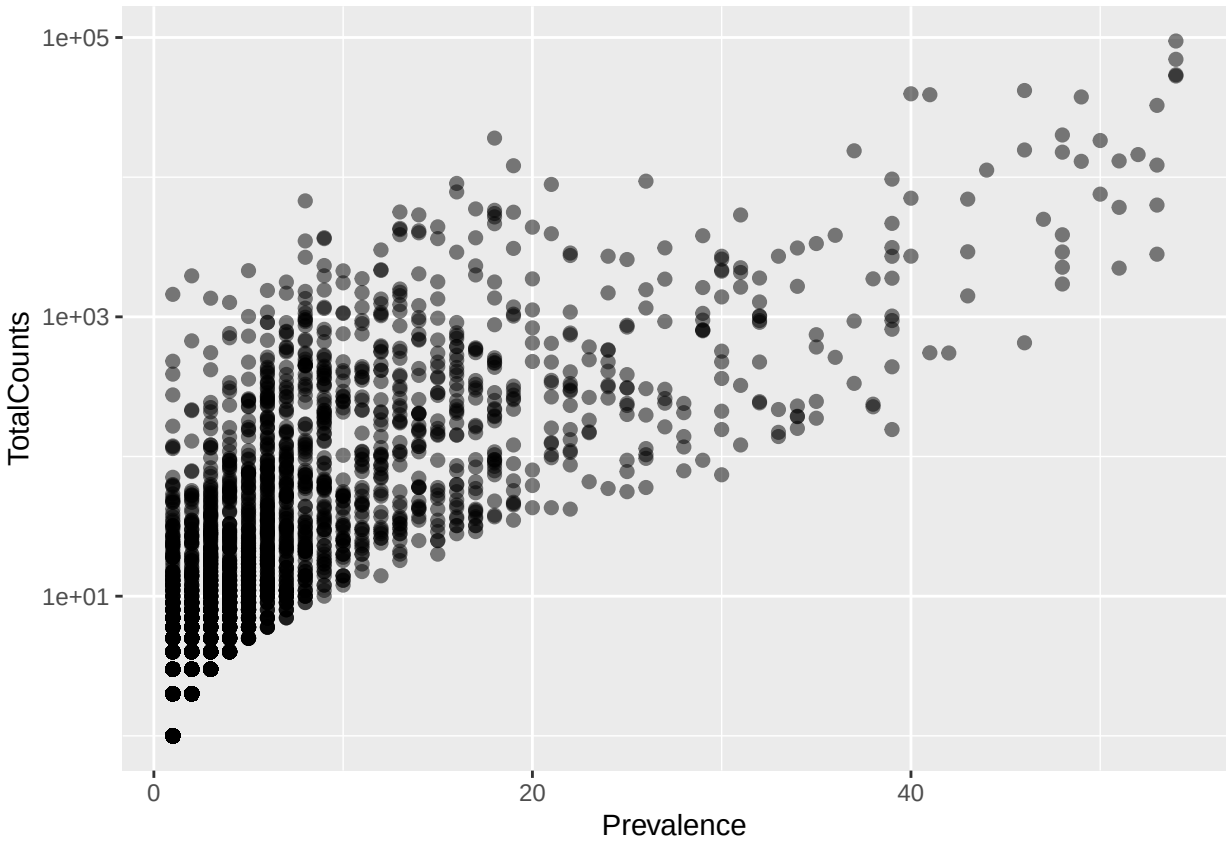
```
prevdtnifH[(Prevalence >= 54), .N]#how many OTUS are in every sample
```

```
## [1] 4
```

```
prevcumsumnifH = prevdtnifH[, .N, by = Prevalence]
setkey(prevcumsumnifH, Prevalence)
prevcumsumnifH[, CumSum := cumsum(N)]
pPrevCumSumnifH = ggplot(prevcumsumnifH, aes(Prevalence, CumSum)) +
  geom_point() +
  xlab("Filtering Threshold, Prevalence") +
  ylab("OTUs Filtered") +
  ggtitle("OTUs that would be filtered vs. the minimum count threshold")
pPrevCumSumnifH
```




```
ggplot(prevdtnifH, aes(Prevalence, TotalCounts)) +
  geom_point(size = 2, alpha = 0.5) +
  scale_y_log10()
```



There is a good a good distribution of the OTUs the OTUs that occur in low abundnace also occur in low prevlance. By removing some of the low counts (below) we will not be losing on the similarities between sites.

Trimming

Remove less than doublets in data and prevlant in 5% of the sample

First transform to realtive abundance

```
physeq_nifH_trim = filter_taxa(physeq_nifH, function(x) sum(x > 2) > (0.05*length(x)),
                               TRUE)
physeq_nifH_trim
```

```
## phyloseq-class experiment-level object
## otu_table()   OTU Table:         [ 1006 taxa and 54 samples ]
## sample_data() Sample Data:       [ 54 samples by 45 sample variables ]
## tax_table()   Taxonomy Table:    [ 1006 taxa by 8 taxonomic ranks ]
```

```
OTU_nifH_trim_1 = as(otu_table(physeq_nifH_trim), "matrix")
OTU_nifH_trim_df = as.data.frame(OTU_nifH_trim_1)
```

To simplfy ordination and save time we will trim the OTUs more

Remove OTUs that do not show appear more than 5 times in more than 10th of the samples

```
wh0_n = genefilter_sample(physeq_nifH_trim, filterfun_sample(function(x) x > 3),
                          A=0.1*nsamples(physeq_nifH_trim))
physeq_nifH_ord = prune_taxa(wh0_n, physeq_nifH_trim)
physeq_nifH_ord
```

```
## phyloseq-class experiment-level object
## otu_table()   OTU Table:         [ 446 taxa and 54 samples ]
## sample_data() Sample Data:       [ 54 samples by 45 sample variables ]
## tax_table()   Taxonomy Table:    [ 446 taxa by 8 taxonomic ranks ]
```

Transform to even sampling depth

```
physeq_nifH_ord = transform_sample_counts(physeq_nifH_ord, function(x) 1E6 * x/sum(x))
physeq_nifH_ord
```

```
## phyloseq-class experiment-level object
## otu_table()   OTU Table:         [ 446 taxa and 54 samples ]
## sample_data() Sample Data:       [ 54 samples by 45 sample variables ]
## tax_table()   Taxonomy Table:    [ 446 taxa by 8 taxonomic ranks ]
```

We have removed the majorty of the low abundance data with a remaining 804 taxa which make the data analysis much more managable.

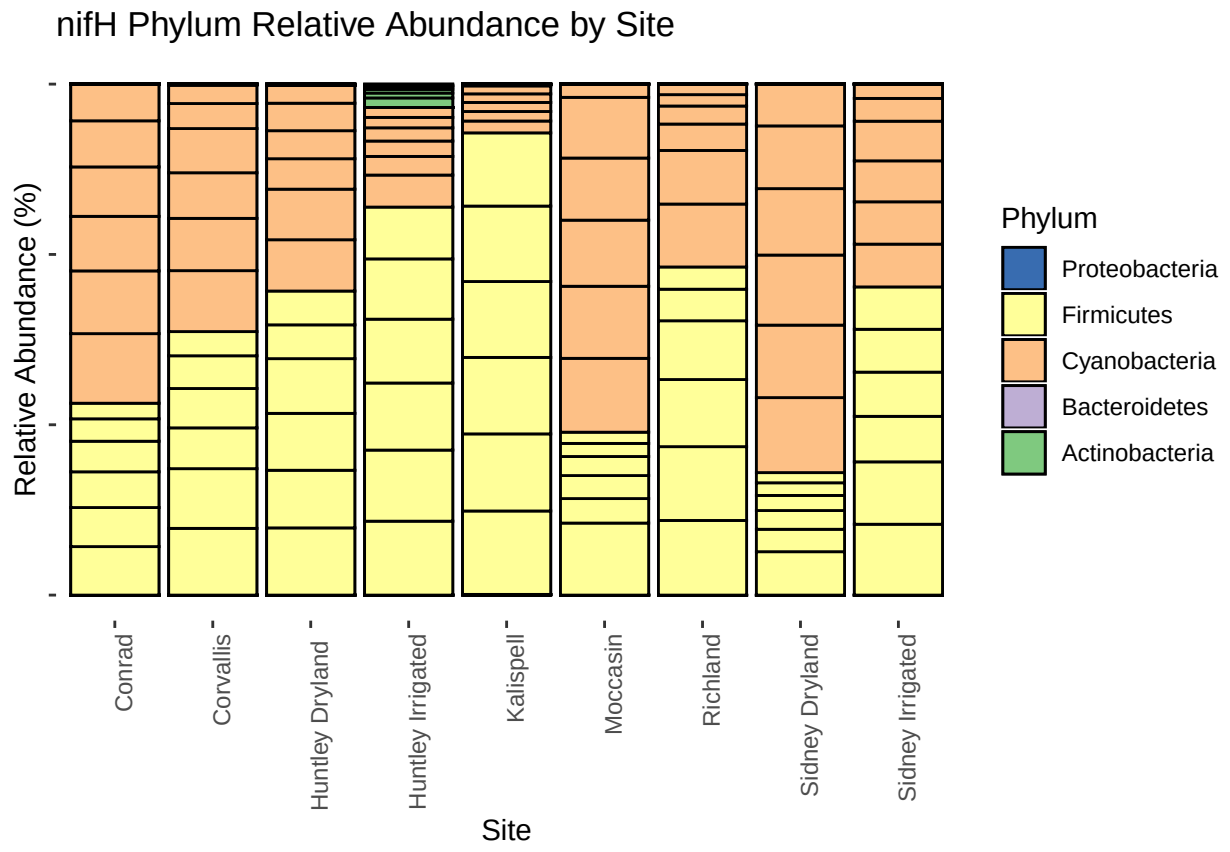
Analysis

Alpha Diveristy Analysis

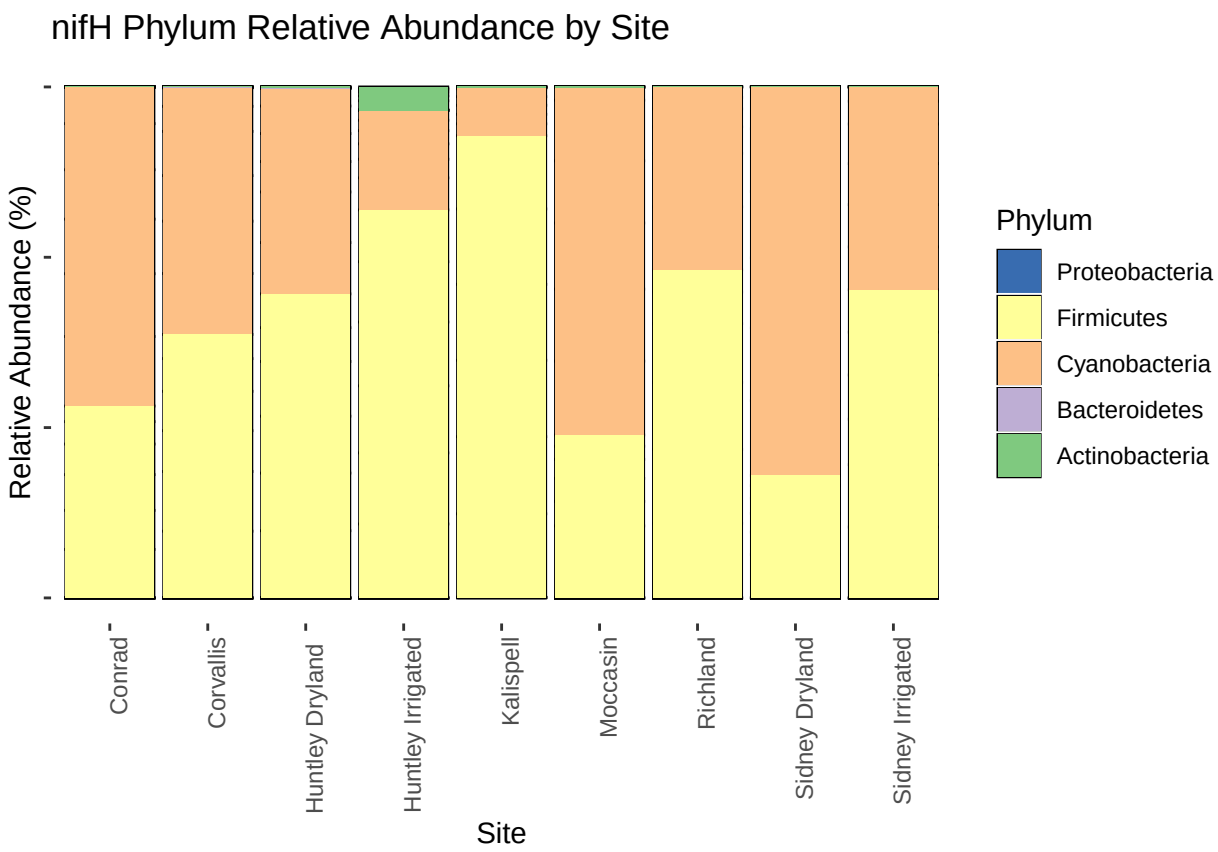
Bar plots

```
physeq_nifH_ord_2_phylum <- tax_glom(physeq_nifH_ord, "phylum")
plot_bar(physeq_nifH_ord_2_phylum, x = "Site", fill = "phylum")+
geom_bar(aes(fill = phylum), color = "black", stat = "identity", position = "stack",
         show.legend = TRUE)+
scale_fill_manual(name = "Phylum",
                  values=c("#7fc97f", "#beaed4", "#fdc086", "#ffff99", "#386cb0",
                           "#f0027f",'#e31a1c'),
                  labels = c("Actinobacteria", "Bacteroidetes", "Cyanobacteria",
                             "Firmicutes", "Proteobacteria", "Verrucomicrobia"),
```

```
                  guide = guide_legend(reverse = TRUE)
                    )+
ggtitle("nifH Phylum Relative Abundance by Site")+
ylab("Relative Abundance (%)")+
theme(axis.text.x = element_text(angle = 90, hjust = 1), axis.text.y = element_blank(),
      panel.background = element_blank())
```




```
physeq_nifH_ord_2_phylum <- tax_glom(physeq_nifH_ord, "phylum")
plot_bar(physeq_nifH_ord_2_phylum, x = "Site", fill = "phylum")+
geom_bar(aes(fill = phylum), stat = "identity", position = "stack", show.legend = TRUE)+
scale_fill_manual(name = "Phylum",
                  values=c("#7fc97f", "#beaed4", "#fdc086", "#ffff99",
                           "#386cb0", "#f0027f"),
                  labels = c("Actinobacteria", "Bacteroidetes", "Cyanobacteria",
                             "Firmicutes", "Proteobacteria", "Verrucomicrobia"),
                  guide = guide_legend(reverse = TRUE)
                    )+
ggtitle("nifH Phylum Relative Abundance by Site")+
ylab("Relative Abundance (%)")+
theme(axis.text.x = element_text(angle = 90, hjust = 1), axis.text.y = element_blank(),
      panel.background = element_blank())
```



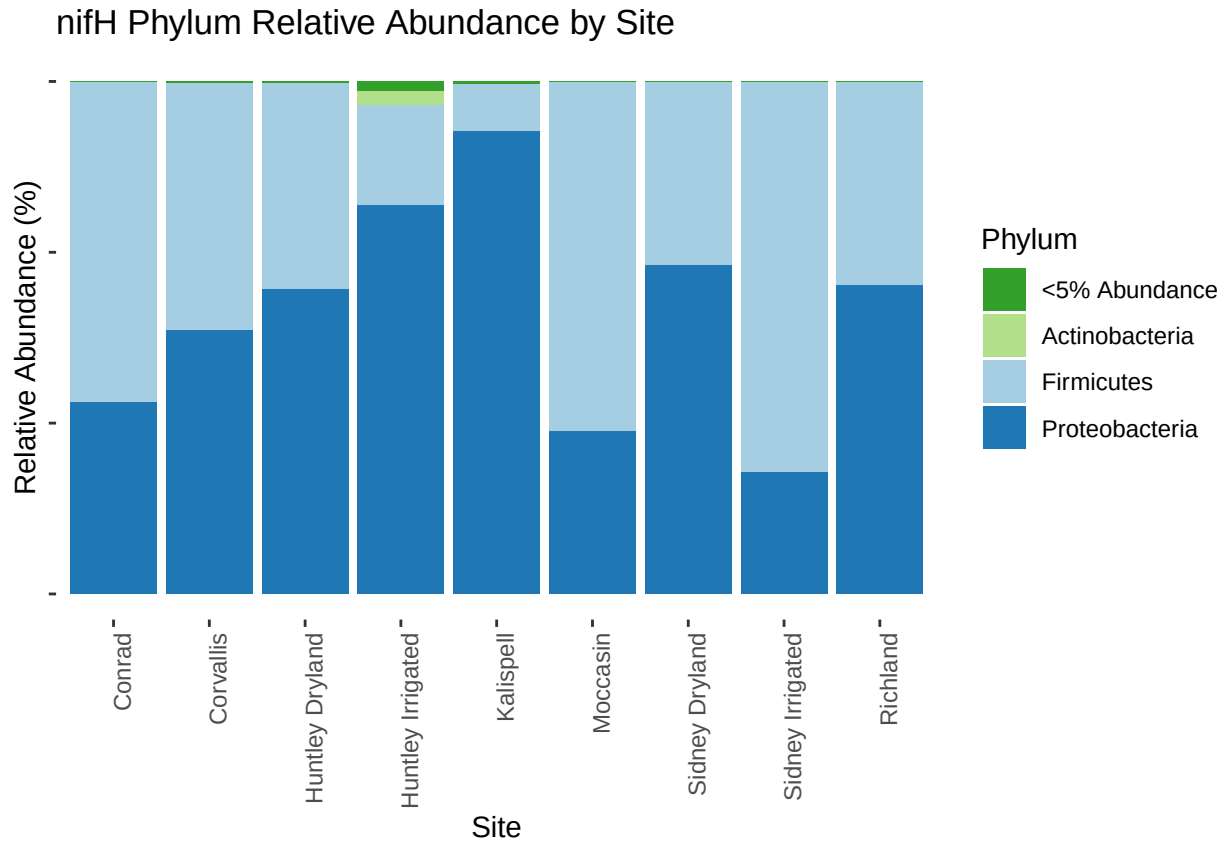
Reduce phylums that contribute the less than 5% of total abundance into one group

```
physeq_nifH_ord_1 = transform_sample_counts(physeq_nifH_ord, function(x) x / sum(x) )
physeq_nifH_ord_phylum <- tax_glom(physeq_nifH_ord_1, "phylum")
data_nifH_phylum <- psmelt(physeq_nifH_ord_phylum)
data_nifH_phylum$phylum<-as.character(data_nifH_phylum$phylum)
data_nifH_phylum$phylum[data_nifH_phylum$Abundance<0.05]<-"<5% abdund"

#list new phylums
unique(data_nifH_phylum$phylum)
```

```
## [1] "p__proteobacteria" "p__firmicutes"     "p__actinobacteria"
## [4] "<5% abdund"
```

```
ggplot(data = data_nifH_phylum, aes(x = Site, y = Abundance, fill = phylum))+
geom_bar(aes(fill = phylum), stat = "identity", position = "stack", show.legend = TRUE)+
scale_fill_manual(name = "Phylum",
                  values=c( "#33a02c",  "#b2df8a","#a6cee3", "#1f78b4"),
                  breaks=c("p__proteobacteria", "p__firmicutes",
                           "p__actinobacteria","<5% abdund" ),
                  labels = c( "Proteobacteria", "Firmicutes",
                              "Actinobacteria", "<5% Abundance"),
                 guide = guide_legend(reverse = TRUE)
                   )+
ggtitle("nifH Phylum Relative Abundance by Site")+
```

```
ylab("Relative Abundance (%)")+
scale_x_discrete(labels = c("Conrad", "Corvallis", "Huntley Dryland",
                            "Huntley Irrigated", "Kalispell", "Moccasin",
                            "Sidney Dryland", "Sidney Irrigated", "Richland"))+
theme(axis.text.x = element_text(angle = 90, hjust = 1),
      axis.text.y = element_blank(), panel.background = element_blank())
```



Publish plot to tiff

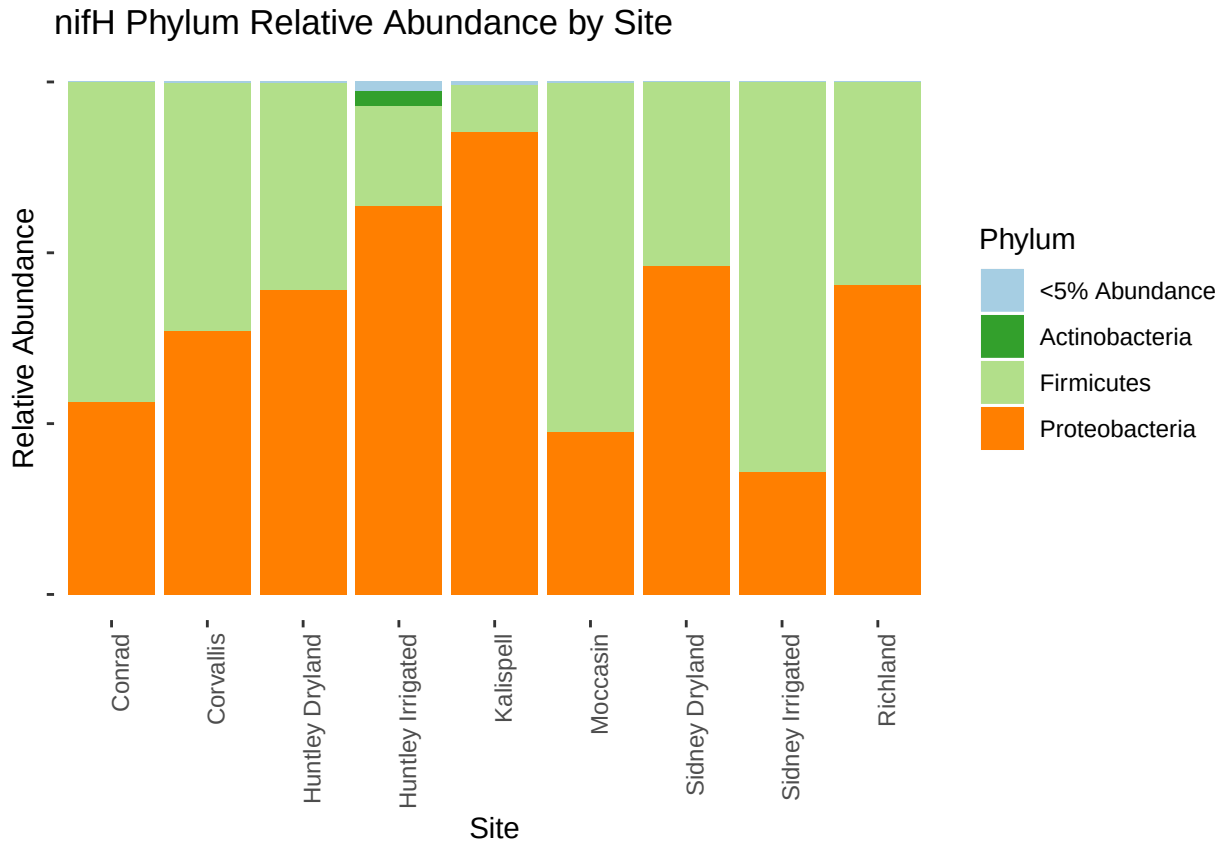
When published bars lose little white lines

```
## pdf
##   2
```

Different Colors

```
ggplot(data = data_nifH_phylum, aes(x = Site, y = Abundance, fill = phylum))+
geom_bar(aes(fill = phylum), stat = "identity", position = "stack", show.legend = TRUE)+
scale_fill_manual(name = "Phylum",
                  values=c("#a6cee3", "#33a02c",  "#b2df8a", "#ff7f00"),
                  breaks=c("p__proteobacteria", "p__firmicutes",
                           "p__actinobacteria","<5% abdund" ),
                  labels = c( "Proteobacteria", "Firmicutes",
                              "Actinobacteria", "<5% Abundance"),
                 guide = guide_legend(reverse = TRUE)
```

```
                    )+
ggtitle("nifH Phylum Relative Abundance by Site")+
ylab("Relative Abundance")+
scale_x_discrete(labels = c("Conrad", "Corvallis",
                            "Huntley Dryland", "Huntley Irrigated", "Kalispell",
                            "Moccasin", "Sidney Dryland", "Sidney Irrigated", "Richland"))+
theme(axis.text.x = element_text(angle = 90, hjust = 1),
      axis.text.y = element_blank(), panel.background = element_blank())
```

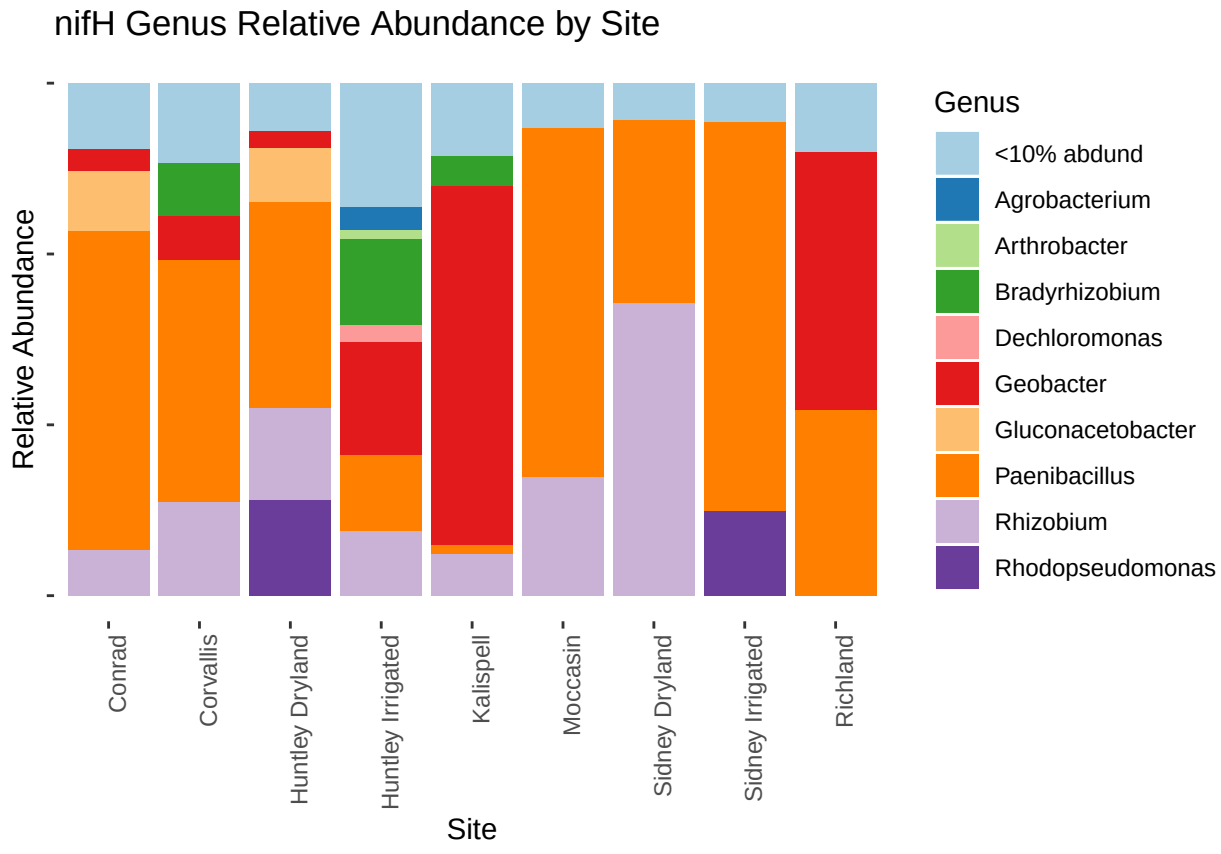


nifH genus barplot

```
physeq_nifH_ord_1 = transform_sample_counts(physeq_nifH_ord, function(x) x / sum(x) )
physeq_nifH_ord_genus <- tax_glom(physeq_nifH_ord_1, "genus")
data_nifH_genus <- psmelt(physeq_nifH_ord_genus)
data_nifH_genus$genus<-as.character(data_nifH_genus$genus)
data_nifH_genus$genus[data_nifH_genus$Abundance<0.1]<-"<10% abdund"
unique(data_nifH_genus$genus)
```

```
##  [1] "g__paenibacillus"     "g__rhizobium"         "g__geobacter"
##  [4] "g__rhodopseudomonas"  "g__bradyrhizobium"    "g__gluconacetobacter"
##  [7] "g__dechloromonas"     "g__agrobacterium"     "g__arthrobacter"
## [10] "<10% abdund"
```

```
ggplot(data = data_nifH_genus, aes(x = Site, y = Abundance, fill = genus))+
geom_bar(aes(fill = genus), stat = "identity", position = "stack", show.legend = TRUE)+
scale_fill_manual(name = "Genus",
                  values=c('#a6cee3','#1f78b4','#b2df8a','#33a02c',
                           '#fb9a99','#e31a1c','#fdbf6f','#ff7f00',
                           '#cab2d6','#6a3d9a','#ffff99','#b15928'),
                  breaks=c("<10% abdund", "g__agrobacterium", "g__arthrobacter",
                           "g__bradyrhizobium","g__dechloromonas","g__geobacter",
                           "g__gluconacetobacter", "g__paenibacillus",
                           "g__rhizobium", "g__rhodopseudomonas"),
                  labels=c("<10% abdund", "Agrobacterium", "Arthrobacter",
                           "Bradyrhizobium","Dechloromonas","Geobacter",
                           "Gluconacetobacter", "Paenibacillus",
                           "Rhizobium", "Rhodopseudomonas"),
                  guide = guide_legend(reverse = FALSE)
                    )+
ggtitle("nifH Genus Relative Abundance by Site")+
ylab("Relative Abundance")+
scale_x_discrete(labels = c("Conrad", "Corvallis", "Huntley Dryland",
                            "Huntley Irrigated", "Kalispell", "Moccasin",
                            "Sidney Dryland", "Sidney Irrigated", "Richland"))+
theme(axis.text.x = element_text(angle = 90, hjust = 1), axis.text.y = element_blank(),
      panel.background = element_blank())
```



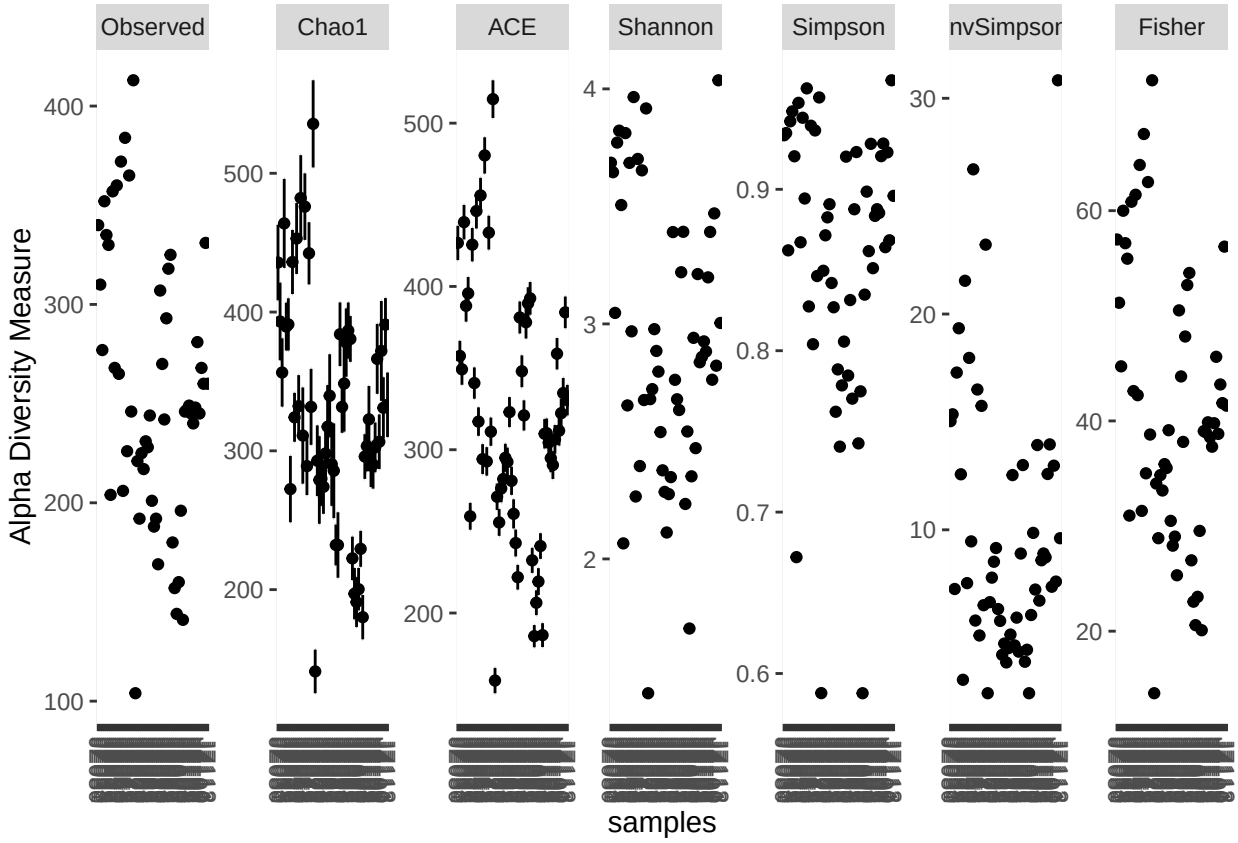
Publish to a tiff image

```
## pdf
##   2
```

Alpha diversity metrics

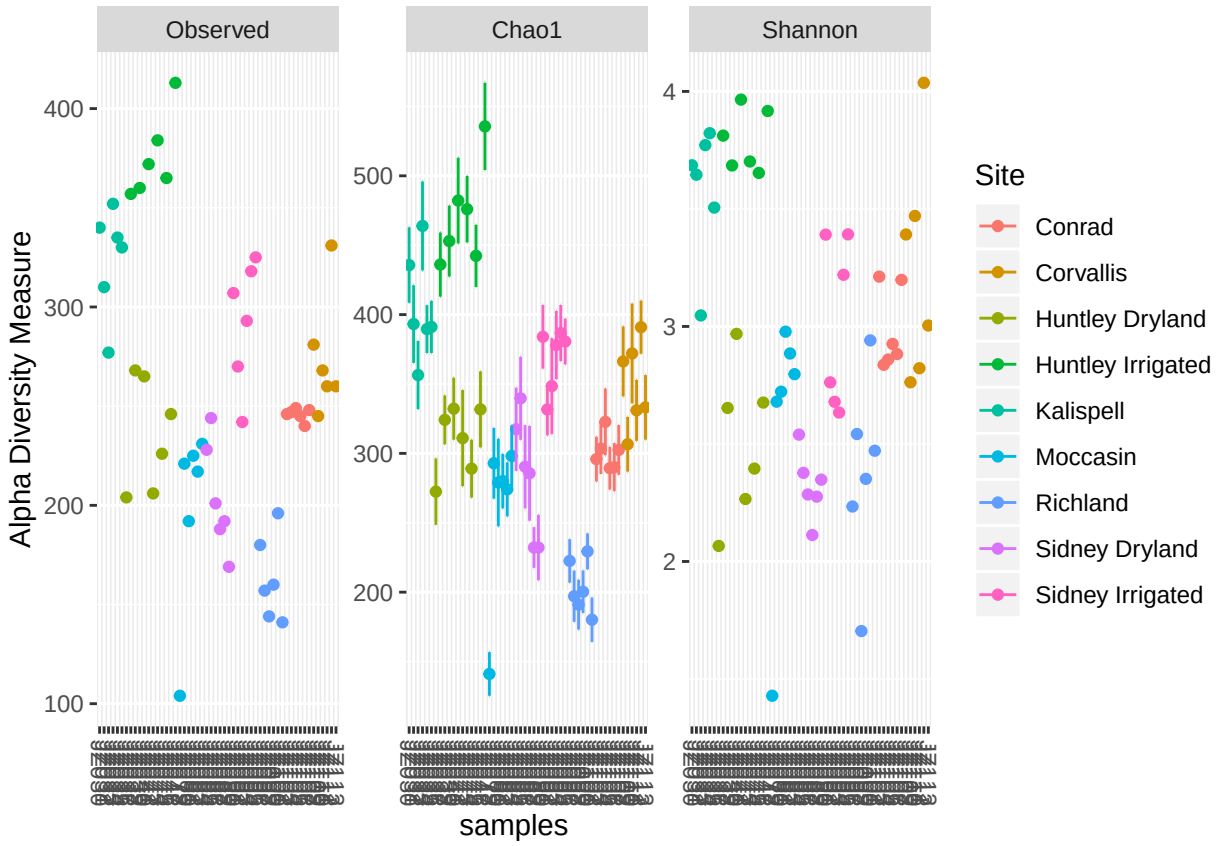
Use phyloseq internal packages to calculate the alpha diversity

```
plot_richness(physeq_nifH_trim)
```



Simplify to just Observed, Chao1, and Shannon

```
plot_richness(physeq_nifH_trim, measures = c("Observed","Chao1", "Shannon"), color = "Site")
```




```
rich_nifH<-estimate_richness(physeq_nifH_trim, split = TRUE)


#First merge data sets with meta2
meta2$sample_names<-rownames(meta2)
rich_nifH$sample_names<-rownames(rich_nifH)
meta_nifH<-merge(meta2, rich_nifH, by = "sample_names")
rownames(meta_nifH)<-meta_nifH$sample_names
meta_nifH<-meta_nifH[,-1]
head(meta_nifH)[,49:54]
```

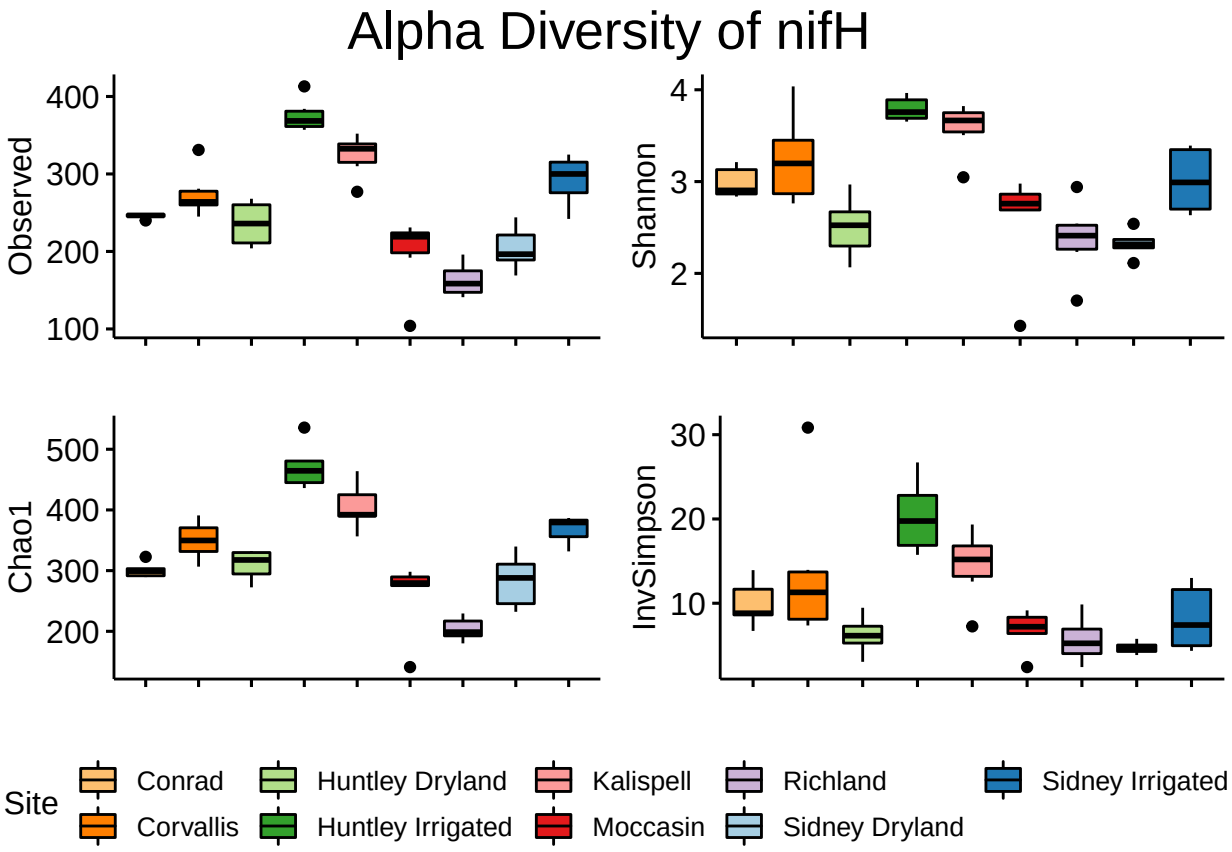
	ACE	se.ACE	Shannon	Simpson	InvSimpson	Fisher
JZ030	426.6245	10.472146	3.685694	0.9334978	15.037099	57.25766
JZ031	357.4261	9.265222	3.645484	0.9348672	15.353248	51.23836
JZ032	349.2533	9.483790	3.046774	0.8621953	7.256646	45.19042
JZ033	439.3598	10.570712	3.771445	0.9421562	17.287932	59.99256
JZ034	388.1394	9.716513	3.822216	0.9482979	19.341566	56.90461
JZ035	395.7359	10.022588	3.505772	0.9204658	12.573215	55.43243

```
mean(meta_nifH$Observed)
```

```
## [1] 256.9444
```

Make boxplots with Observed, Shannon, chao1, and inverse simpson

```r
#use ggpubr for plot
nifH_Observ<-ggboxplot(meta_nifH, x = "Site", y = "Observed",
   rug = TRUE,
   fill = "Site", xlab = " ",
   palette = farm_col_paired)+
  rremove("x.text")

nifH_Shannon<-ggboxplot(meta_nifH, x = "Site", y = "Shannon",
    rug = TRUE,
   fill = "Site", xlab = " ",
   palette = farm_col_paired)+
  rremove("x.text")

nifH_Chao<- ggboxplot(meta_nifH, x = "Site", y = "Chao1",
    rug = TRUE,
   fill = "Site", xlab = " ",
   palette = farm_col_paired)+
  rremove("x.text")

nifH_InvSim<- ggboxplot(meta_nifH, x = "Site", y = "InvSimpson",
   rug = TRUE,
   fill = "Site", xlab = " ",
   palette = farm_col_paired)+
  rremove("x.text")

alpha_nifH_fig<-ggarrange(nifH_Observ, nifH_Shannon, nifH_Chao, nifH_InvSim, ncol = 2, nrow = 2, common

annotate_figure(alpha_nifH_fig, top = text_grob("Alpha Diversity of nifH ", size = 20))
```




```
nifH_Observ_stats<-ggplot_build(nifH_Observ)
nifH_Observ_stats$data
```

```
## [[1]]
##      fill ymin  lower middle  upper ymax outliers notchupper notchlower x
## 1 #fdbf6f  245 245.25  246.5 247.75  249      240   248.1126   244.8874 1
## 2 #ff7f00  245 260.00  264.0 277.75  281      331   275.4493   252.5507 2
## 3 #b2df8a  204 211.00  236.0 260.25  268           267.7678   204.2322 3
## 4 #33a02c  357 361.25  368.5 381.00  384      413   381.2394   355.7606 4
## 5 #fb9a99  310 315.00  332.5 338.75  352      277   347.8195   317.1805 5
## 6 #e31a1c  192 198.25  219.0 224.00  231      104   235.6096   202.3904 6
## 7 #cab2d6  141 147.25  158.5 175.00  196           176.3996   140.6004 7
## 8 #a6cee3  169 189.00  196.5 221.25  244           217.3023   175.6977 8
## 9 #1f78b4  242 275.75  300.0 315.25  325           325.4788   274.5212 9
##   PANEL group ymin_final ymax_final xmin xmax weight colour size alpha
## 1     1     1         240         249 0.65 1.35      1  black  0.5    NA
## 2     1     2         245         331 1.65 2.35      1  black  0.5    NA
## 3     1     3         204         268 2.65 3.35      1  black  0.5    NA
## 4     1     4         357         413 3.65 4.35      1  black  0.5    NA
## 5     1     5         277         352 4.65 5.35      1  black  0.5    NA
## 6     1     6         104         231 5.65 6.35      1  black  0.5    NA
## 7     1     7         141         196 6.65 7.35      1  black  0.5    NA
## 8     1     8         169         244 7.65 8.35      1  black  0.5    NA
## 9     1     9         242         325 8.65 9.35      1  black  0.5    NA
##   shape linetype
## 1    19    solid
```

```
## 2    19    solid
## 3    19    solid
## 4    19    solid
## 5    19    solid
## 6    19    solid
## 7    19    solid
## 8    19    solid
## 9    19    solid
```

Publish to .tiff

```
## pdf
##   2
```

Observed- total observed OTUs

Chao1- estimate diversity and assumes that the number of observations for a taxa has a Poisson distribution and corrects for variance

Shannon- Number of OTUs (richness) scaled to the evenness

Simpson- scale of dominace probabilty of any two indviduals drawn at random beloging to the same species

Source: http://biology.kenyon.edu/courses/biol229/diversity.pdf

```
nifH_shannon<-ggboxplot(meta_nifH, x = "Site", y = "Shannon",
   rug = TRUE,
   fill = "Site", xlab = " ", ylab = " ", width = 0.4, title = "nifH",
   palette = farm_col_paired,
   legend = "right"
   )+
  rremove("x.text")
```

```
tiff("shannon_nifH.tiff", width = 5, height = 8, units = 'in', res = 600)
nifH_shannon

dev.off()
```

```
## pdf
##   2
```

```
nifH_Observ_stats<-ggplot_build(nifH_Observ)
nifH_Observ_stats$data
```

```
## [[1]]
##      fill ymin  lower middle  upper ymax outliers notchupper notchlower x
## 1 #fdbf6f  245 245.25  246.5 247.75  249      240   248.1126   244.8874 1
## 2 #ff7f00  245 260.00  264.0 277.75  281      331   275.4493   252.5507 2
## 3 #b2df8a  204 211.00  236.0 260.25  268           267.7678   204.2322 3
```

```
## 4 #33a02c  357 361.25  368.5 381.00  384      413   381.2394   355.7606 4
## 5 #fb9a99  310 315.00  332.5 338.75  352      277   347.8195   317.1805 5
## 6 #e31a1c  192 198.25  219.0 224.00  231      104   235.6096   202.3904 6
## 7 #cab2d6  141 147.25  158.5 175.00  196            176.3996   140.6004 7
## 8 #a6cee3  169 189.00  196.5 221.25  244            217.3023   175.6977 8
## 9 #1f78b4  242 275.75  300.0 315.25  325            325.4788   274.5212 9
##   PANEL group ymin_final ymax_final xmin xmax weight colour size alpha
## 1     1     1        240        249 0.65 1.35      1  black  0.5    NA
## 2     1     2        245        331 1.65 2.35      1  black  0.5    NA
## 3     1     3        204        268 2.65 3.35      1  black  0.5    NA
## 4     1     4        357        413 3.65 4.35      1  black  0.5    NA
## 5     1     5        277        352 4.65 5.35      1  black  0.5    NA
## 6     1     6        104        231 5.65 6.35      1  black  0.5    NA
## 7     1     7        141        196 6.65 7.35      1  black  0.5    NA
## 8     1     8        169        244 7.65 8.35      1  black  0.5    NA
## 9     1     9        242        325 8.65 9.35      1  black  0.5    NA
##   shape linetype
## 1    19    solid
## 2    19    solid
## 3    19    solid
## 4    19    solid
## 5    19    solid
## 6    19    solid
## 7    19    solid
## 8    19    solid
## 9    19    solid
```

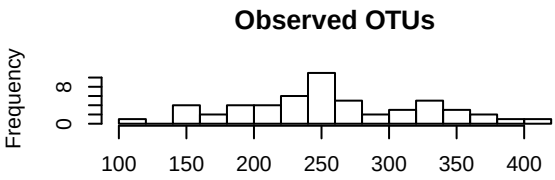
```
nifH_otu_count<-as.data.frame(sample_sums(physeq_nifH_trim))
nifH_otu_count$sample_names<-rownames(nifH_otu_count)
```

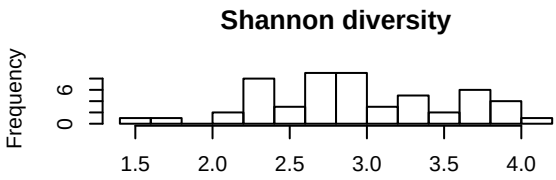
Determine if distrubution is normal for each diversity Metric

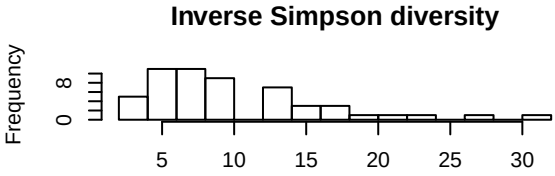
Used the following protocol :

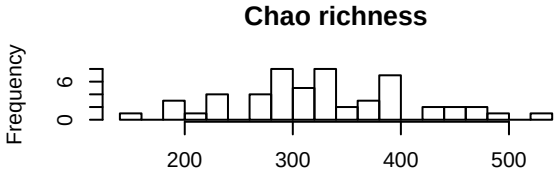
https://rpubs.com/dillmcfarlan/R_microbiotaSOP

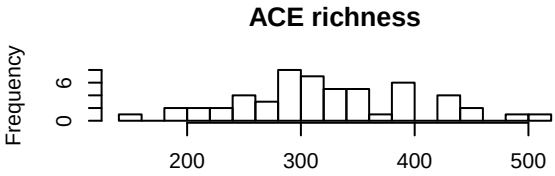
```
#Create 2x2 plot environment so that we can see all 4 metrics at once.
par(mfrow = c(3, 2))

#Then plot each metric.
hist(rich_nifH$Observed, main="Observed OTUs", xlab="", breaks=15)
hist(rich_nifH$Shannon, main="Shannon diversity", xlab="", breaks=10)
hist(rich_nifH$InvSimpson, main="Inverse Simpson diversity", xlab="", breaks=15)
hist(rich_nifH$Chao1, main="Chao richness", xlab="", breaks=15)
hist(rich_nifH$ACE, main="ACE richness", xlab="", breaks=15)
```











Test for normalcy using the shapiro test. The null hypothesis for this test is that the data are normally distributed, if the p-value is greater than 0.05, then the null hypothesis is not rejected.

```
shapiro.test(rich_nifH$Observed)
```

```
##
##  Shapiro-Wilk normality test
##
## data:  rich_nifH$Observed
## W = 0.98368, p-value = 0.6687
```

```
shapiro.test(rich_nifH$Shannon)
```

```
##
##  Shapiro-Wilk normality test
##
## data:  rich_nifH$Shannon
## W = 0.97519, p-value = 0.3226
```

```
shapiro.test(rich_nifH$InvSimpson)
```

```
##
##  Shapiro-Wilk normality test
##
## data:  rich_nifH$InvSimpson
## W = 0.87892, p-value = 5.746e-05
```

```r
shapiro.test(rich_nifH$Chao1)
```

```
##
##  Shapiro-Wilk normality test
##
## data:  rich_nifH$Chao1
## W = 0.98578, p-value = 0.768
```

```r
shapiro.test(rich_nifH$ACE)
```

```
##
##  Shapiro-Wilk normality test
##
## data:  rich_nifH$ACE
## W = 0.98743, p-value = 0.8402
```

Use ANOVA on alpha diveristy metrics for Shannon variables becuase the other are not normal

Shannon anova

```r
aov_shannon_site_nifH <- aov(Shannon ~ Site, meta_nifH)
summary(aov_shannon_site_nifH)
```

```
##             Df Sum Sq Mean Sq F value   Pr(>F)
## Site         8 13.506  1.6882   13.67 8.29e-10 ***
## Residuals   45  5.558  0.1235
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Site location has a signifcant effect on shannon diversity

Correct for multiple comparisons

```r
shannon_nifH_site<-TukeyHSD(aov_shannon_site_nifH, "Site", ordered = TRUE)
shannon_nifH_site
```

```
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##     factor levels have been ordered
##
## Fit: aov(formula = Shannon ~ Site, data = meta_nifH)
##
## $Site
##                                       diff          lwr       upr
## Richland-Sidney Dryland           0.05089297 -0.609986678 0.7117726
## Huntley Dryland -Sidney Dryland   0.18109141 -0.479788234 0.8419711
## Moccasin-Sidney Dryland           0.25873022 -0.402149425 0.9196099
```

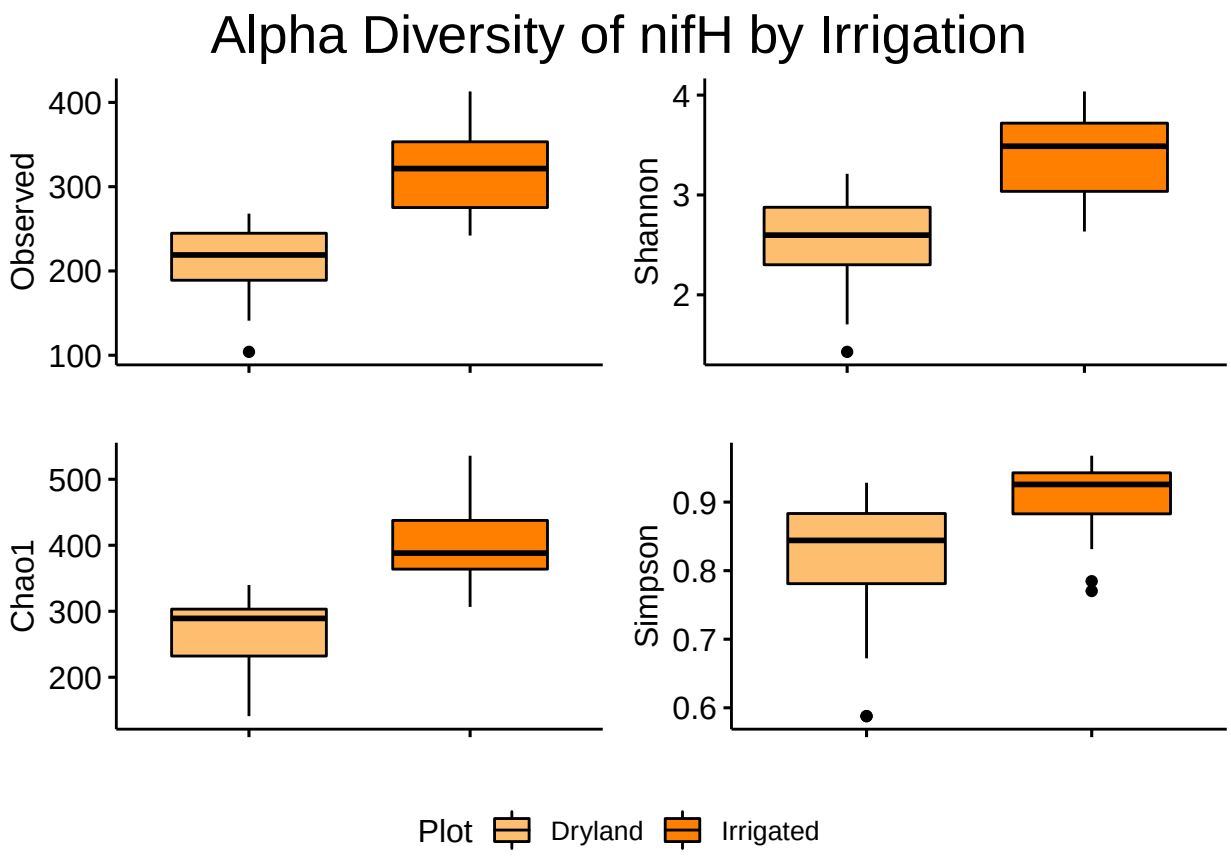
```
## Conrad-Sidney Dryland              0.66272066  0.001841014 1.3236003
## Sidney Irrigated-Sidney Dryland    0.68988515  0.029005499 1.3507648
## Corvallis-Sidney Dryland           0.92476069  0.263881042 1.5856403
## Kalispell-Sidney Dryland           1.25652759  0.595647944 1.9174072
## Huntley Irrigated-Sidney Dryland   1.46594982  0.805070172 2.1268295
## Huntley Dryland -Richland          0.13019844 -0.530681204 0.7910781
## Moccasin-Richland                  0.20783725 -0.453042395 0.8687169
## Conrad-Richland                    0.61182769 -0.049051955 1.2727073
## Sidney Irrigated-Richland          0.63899218 -0.021887471 1.2998718
## Corvallis-Richland                 0.87386772  0.212988073 1.5347474
## Kalispell-Richland                 1.20563462  0.544754974 1.8665143
## Huntley Irrigated-Richland         1.41505685  0.754177202 2.0759365
## Moccasin-Huntley Dryland           0.07763881 -0.583240839 0.7385185
## Conrad-Huntley Dryland             0.48162925 -0.179250399 1.1425089
## Sidney Irrigated-Huntley Dryland   0.50879373 -0.152085915 1.1696734
## Corvallis-Huntley Dryland          0.74366928  0.082789629 1.4045489
## Kalispell-Huntley Dryland          1.07543618  0.414556530 1.7363158
## Huntley Irrigated-Huntley Dryland  1.28485841  0.623978758 1.9457381
## Conrad-Moccasin                    0.40399044 -0.256889209 1.0648701
## Sidney Irrigated-Moccasin          0.43115492 -0.229724724 1.0920346
## Corvallis-Moccasin                 0.66603047  0.005150819 1.3269101
## Kalispell-Moccasin                 0.99779737  0.336917720 1.6586770
## Huntley Irrigated-Moccasin         1.20721960  0.546339948 1.8680992
## Sidney Irrigated-Conrad            0.02716448 -0.633715164 0.6880441
## Corvallis-Conrad                   0.26204003 -0.398839620 0.9229197
## Kalispell-Conrad                   0.59380693 -0.067072719 1.2546866
## Huntley Irrigated-Conrad           0.80322916  0.142349509 1.4641088
## Corvallis-Sidney Irrigated         0.23487554 -0.426004105 0.8957552
## Kalispell-Sidney Irrigated         0.56664244 -0.094237204 1.2275221
## Huntley Irrigated-Sidney Irrigated 0.77606467  0.115185024 1.4369443
## Kalispell-Corvallis                0.33176690 -0.329112747 0.9926465
## Huntley Irrigated-Corvallis        0.54118913 -0.119690519 1.2020688
## Huntley Irrigated-Kalispell        0.20942223 -0.451457420 0.8703019
##                                        p adj
## Richland-Sidney Dryland            0.9999994
## Huntley Dryland -Sidney Dryland    0.9923049
## Moccasin-Sidney Dryland            0.9333949
## Conrad-Sidney Dryland              0.0488792
## Sidney Irrigated-Sidney Dryland    0.0347461
## Corvallis-Sidney Dryland           0.0012087
## Kalispell-Sidney Dryland           0.0000055
## Huntley Irrigated-Sidney Dryland   0.0000002
## Huntley Dryland -Richland          0.9992243
## Moccasin-Richland                  0.9813852
## Conrad-Richland                    0.0893804
## Sidney Irrigated-Richland          0.0651612
## Corvallis-Richland                 0.0026283
## Kalispell-Richland                 0.0000128
## Huntley Irrigated-Richland         0.0000004
## Moccasin-Huntley Dryland           0.9999841
## Conrad-Huntley Dryland             0.3228127
## Sidney Irrigated-Huntley Dryland   0.2557929
## Corvallis-Huntley Dryland          0.0170802
## Kalispell-Huntley Dryland          0.0001098
```

```
## Huntley Irrigated-Huntley Dryland  0.0000034
## Conrad-Moccasin                    0.5572599
## Sidney Irrigated-Moccasin          0.4701256
## Corvallis-Moccasin                 0.0469200
## Kalispell-Moccasin                 0.0003838
## Huntley Irrigated-Moccasin         0.0000125
## Sidney Irrigated-Conrad            1.0000000
## Corvallis-Conrad                   0.9286970
## Kalispell-Conrad                   0.1093235
## Huntley Irrigated-Conrad           0.0074286
## Corvallis-Sidney Irrigated         0.9612154
## Kalispell-Sidney Irrigated         0.1461472
## Huntley Irrigated-Sidney Irrigated 0.0109195
## Kalispell-Corvallis                0.7805875
## Huntley Irrigated-Corvallis        0.1888886
## Huntley Irrigated-Kalispell        0.9804879
```

Write to table

Plot Irrigation managment against diffrent diveristies

```
nifH_irr_Observ<-ggboxplot(meta_nifH, x = "Plot", y = "Observed",
   rug = TRUE,
   fill = "Plot", xlab = " ",
   palette = farm_col_paired)+
  rremove("x.text")

nifH_irr_Shannon<-ggboxplot(meta_nifH, x = "Plot", y = "Shannon",
   rug = TRUE,
   fill = "Plot", xlab = " ",
   palette = farm_col_paired)+
  rremove("x.text")

nifH_irr_Chao<- ggboxplot(meta_nifH, x = "Plot", y = "Chao1",
    rug = TRUE,
   fill = "Plot", xlab = " ",
   palette = farm_col_paired)+
  rremove("x.text")

nifH_irr_InvSim<- ggboxplot(meta_nifH, x = "Plot", y = "Simpson",
   rug = TRUE,
   fill = "Plot", xlab = " ",
   palette = farm_col_paired)+
  rremove("x.text")

alpha_nifH_irr_fig<-ggarrange(nifH_irr_Observ, nifH_irr_Shannon, nifH_irr_Chao, nifH_irr_InvSim, ncol =

annotate_figure(alpha_nifH_irr_fig, top = text_grob("Alpha Diversity of nifH by Irrigation", size = 20))
```



Check for significance in the other metrics to for irrigation (Dryland vs irrigated)

Since Shannon was the only normally distributed data set I will use Kruskal Wallis test for others

```
KW_observed_irr_nifH <- kruskal.test(Observed ~ Plot, meta_nifH)
KW_observed_irr_nifH
```

```
##
##  Kruskal-Wallis rank sum test
##
## data:  Observed by Plot
## Kruskal-Wallis chi-squared = 34.831, df = 1, p-value = 3.596e-09
```

```
aov_observed_irr_nifH <- aov(Observed ~ Plot, meta_nifH)
summary(aov_observed_irr_nifH)
```

```
##             Df Sum Sq Mean Sq F value   Pr(>F)
## Plot         1 153010  153010      81 3.47e-12 ***
## Residuals   52  98225    1889
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Significance in observed

```
aov_observed_irr_nifH <- aov(Observed ~ Plot/Site, meta_nifH)
summary(aov_observed_irr_nifH)
```

```
##             Df Sum Sq Mean Sq F value   Pr(>F)
## Plot         1 153010  153010  185.99  < 2e-16 ***
## Plot:Site    7  61203    8743   10.63 8.31e-08 ***
## Residuals   45  37021     823
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
capture.output(aov_observed_irr_nifH,file="aov_nifH_plot_state.txt")
```

Plot is significant but the interaction of Plot:Site is also significant so we cannot say there is actuall correlation

```
aov_Chao1_irr_nifH <- aov(Chao1 ~ Plot, meta_nifH)
summary(aov_Chao1_irr_nifH)
```

```
##             Df Sum Sq Mean Sq F value   Pr(>F)
## Plot         1 214872  214872      75 1.17e-11 ***
## Residuals   52 148968    2865
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Signifcant in chao1

```
aov_Chao1_irr_nifH <- aov(Chao1 ~ Plot/Site, meta_nifH)
summary(aov_Chao1_irr_nifH)
```

```
##             Df Sum Sq Mean Sq F value   Pr(>F)
## Plot         1 214872  214872  177.83  < 2e-16 ***
## Plot:Site    7  94595   13514   11.18 4.22e-08 ***
## Residuals   45  54373    1208
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
aov_Simpson_irr_nifH <- aov(Simpson ~ Plot, meta_nifH)
summary(aov_Simpson_irr_nifH)
```

```
##             Df Sum Sq Mean Sq F value   Pr(>F)
## Plot         1 0.1044 0.10442   19.29 5.55e-05 ***
## Residuals   52 0.2815 0.00541
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
aov_Simpson_irr_nifH <- aov(Simpson ~ Plot/Site, meta_nifH)
summary(aov_Simpson_irr_nifH)
```

```
##             Df  Sum Sq Mean Sq F value   Pr(>F)
## Plot         1 0.10442 0.10442   23.02 1.79e-05 ***
## Plot:Site    7 0.07744 0.01106    2.44    0.033 *
## Residuals   45 0.20407 0.00453
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

All alpha diversities are significant with irrigation method but they are nested in Site so we must look within site (Huntley and Sidney) later. It is hard to prove anything in the statewide survey with univariate ANOVAs

```
aov_shannon_pea_nifH <- aov(Shannon ~ Pea_variety + Site, meta_nifH)
summary(aov_shannon_pea_nifH)
```

```
##             Df Sum Sq Mean Sq F value   Pr(>F)
## Pea_variety  6  0.898  0.1497   1.178    0.338
## Site         8 13.209  1.6511  12.991 6.73e-09 ***
## Residuals   39  4.957  0.1271
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
aov_shannon_prevcrop_nifh<-aov(Shannon ~ prev_crop, meta_nifH)
summary(aov_shannon_prevcrop_nifh)
```

```
##             Df Sum Sq Mean Sq F value   Pr(>F)
## prev_crop    3 10.965   3.655   22.57 2.23e-09 ***
## Residuals   50  8.098   0.162
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
aov_shannon_prevcrop_site_nifh<-aov(Shannon ~ prev_crop/Site, meta_nifH)
summary(aov_shannon_prevcrop_site_nifh)
```

```
##                Df Sum Sq Mean Sq F value   Pr(>F)
## prev_crop       3 10.965   3.655  29.594 1.01e-10 ***
## prev_crop:Site  5  2.540   0.508   4.114  0.00367 **
## Residuals      45  5.558   0.124
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

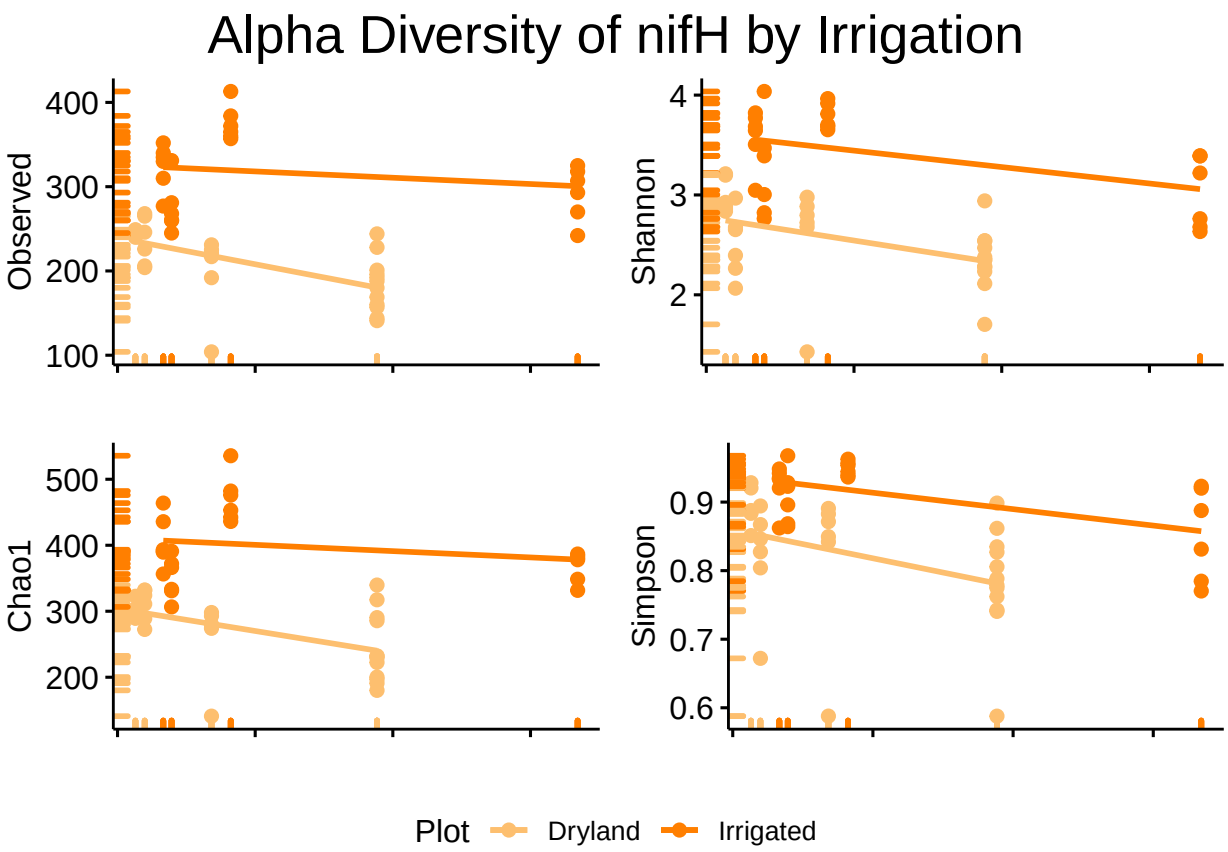
```
aov_shannon_till_nifh<-aov(Shannon ~ Tillage, meta_nifH)
summary(aov_shannon_till_nifh)
```

```
##             Df Sum Sq Mean Sq F value Pr(>F)
## Tillage      2  0.844  0.4220   1.181  0.315
## Residuals   51 18.219  0.3572
```

```r
aov_shannon_till_site_nifh<-aov(Shannon ~ Tillage/Site, meta_nifH)
summary(aov_shannon_till_site_nifh)
```

```
##              Df Sum Sq Mean Sq F value   Pr(>F)
## Tillage       2  0.844  0.4220   3.417   0.0415 *
## Tillage:Site  6 12.662  2.1103  17.086 3.61e-10 ***
## Residuals    45  5.558  0.1235
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```r
nifH_irr_Observ_scatter<-ggscatter(meta_nifH, x = "total_precip_irr", y = "Observed",
   rug = TRUE,
   add = "reg.line",
   color = "Plot", xlab = " ",
   palette = farm_col_paired)+
  rremove("x.text")

nifH_irr_Shannon_scatter<-ggscatter(meta_nifH, x = "total_precip_irr", y = "Shannon",
   rug = TRUE,
   add = "reg.line",
   color = "Plot", xlab = " ",
   palette = farm_col_paired)+
  rremove("x.text")

nifH_irr_Chao_scatter<- ggscatter(meta_nifH, x = "total_precip_irr", y = "Chao1",
   rug = TRUE,
   add = "reg.line",
   color = "Plot", xlab = " ",
   palette = farm_col_paired)+
  rremove("x.text")

nifH_irr_InvSim_scatter<- ggscatter(meta_nifH, x = "total_precip_irr", y = "Simpson",
   rug = TRUE,
   add = "reg.line",
   color = "Plot", xlab = " ",
   palette = farm_col_paired)+
  rremove("x.text")

alpha_nifH_irr_fig<-ggarrange(nifH_irr_Observ_scatter, nifH_irr_Shannon_scatter,
                              nifH_irr_Chao_scatter, nifH_irr_InvSim_scatter,
                              ncol = 2, nrow = 2, common.legend = TRUE, legend = "bottom")

annotate_figure(alpha_nifH_irr_fig,
                top = text_grob("Alpha Diversity of nifH by Irrigation", size = 20))
```



Plot ordination

Used the following protocol from the phyloseq tutorial:

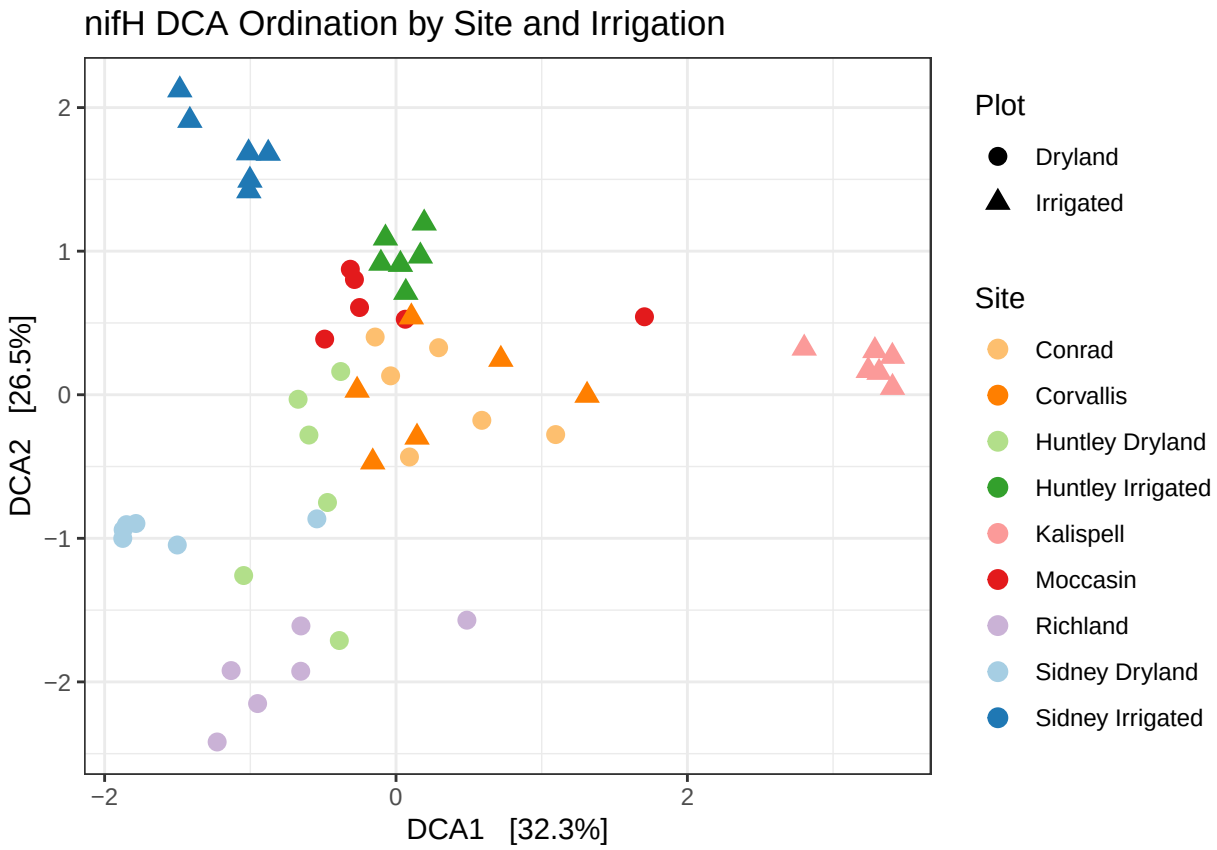
https://joey711.github.io/phyloseq/plot_ordination-examples.html

Resources for ordination statistics

http://ordination.okstate.edu/overview.htm#Principal_Components_Analysis Application of multivariate statistical techniques in microbial ecology: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4769650/pdf/nihms752912.pdf Linking multidimensional functional diversity to quantitative methods: a graphical hypothesis-evaluation framework https://esajournals.onlinelibrary.wiley.com/doi/full/10.1890/15-0688

DCA Ordination

```
phynifH_ord_DCA <- ordinate(physeq_nifH_ord, "DCA", "bray")
plot_ordination(physeq_nifH_ord, phynifH_ord_DCA, color = "Site", shape = "Plot")+
    geom_point(size = 3)+
  scale_color_manual(values =  farm_col_paired)+
  #stat_ellipse(type = "norm", linetype = 2, aes(color ="Plot"), show.legend = TRUE) +
  ggtitle("nifH DCA Ordination by Site and Irrigation")+
  theme_bw()
```



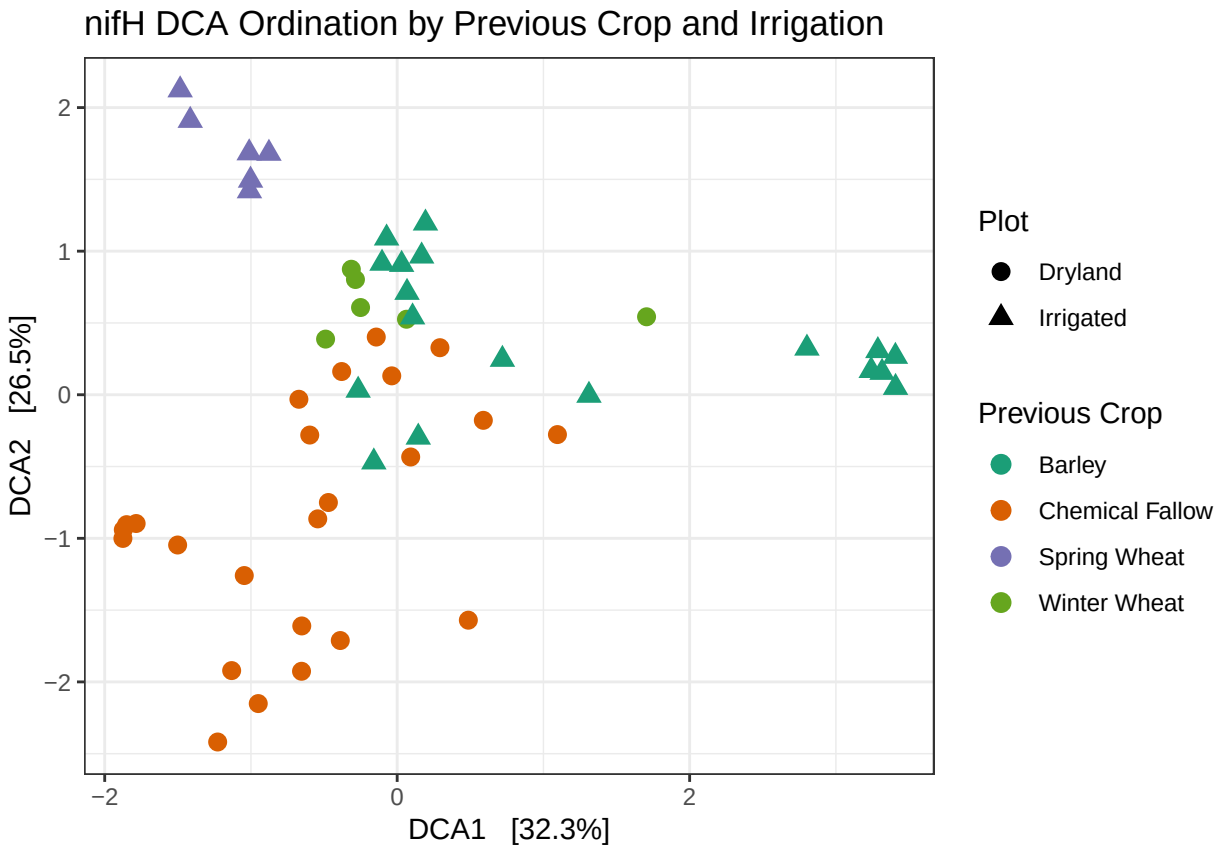
Irrigation is influencing the ordination of the principle components (DCA2 is most likely comprsied of irrigation / other farm Managment)

Publish to the tiff

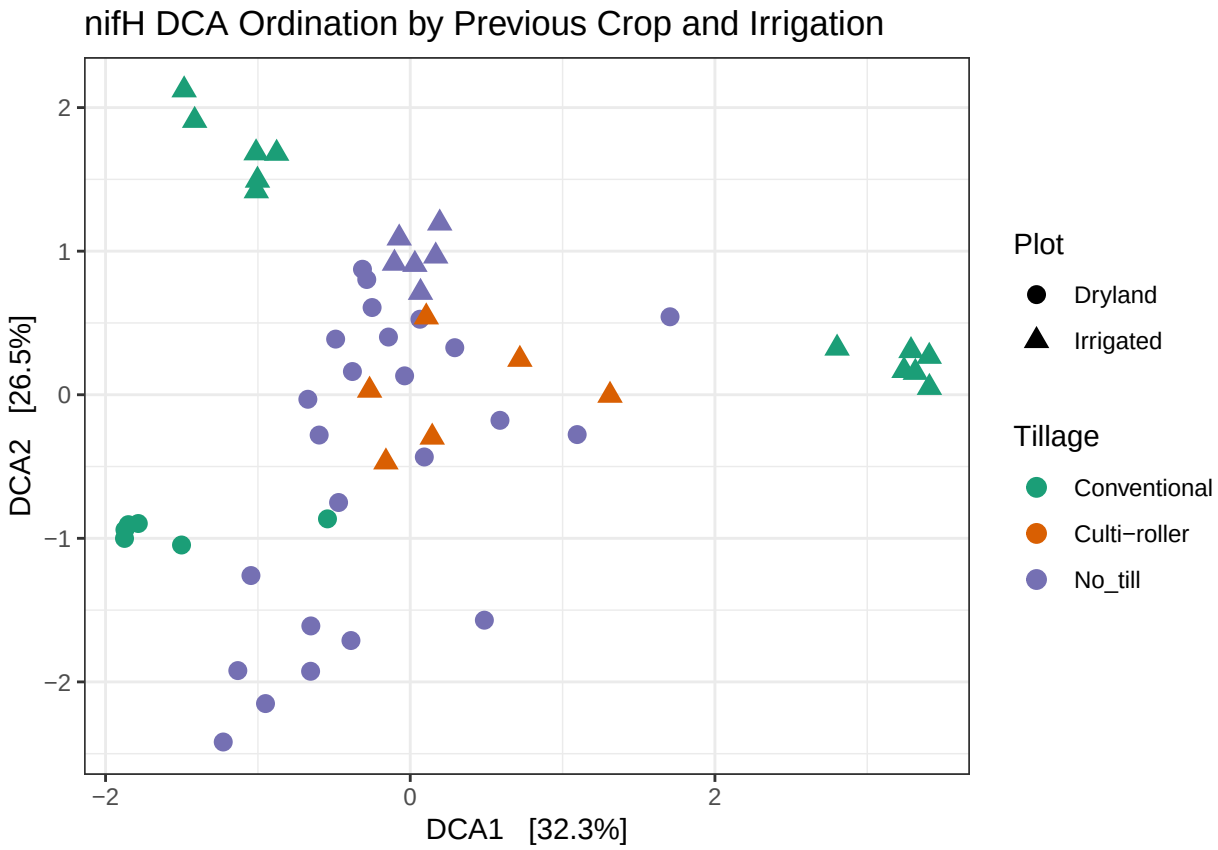
```
## pdf
##   2
```

Will color with other farm managment to see if anytning is intresting.

```
plot_ordination(physeq_nifH_ord, phynifH_ord_DCA, color = "prev_crop", shape = "Plot")+
    geom_point(size = 3)+
  scale_color_manual(values =  c("#1B9E77", "#D95F02", "#7570B3", "#66a61E"),
                     name = "Previous Crop",
                     breaks=c("barley",  "Chem_fallow", "Spring_wheat", "winter_wheat"),
                     labels=c("Barley", "Chemical Fallow", "Spring Wheat", "Winter Wheat"))+
  ggtitle("nifH DCA Ordination by Previous Crop and Irrigation")+
  theme_bw()
```



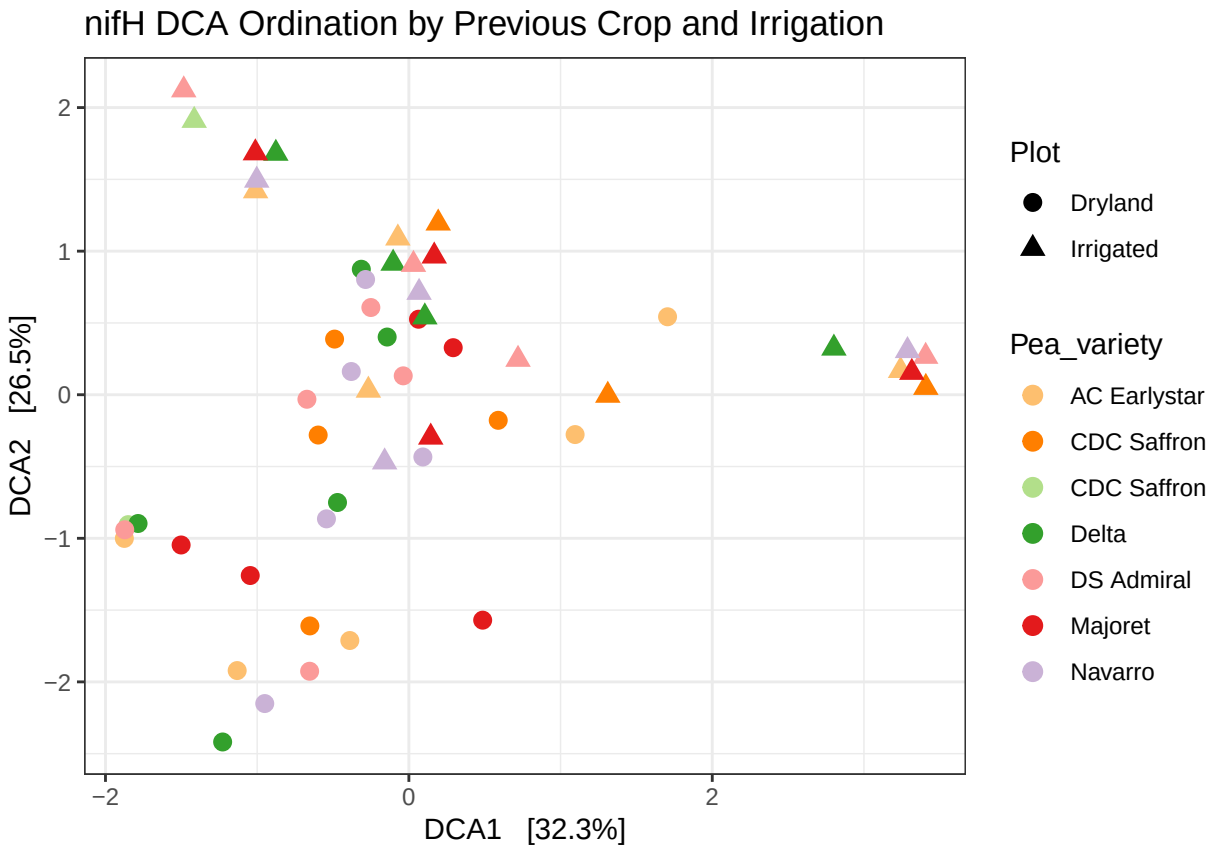

```
plot_ordination(physeq_nifH_ord, phynifH_ord_DCA, color = "Tillage", shape = "Plot")+
    geom_point(size = 3)+
  scale_color_manual(values = farm_col_dark)+
  ggtitle("nifH DCA Ordination by Previous Crop and Irrigation")+
  theme_bw()
```



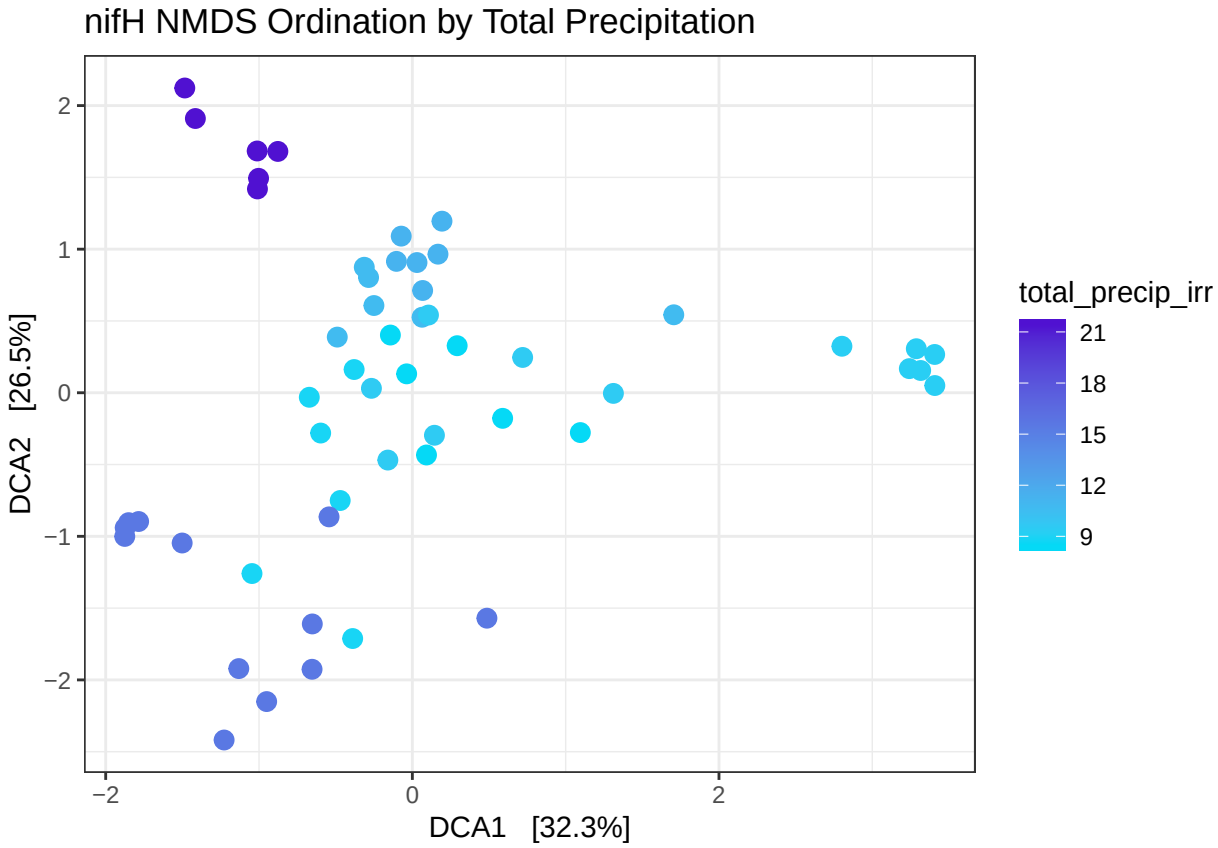
These DCA plot show a distinct seperation for Tillage but not as much for previous crop

Pea variety has no correlation or ordinaiton to bacterial community bray-curtis distance

```
plot_ordination(physeq_nifH_ord, phynifH_ord_DCA, color = "Pea_variety", shape = "Plot")+
    geom_point(size = 3)+
  scale_color_manual(values =  farm_col_paired)+
  ggtitle("nifH DCA Ordination by Previous Crop and Irrigation")+
  theme_bw()
```




```
plot_ordination(physeq_nifH_ord, phynifH_ord_DCA, color = "total_precip_irr")+
  geom_point(size = 3)+
  scale_color_gradient(low='#05D9F6', high='#5011D1')+
  ggtitle("nifH NMDS Ordination by Total Precipitation")+
  theme_bw()
```



There might be some Total water gradient effect in the DCA plot

DCA is good but we can also look in nonmetric multidimensional scaling

Contrast between DCA and NMDS

> DCA and NMDS are the two most popular methods for indirect gradient analysis. The reason they have remained side-by-side for so long is because, in part, they have different strengths and weaknesses... Some of the issues are relatively minor: for example, computation time is rarely an important consideration, except for the hugest data sets. Some issues are not entirely resolved: the degree to which noise affects NMDS, and the degree to which NMDS finds local rather than global options still need to be determined ... Since NMDS is a distance-based method, all information about species identities is hidden once the distance matrix is created. For many, this is the biggest disadvantage of NMDS... perhaps the biggest difference between the two methods: DCA is based on an underlying model of species distributions, the unimodal model, while NMDS is not. Thus, DCA is closer to a theory of community ecology. However, NMDS may be a method of choice if species composition is determined by factors other than position along a gradient: For example, the species present on islands may have more to do with vicariance biogeography and chance extinction events than with environmental preferences – and for such a system, NMDS would be a better a priori choice. As De'ath (1999) points out, there are two classes of ordination methods - 'species composition restoration' (e.g. NMDS) and 'gradient analysis' (e.g. DCA). The choice between the methods should ultimately be governed by this philosophical distinction. - http://ordination.okstate.edu/overview.htm#Principal_Components_Analysis

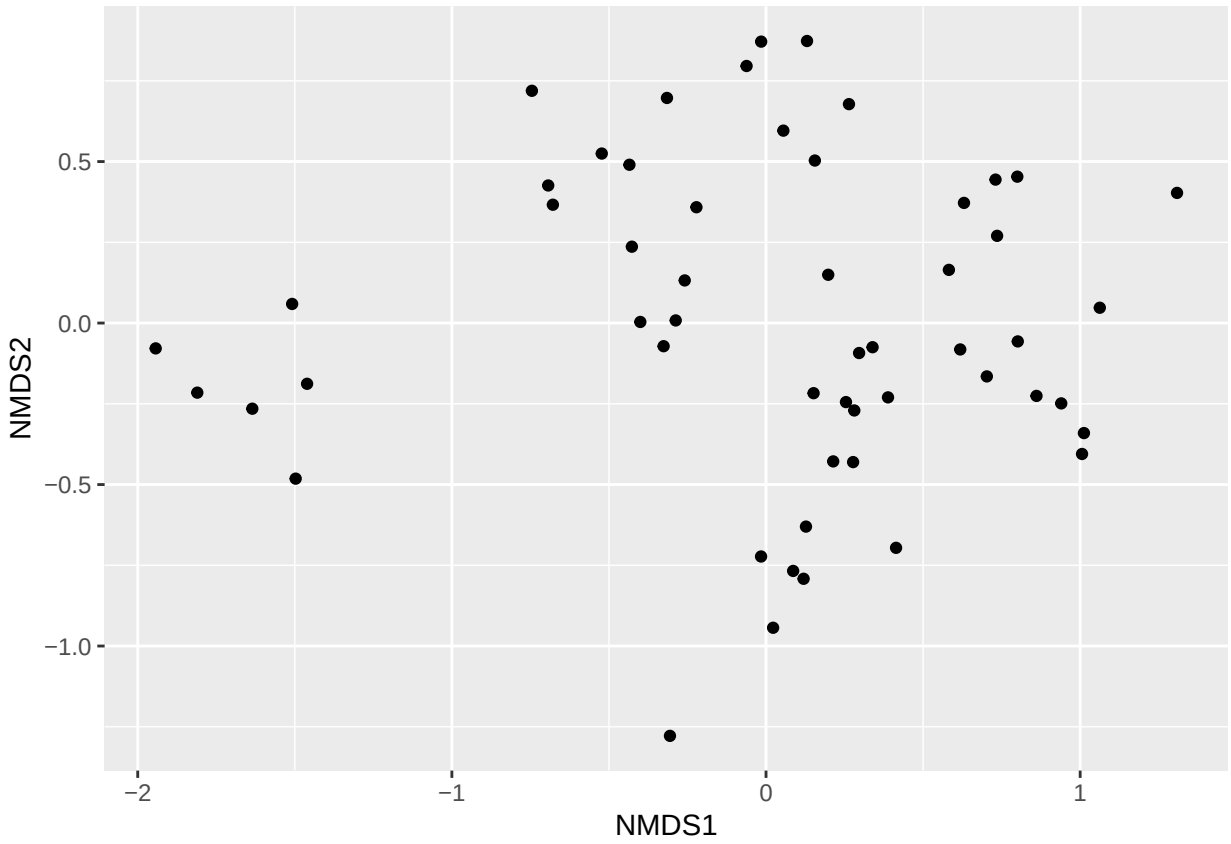
NMDS might be a better choice since we have non gradient determining facotrs site and farm managment effecting the bacteria community

NMDS ordination

```
phynifH_ord_NMDS <- ordinate(physeq_nifH_ord, "NMDS", "bray")
```

```
## Square root transformation
## Wisconsin double standardization
## Run 0 stress 0.1929531
## Run 1 stress 0.1866013
## ... New best solution
## ... Procrustes: rmse 0.0548474  max resid 0.1690312
## Run 2 stress 0.192952
## Run 3 stress 0.201325
## Run 4 stress 0.2186671
## Run 5 stress 0.1929529
## Run 6 stress 0.192428
## Run 7 stress 0.1866138
## ... Procrustes: rmse 0.002278817  max resid 0.009262113
## ... Similar to previous best
## Run 8 stress 0.1924281
## Run 9 stress 0.2013283
## Run 10 stress 0.1903583
## Run 11 stress 0.1986486
## Run 12 stress 0.1924274
## Run 13 stress 0.1937887
## Run 14 stress 0.1927817
## Run 15 stress 0.2300822
## Run 16 stress 0.1929299
## Run 17 stress 0.1880774
## Run 18 stress 0.1927839
## Run 19 stress 0.1929304
## Run 20 stress 0.1865915
## ... New best solution
## ... Procrustes: rmse 0.003521058  max resid 0.02043394
## *** No convergence -- monoMDS stopping criteria:
##     20: stress ratio > sratmax
```

```
plot_ordination(physeq_nifH_ord, phynifH_ord_NMDS)
```



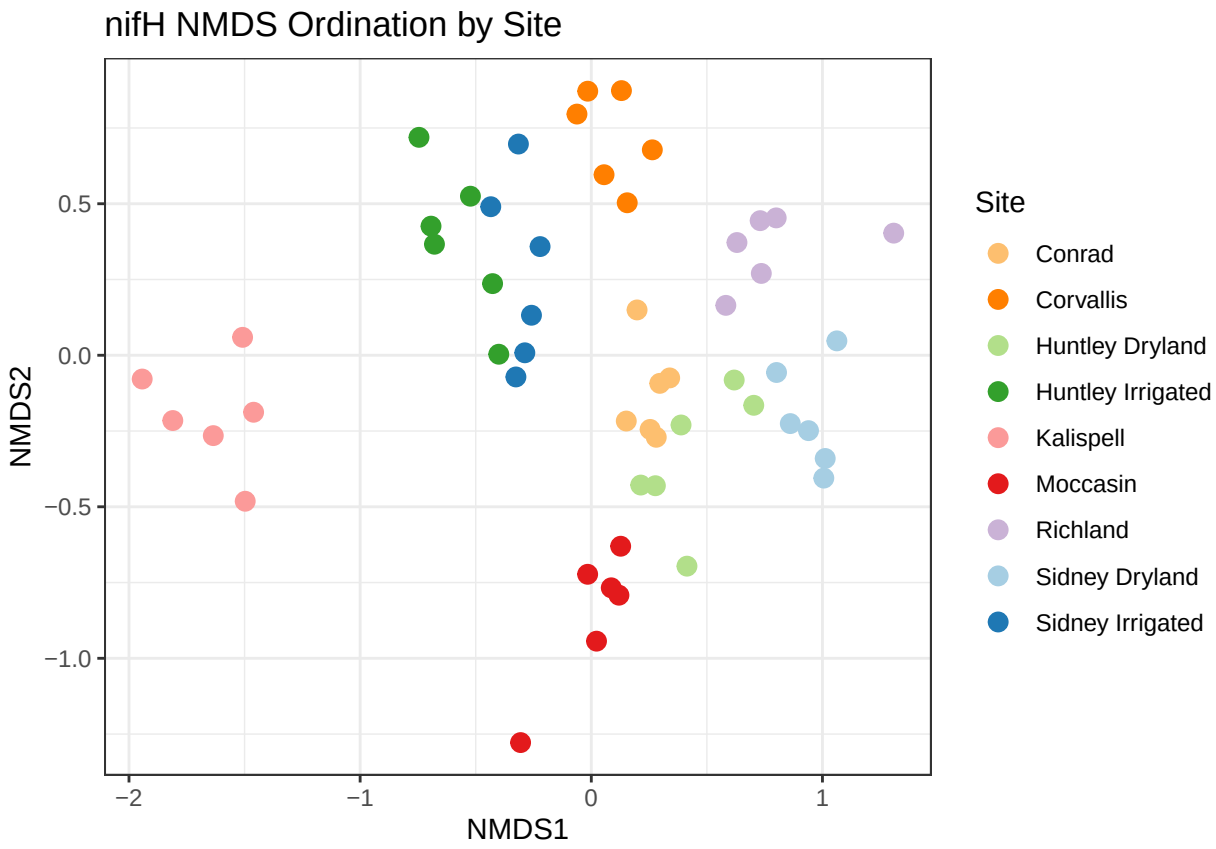

```
phynifH_ord_NMDS
```

```
##
## Call:
## metaMDS(comm = veganifyOTU(physeq), distance = distance)
##
## global Multidimensional Scaling using monoMDS
##
## Data:     wisconsin(sqrt(veganifyOTU(physeq)))
## Distance: bray
##
## Dimensions: 2
## Stress:     0.1865915
## Stress type 1, weak ties
## No convergent solutions - best solution after 20 tries
## Scaling: centring, PC rotation, halfchange scaling
## Species: expanded scores based on 'wisconsin(sqrt(veganifyOTU(physeq)))'
```

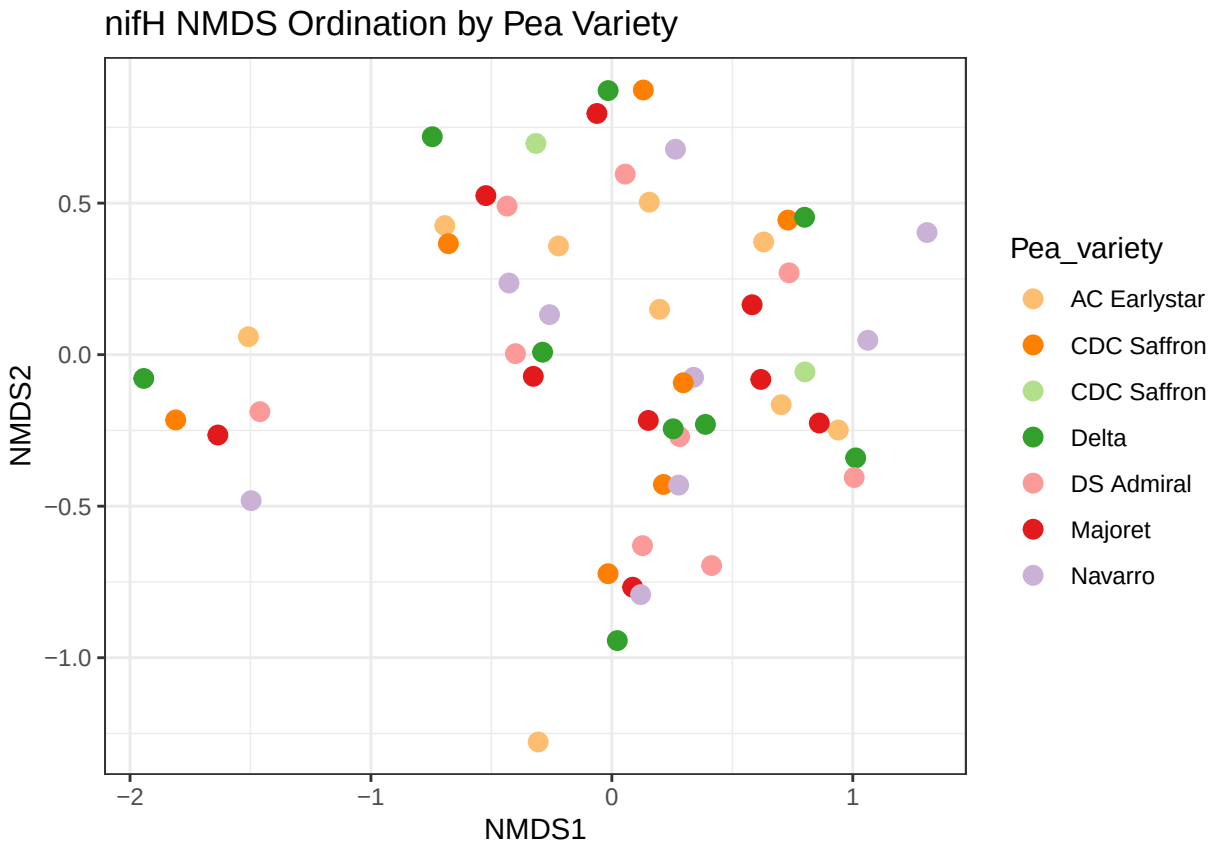
After stress test run, we get a value of 0.16 which is considered good, anything below 0.2 is acceptable.

Plot NMDS with Site

```
plot_ordination(physeq_nifH_ord, phynifH_ord_NMDS, color = "Site")+
  geom_point(size = 3)+
  scale_color_manual(values =  farm_col_paired)+
  ggtitle("nifH NMDS Ordination by Site")+
  theme_bw()
```

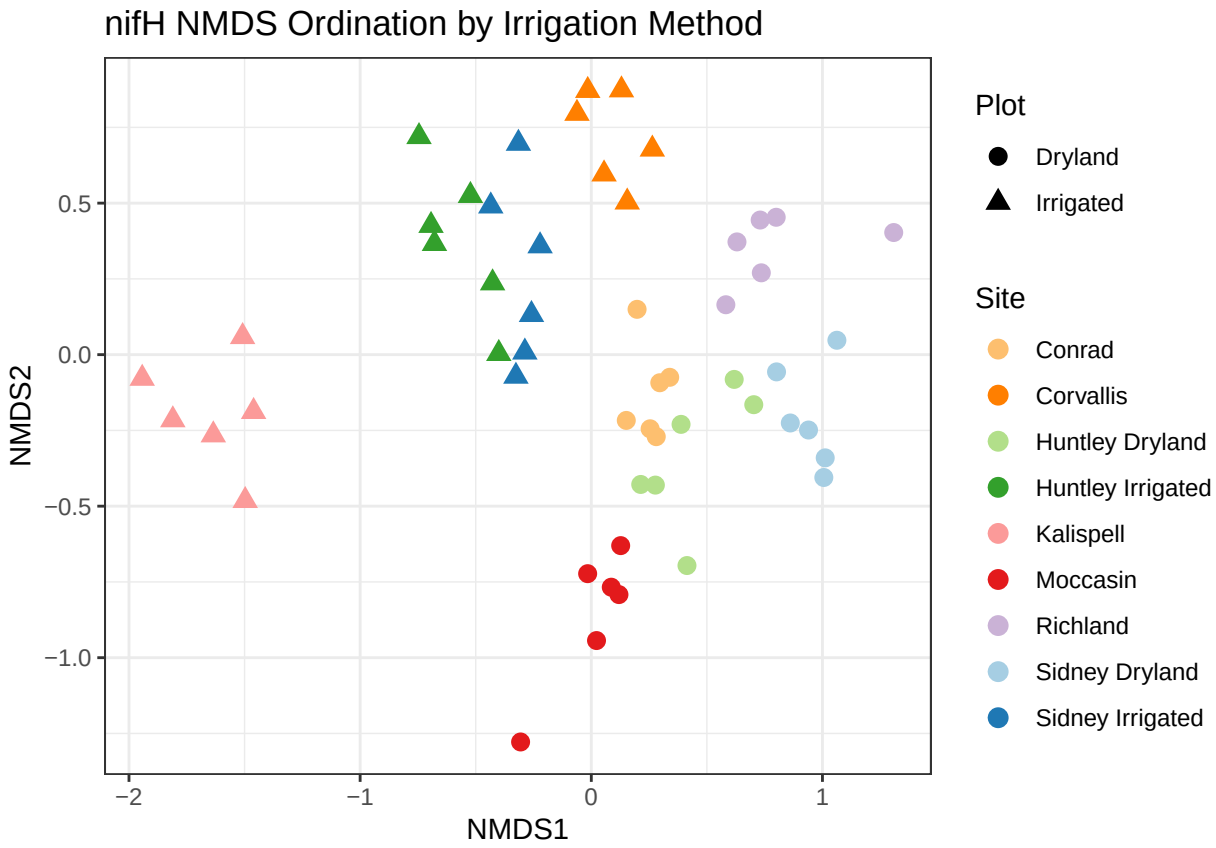



```
plot_ordination(physeq_nifH_ord, phynifH_ord_NMDS, color = "Pea_variety")+
  geom_point(size = 3)+
  scale_color_manual(values =  farm_col_paired)+
  ggtitle("nifH NMDS Ordination by Pea Variety")+
  theme_bw()
```



Pea variety does not influence the microbiome compostion in NMDS as well

```
plot_ordination(physeq_nifH_ord, phynifH_ord_NMDS, shape = "Plot", color = "Site")+
  geom_point(size = 3)+
  scale_color_manual(values =  farm_col_paired)+
  ggtitle("nifH NMDS Ordination by Irrigation Method")+
  #stat_ellipse(type = "norm", linetype = 2, aes(color = "Plot")) +
  theme_bw()
```




```
NMDS_nifH<-plot_ordination(physeq_nifH_ord, phynifH_ord_NMDS, shape = "Plot", color = "Site")+
  geom_point(size = 3)+
  scale_color_manual(values =  farm_col_paired)+
  #ggtitle("nifH NMDS Ordination by Irrigation Method")+
  #stat_ellipse(type = "norm", linetype = 2, aes(color = "Plot")) +
  theme_bw()
```

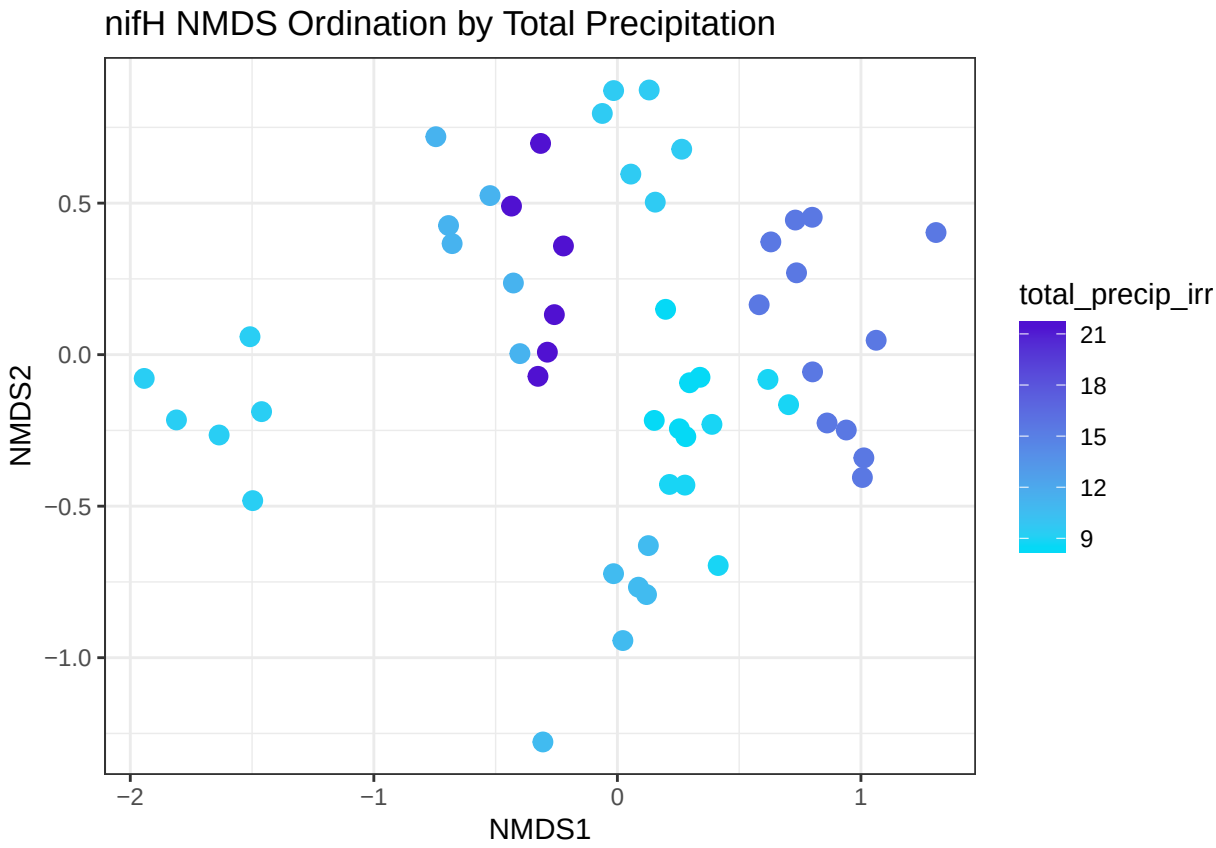
Publish to tiff

```
## pdf
##   2
```

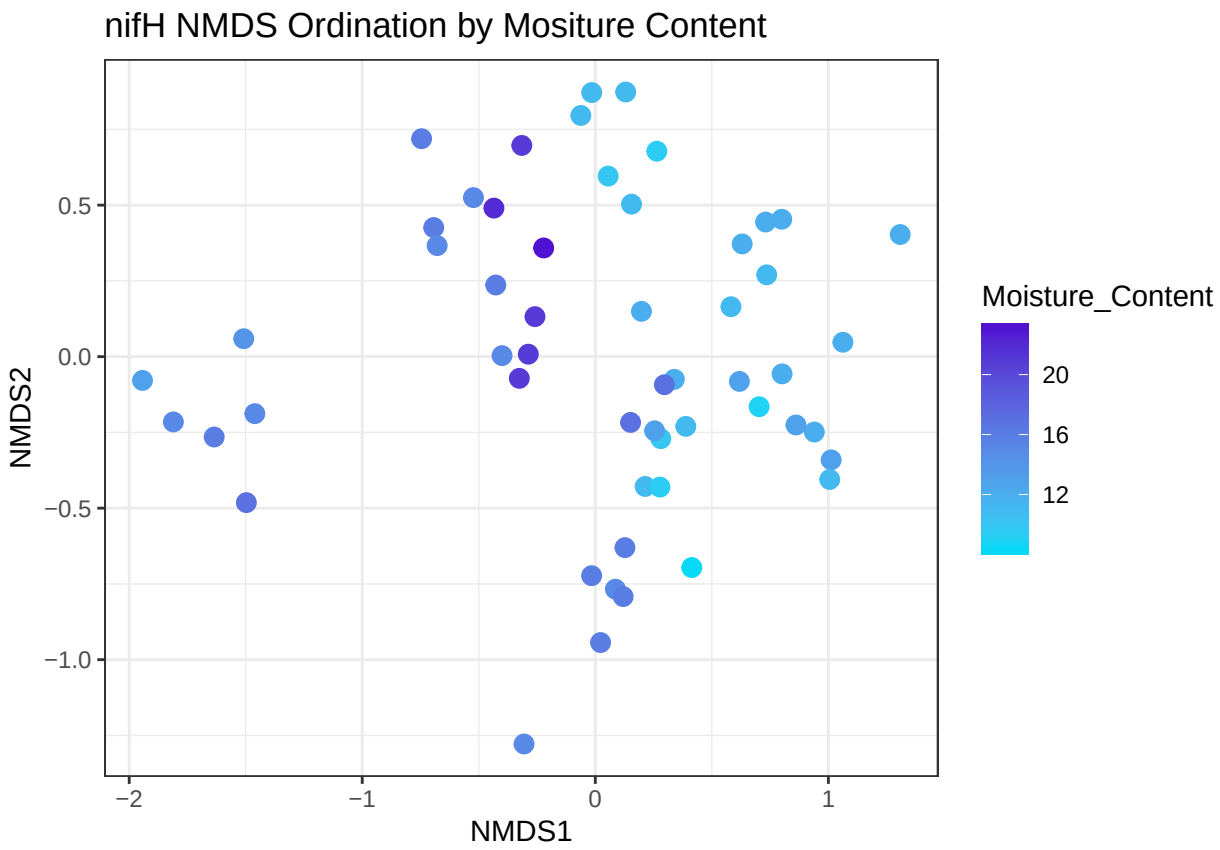
There is again a nice spliting pattern between irrgated and dryland

Total water added

```
plot_ordination(physeq_nifH_ord, phynifH_ord_NMDS, color = "total_precip_irr")+
  geom_point(size = 3)+
  scale_color_gradient(low='#05D9F6', high='#5011D1')+
  ggtitle("nifH NMDS Ordination by Total Precipitation")+
  theme_bw()
```



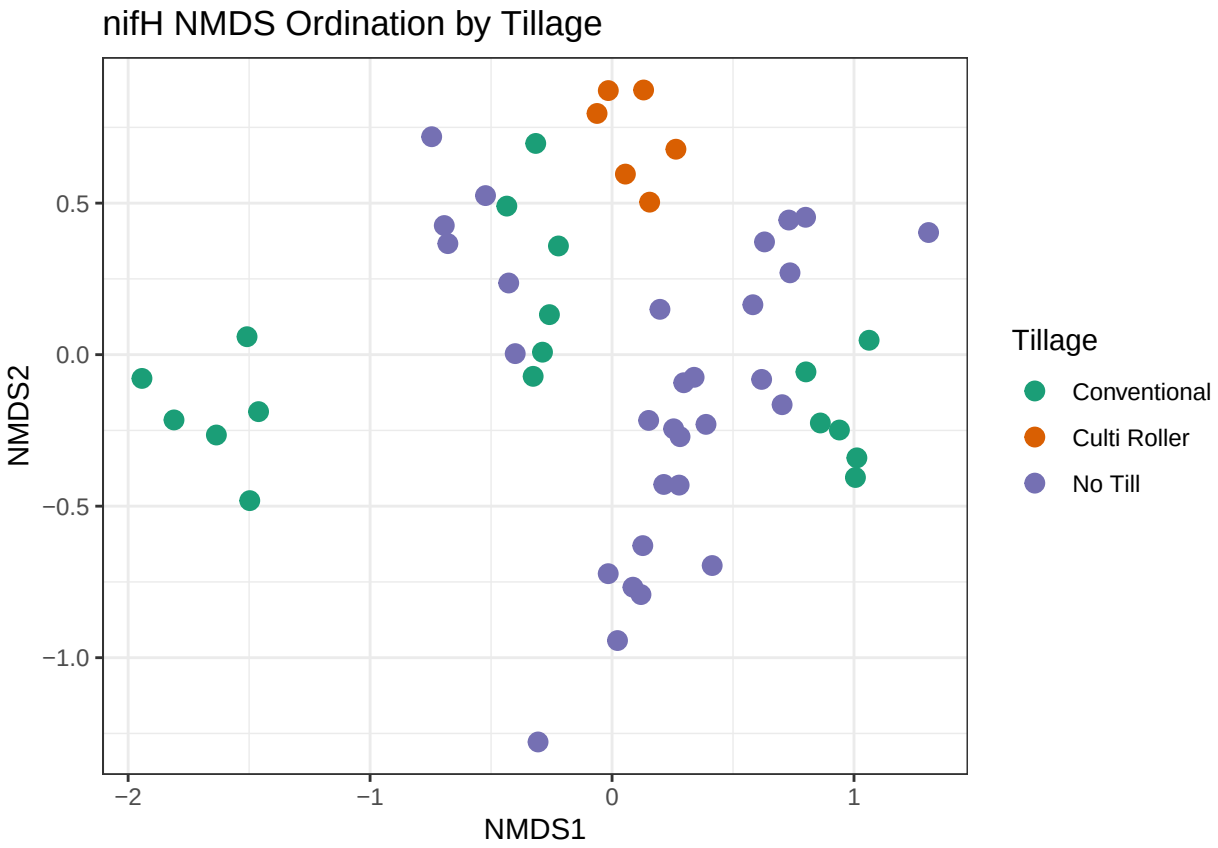

```
plot_ordination(physeq_nifH_ord, phynifH_ord_NMDS, color = "Moisture_Content")+
  geom_point(size = 3)+
  scale_color_continuous(low='#05D9F6', high='#5011D1')+
  ggtitle("nifH NMDS Ordination by Mositure Content")+
  theme_bw()
```



Total water does not ordinate well in NMDS as compared to DCA.

Lets try some of the other variables like till, fallow, and previous crop.

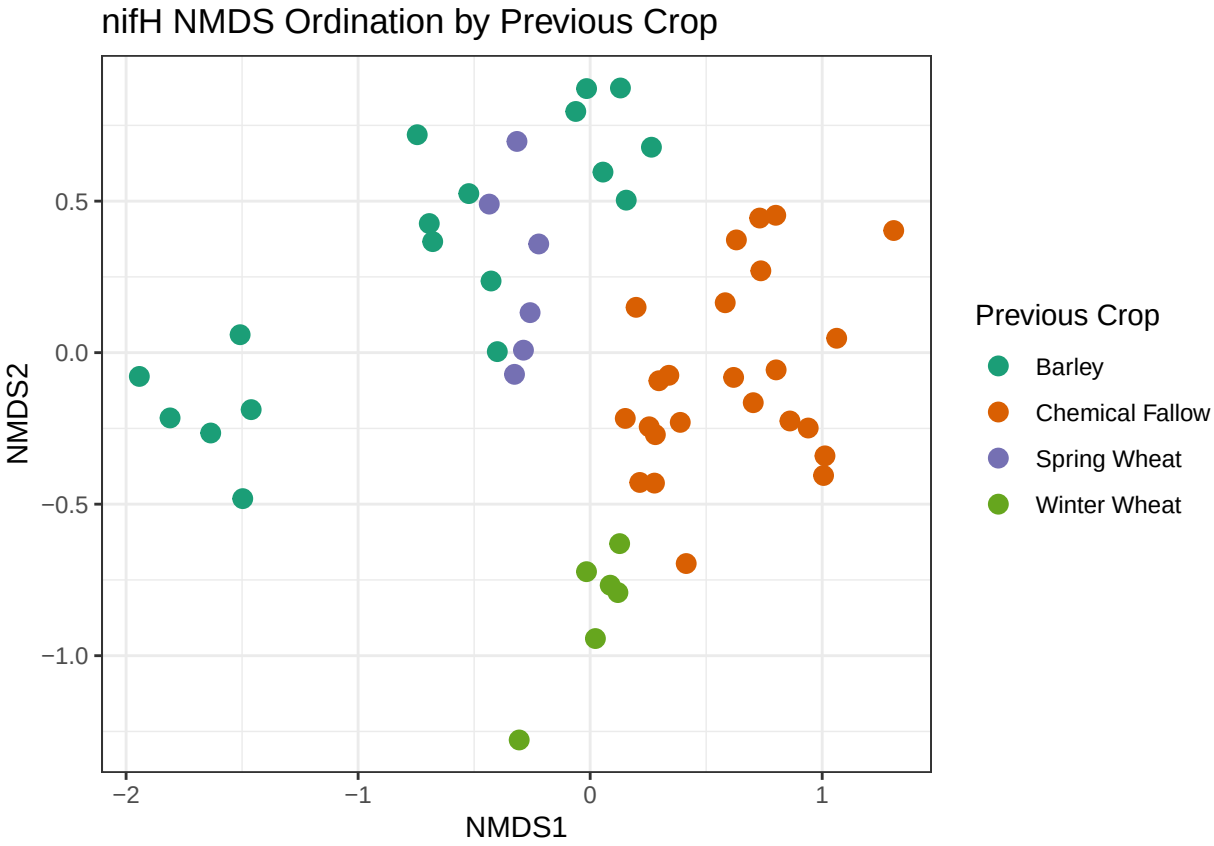
```
plot_ordination(physeq_nifH_ord, phynifH_ord_NMDS,color = "Tillage")+
  geom_point(size = 3)+
  scale_color_manual(values =  farm_col_dark, breaks=c("Conventional", "Culti-roller", "No_till"),
                     labels=c("Conventional", "Culti Roller", "No Till"))+
  ggtitle("nifH NMDS Ordination by Tillage")+
  theme_bw()
```



Publish to .tiff

```
## pdf
##   2
```

```
plot_ordination(physeq_nifH_ord, phynifH_ord_NMDS, color = "prev_crop")+
  geom_point(size = 3)+
  scale_color_manual(values =  c("#1B9E77", "#D95F02", "#7570B3", "#66a61E"),
                     name = "Previous Crop",
                     breaks=c("barley",  "Chem_fallow", "Spring_wheat", "winter_wheat"),
                     labels=c("Barley", "Chemical Fallow", "Spring Wheat", "Winter Wheat"))+
  ggtitle("nifH NMDS Ordination by Previous Crop")+
  theme_bw()
```



Publish to .tiff

```
## pdf
##   2
```

Beta dispersions

Test the diffrences in group homogeneities. Do our farm managment factors effect the homogeneitiey of the bray curtis distance?

If a group (Site) in the MDS space are close but have diffrenent dispersion you could have a signifcant results when it is only a diffrence in dispersion.

Anderson (2006)-https://www.ncbi.nlm.nih.gov/pubmed/16542252

https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1461-0248.2006.00926.x

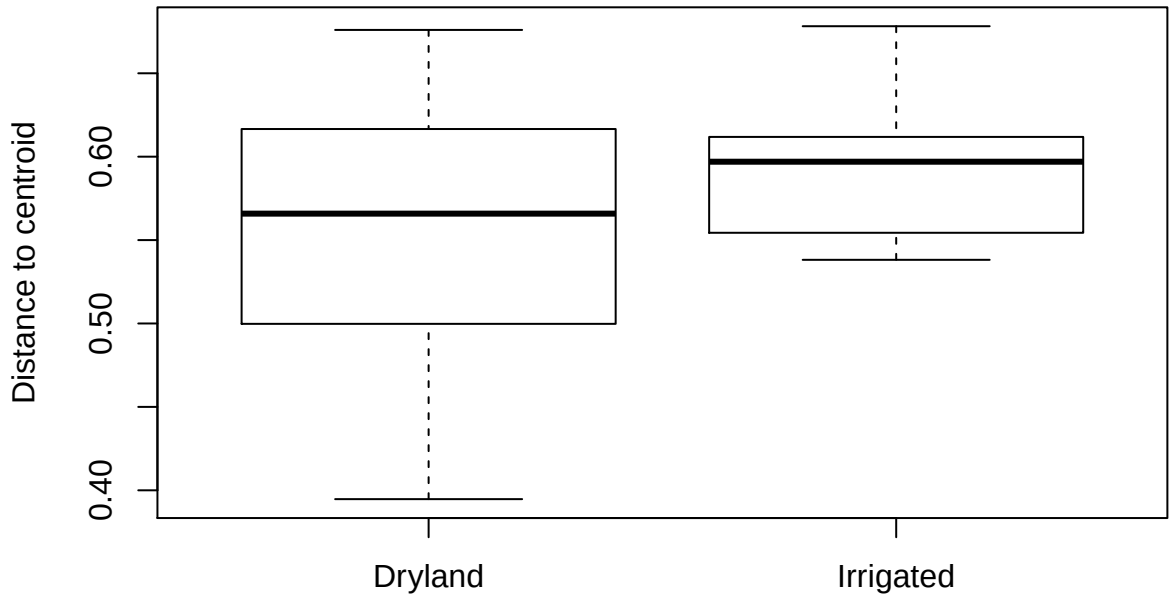
Irrigation beta dispersion

```
nifH_bray<-distance(physeq_nifH_ord, method = "bray")
disp.plot <- betadisper(nifH_bray, meta2$Plot)
permutest(disp.plot, pairwise=TRUE, permutations=1000)
```

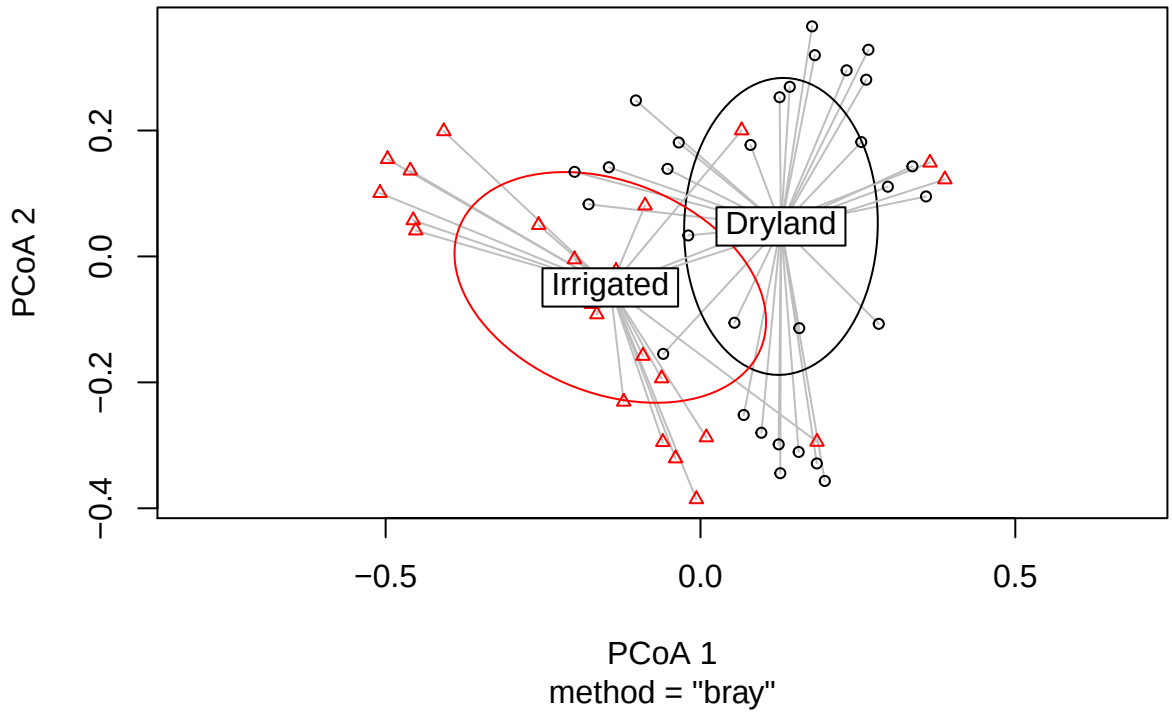
```
##
## Permutation test for homogeneity of multivariate dispersions
```

```
## Permutation: free
## Number of permutations: 1000
##
## Response: Distances
##           Df  Sum Sq  Mean Sq      F N.Perm  Pr(>F)
## Groups     1 0.01565 0.015651 4.0587   1000 0.04396 *
## Residuals 52 0.20051 0.003856
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Pairwise comparisons:
## (Observed p-value below diagonal, permuted p-value above diagonal)
##            Dryland Irrigated
## Dryland                0.049
## Irrigated 0.049129
```

```
boxplot(disp.plot)
```



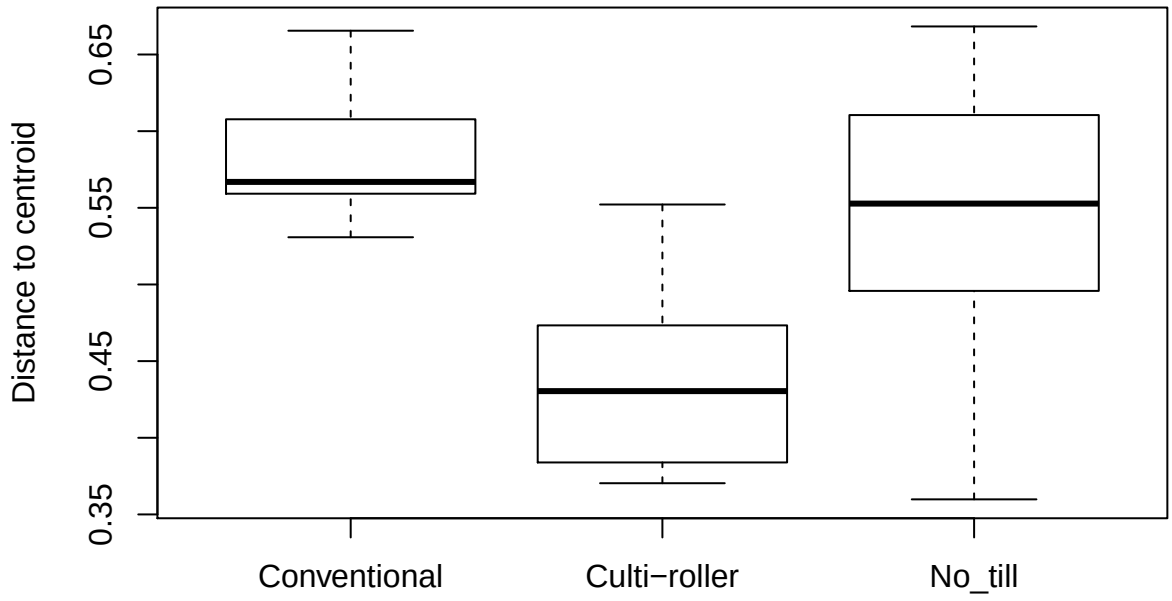

```
plot(disp.plot, hull = FALSE, ellipse = TRUE)
```



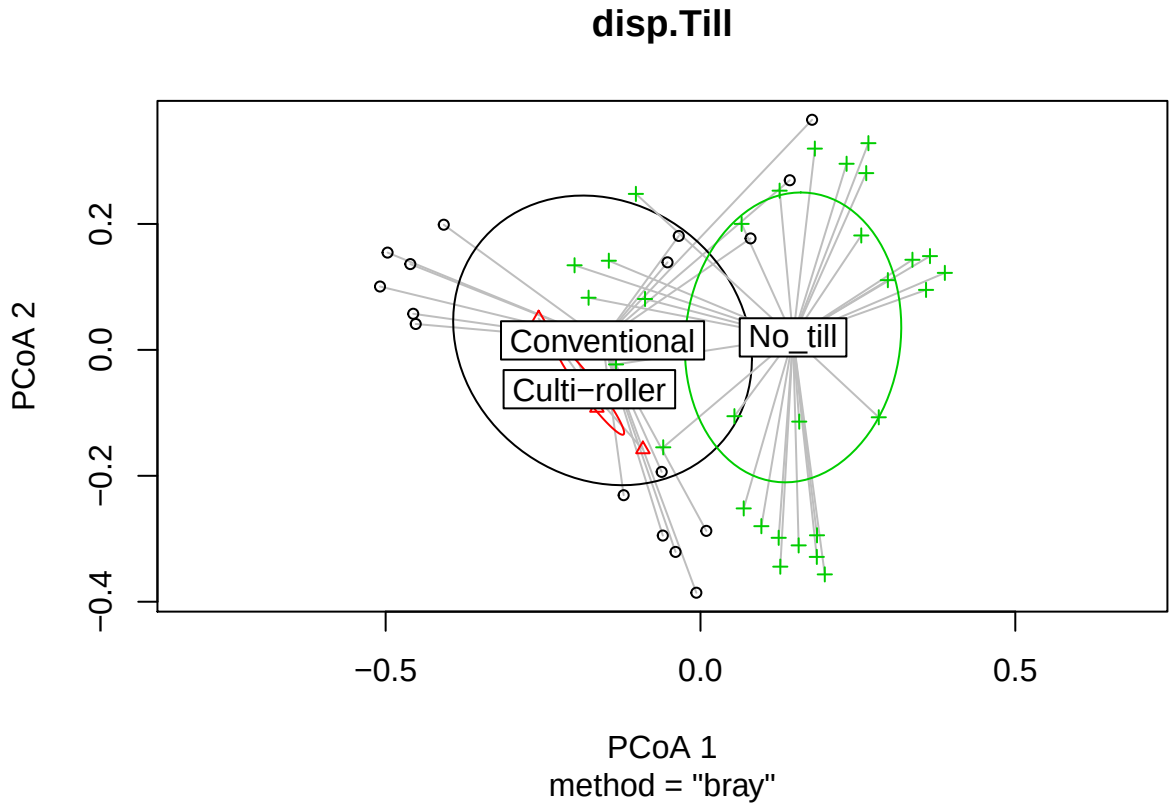

```
disp.Till <- betadisper(distance(physeq_nifH_ord, method = "bray"), meta2$Tillage)
permutest(disp.Till, pairwise=TRUE, permutations=1000)
```

```
##
## Permutation test for homogeneity of multivariate dispersions
## Permutation: free
## Number of permutations: 1000
##
## Response: Distances
##           Df   Sum Sq  Mean Sq      F N.Perm   Pr(>F)
## Groups     2 0.089137 0.044568 8.6297   1000 0.001998 **
## Residuals 51 0.263390 0.005165
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Pairwise comparisons:
## (Observed p-value below diagonal, permuted p-value above diagonal)
##              Conventional Culti-roller No_till
## Conventional                9.9900e-04  0.0669
## Culti-roller   2.0614e-06               0.0050
## No_till        6.7763e-02   1.1376e-02
```

```r
boxplot(disp.Till)
```




```r
plot(disp.Till, hull = FALSE, ellipse = TRUE)
```

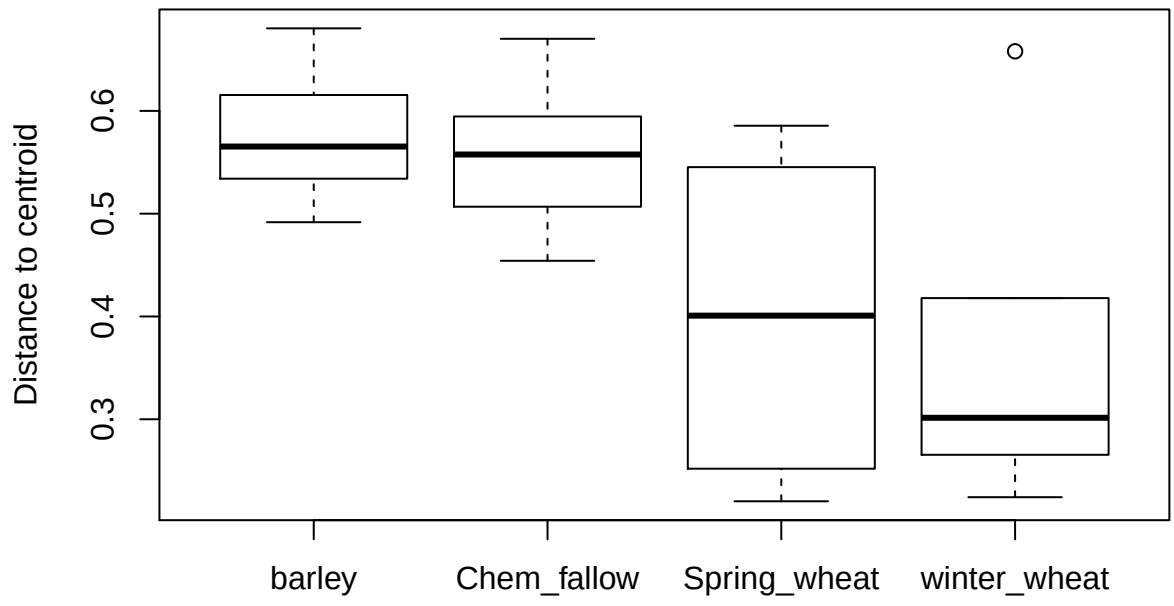


Tillage (Conventional and Cuti-Roller) vs NO till are spliting

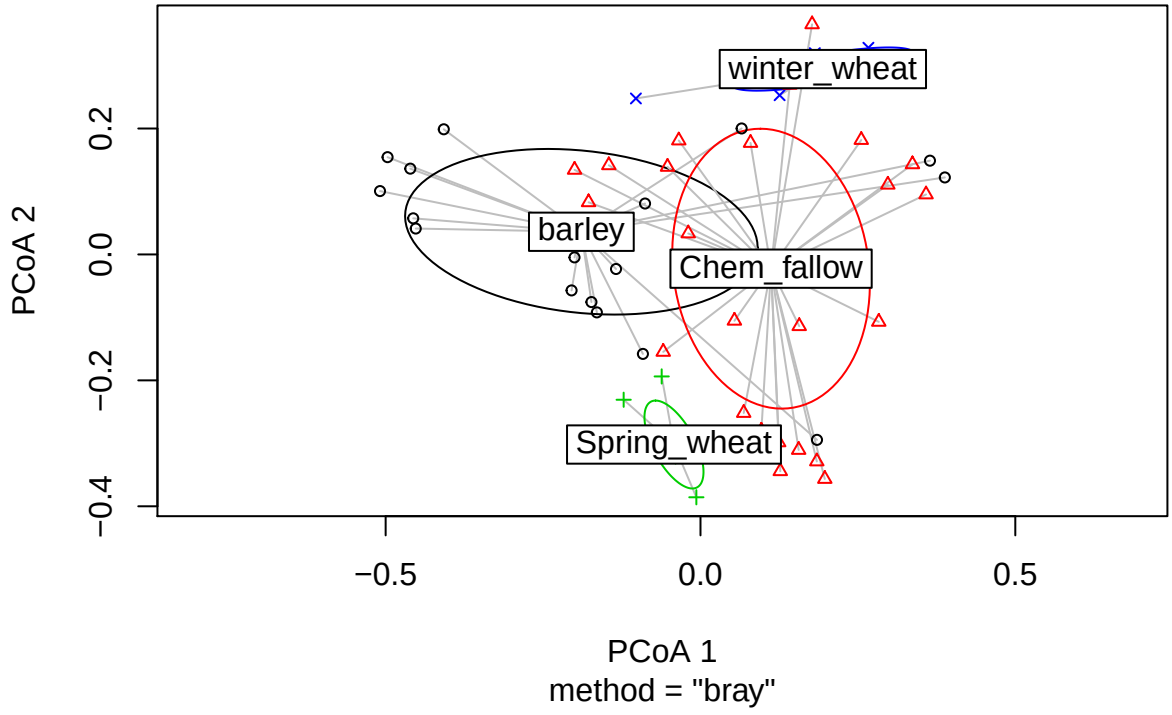
```
disp.prev_crop <- betadisper(distance(physeq_nifH_ord, method = "bray"), meta2$prev_crop)
permutest(disp.prev_crop, pairwise=TRUE, permutations=1000)
```

```
##
## Permutation test for homogeneity of multivariate dispersions
## Permutation: free
## Number of permutations: 1000
##
## Response: Distances
##           Df  Sum Sq  Mean Sq      F N.Perm   Pr(>F)
## Groups     3 0.32146 0.107153 13.645   1000 0.000999 ***
## Residuals 50 0.39265 0.007853
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Pairwise comparisons:
## (Observed p-value below diagonal, permuted p-value above diagonal)
##                  barley Chem_fallow Spring_wheat winter_wheat
## barley                   1.9580e-01   9.9900e-04       0.0010
## Chem_fallow  1.8314e-01               2.9970e-03       0.0020
## Spring_wheat 3.6221e-04  8.3887e-04                    0.7073
## winter_wheat 3.1348e-05  5.3897e-05   6.7995e-01
```

```
boxplot(disp.prev_crop)
```




```
plot(disp.prev_crop, hull = FALSE, ellipse = TRUE)
```




```
TukeyHSD(disp.prev_crop)
```

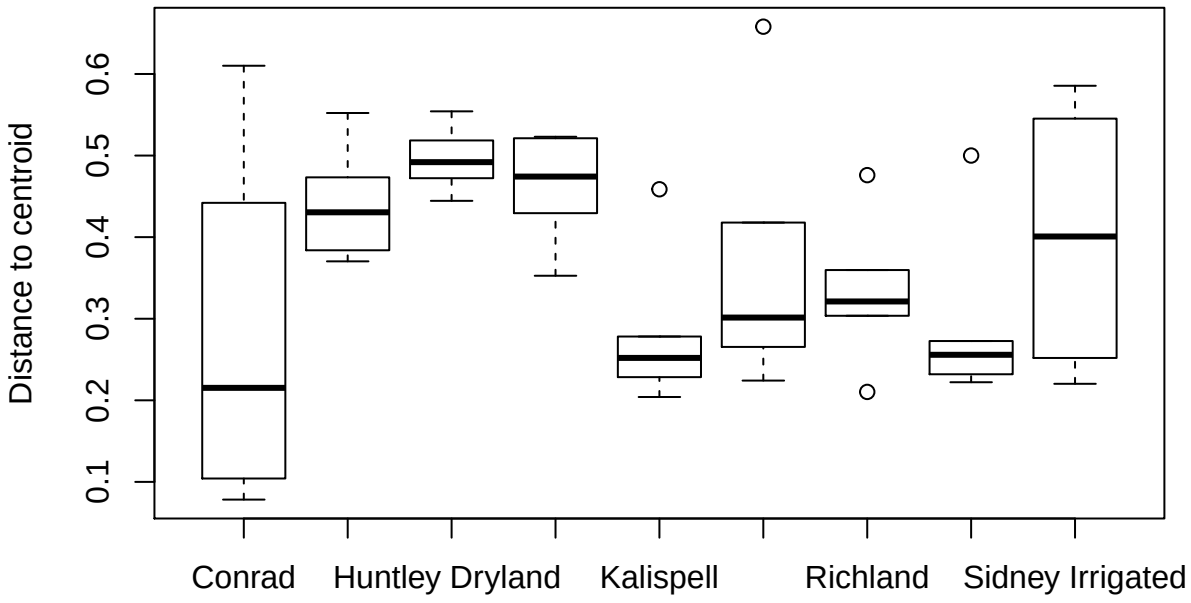
```
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = distances ~ group, data = df)
##
## $group
##                                 diff         lwr         upr     p adj
## Chem_fallow-barley        -0.02453687 -0.09796924  0.04889550 0.8110677
## Spring_wheat-barley       -0.17676352 -0.28778283 -0.06574422 0.0005585
## winter_wheat-barley       -0.21613604 -0.32715535 -0.10511673 0.0000237
## Spring_wheat-Chem_fallow  -0.15222665 -0.25972063 -0.04473267 0.0024172
## winter_wheat-Chem_fallow  -0.19159917 -0.29909315 -0.08410518 0.0001054
## winter_wheat-Spring_wheat -0.03937252 -0.17534285  0.09659781 0.8677798
```

```
disp.site.nifH <- betadisper(distance(physeq_nifH_ord, method = "bray"), meta2$Site)
permutest(disp.site.nifH, pairwise=TRUE, permutations=1000)
```

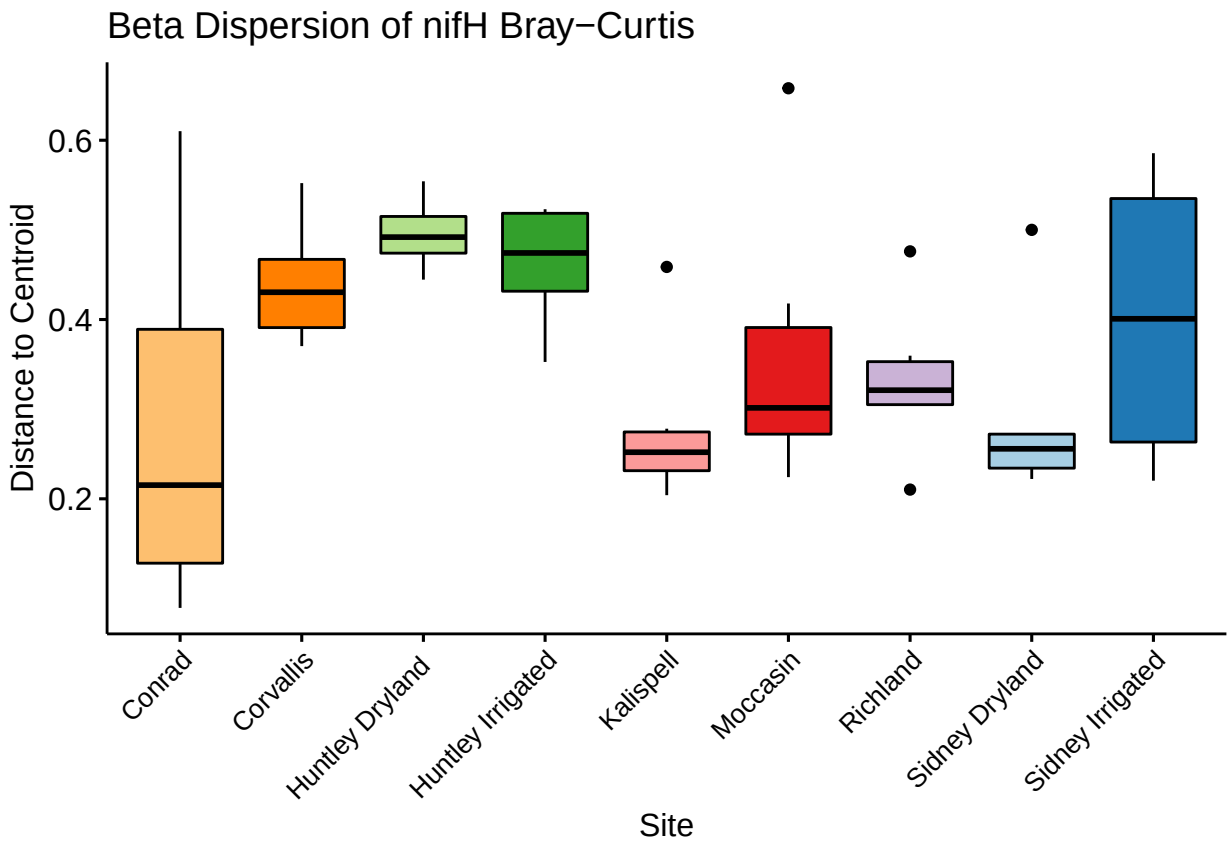
```
##
## Permutation test for homogeneity of multivariate dispersions
## Permutation: free
## Number of permutations: 1000
##
## Response: Distances
##           Df  Sum Sq  Mean Sq      F N.Perm  Pr(>F)
## Groups     8 0.32992 0.041240 2.8058   1000 0.01598 *
```

```
## Residuals 45 0.66143 0.014698
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Pairwise comparisons:
## (Observed p-value below diagonal, permuted p-value above diagonal)
##                      Conrad  Corvallis Huntley Dryland  Huntley Irrigated
## Conrad                       0.08691309       0.03096903        0.05694306
## Corvallis         0.09803153                  0.11988012        0.61138861
## Huntley Dryland   0.02995297 0.10991872                        0.32667333
## Huntley Irrigated 0.06494609 0.57900203       0.32466376
## Kalispell         0.98867876 0.00601294       0.00033484        0.00280268
## Moccasin          0.45050342 0.29029786       0.07247948        0.18292552
## Richland          0.56654632 0.03642784       0.00177420        0.01586284
## Sidney Dryland    0.90035851 0.01441515       0.00113378        0.00698916
## Sidney Irrigated  0.27835921 0.59461068       0.19328302        0.40961155
##                    Kalispell   Moccasin   Richland Sidney Dryland
## Conrad            0.98801199 0.46253746 0.55644356     0.89310689
## Corvallis         0.00699301 0.27372627 0.03796204     0.01998002
## Huntley Dryland   0.00099900 0.07692308 0.00499500     0.00299700
## Huntley Irrigated 0.00799201 0.20279720 0.01898102     0.00899101
## Kalispell                    0.28271728 0.32767233     0.85714286
## Moccasin          0.29701845            0.67632368     0.35464535
## Richland          0.32751945 0.69935339                0.43556444
## Sidney Dryland    0.85273083 0.37883278 0.46494154
## Sidney Irrigated  0.13987695 0.67994777 0.38070532     0.18913329
##                   Sidney Irrigated
## Conrad                      0.2697
## Corvallis                   0.5674
## Huntley Dryland             0.1928
## Huntley Irrigated           0.4176
## Kalispell                   0.1349
## Moccasin                    0.6643
## Richland                    0.3666
## Sidney Dryland              0.2008
## Sidney Irrigated
```

```
boxplot(disp.site.nifH)
```



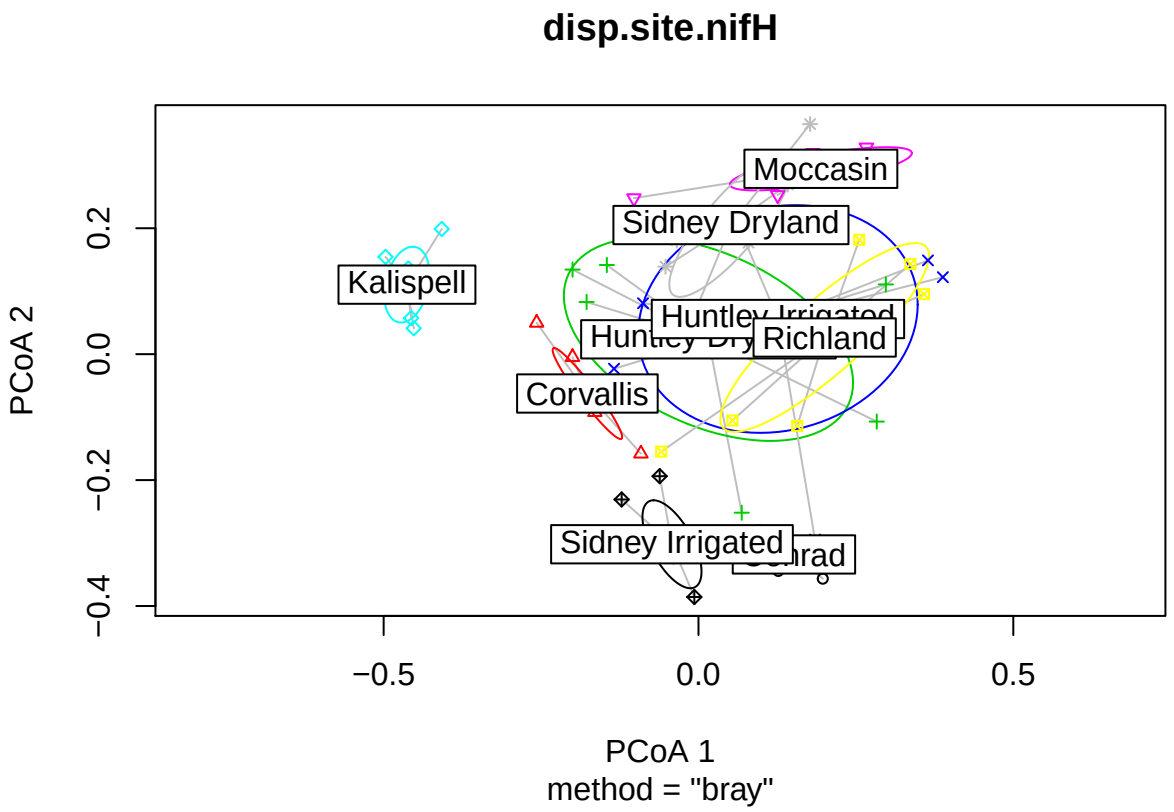

```
nifH_dispersion_site<-data.frame(Distance_to_centroid=disp.site.nifH$distances, Site=disp.site.nifH$grou
ggboxplot(nifH_dispersion_site, x = "Site", y = "Distance_to_centroid",
   rug = TRUE,  legend = "none",
   fill = "Site", ylab = "Distance to Centroid", title = "Beta Dispersion of nifH Bray-Curtis",
   palette = farm_col_paired)+
   rotate_x_text(45, size = 10)
```



Publish to .tiff

```
## pdf
##   2
```

```
plot(disp.site.nifH, hull = FALSE, ellipse = TRUE)
```




```
TukeyHSD(disp.site.nifH)
```

```
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = distances ~ group, data = df)
##
## $group
##                                          diff          lwr        upr
## Corvallis-Conrad                    0.162574317 -0.065413487 0.39056212
## Huntley Dryland -Conrad             0.218030908 -0.009956896 0.44601871
## Huntley Irrigated-Conrad            0.184950208 -0.043037596 0.41293801
## Kalispell-Conrad                    0.001348719 -0.226639086 0.22933652
## Moccasin-Conrad                     0.083865359 -0.144122446 0.31185316
## Richland-Conrad                     0.054458523 -0.173529281 0.28244633
## Sidney Dryland-Conrad               0.012201487 -0.215786317 0.24018929
## Sidney Irrigated-Conrad             0.123237876 -0.104749928 0.35122568
## Huntley Dryland -Corvallis          0.055456591 -0.172531214 0.28344440
## Huntley Irrigated-Corvallis         0.022375890 -0.205611914 0.25036369
## Kalispell-Corvallis                -0.161225599 -0.389213403 0.06676221
## Moccasin-Corvallis                 -0.078708959 -0.306696763 0.14927885
## Richland-Corvallis                 -0.108115794 -0.336103598 0.11987201
```

```
## Sidney Dryland-Corvallis           -0.150372830 -0.378360635 0.07761497
## Sidney Irrigated-Corvallis         -0.039336441 -0.267324246 0.18865136
## Huntley Irrigated-Huntley Dryland  -0.033080700 -0.261068505 0.19490710
## Kalispell-Huntley Dryland          -0.216682189 -0.444669994 0.01130561
## Moccasin-Huntley Dryland           -0.134165550 -0.362153354 0.09382225
## Richland-Huntley Dryland           -0.163572385 -0.391560189 0.06441542
## Sidney Dryland-Huntley Dryland     -0.205829421 -0.433817225 0.02215838
## Sidney Irrigated-Huntley Dryland   -0.094793032 -0.322780836 0.13319477
## Kalispell-Huntley Irrigated        -0.183601489 -0.411589293 0.04438632
## Moccasin-Huntley Irrigated         -0.101084849 -0.329072654 0.12690296
## Richland-Huntley Irrigated         -0.130491684 -0.358479489 0.09749612
## Sidney Dryland-Huntley Irrigated   -0.172748721 -0.400736525 0.05523908
## Sidney Irrigated-Huntley Irrigated -0.061712332 -0.289700136 0.16627547
## Moccasin-Kalispell                  0.082516640 -0.145471164 0.31050444
## Richland-Kalispell                  0.053109805 -0.174878000 0.28109761
## Sidney Dryland-Kalispell            0.010852768 -0.217135036 0.23884057
## Sidney Irrigated-Kalispell          0.121889157 -0.106098647 0.34987696
## Richland-Moccasin                  -0.029406835 -0.257394639 0.19858097
## Sidney Dryland-Moccasin            -0.071663871 -0.299651676 0.15632393
## Sidney Irrigated-Moccasin           0.039372517 -0.188615287 0.26736032
## Sidney Dryland-Richland            -0.042257036 -0.270244841 0.18573077
## Sidney Irrigated-Richland           0.068779353 -0.159208452 0.29676716
## Sidney Irrigated-Sidney Dryland     0.111036389 -0.116951415 0.33902419
##                                        p adj
## Corvallis-Conrad                   0.3508722
## Huntley Dryland -Conrad            0.0707667
## Huntley Irrigated-Conrad           0.1984083
## Kalispell-Conrad                   1.0000000
## Moccasin-Conrad                    0.9527776
## Richland-Conrad                    0.9969598
## Sidney Dryland-Conrad              1.0000000
## Sidney Irrigated-Conrad            0.7065213
## Huntley Dryland -Corvallis         0.9965542
## Huntley Irrigated-Corvallis        0.9999961
## Kalispell-Corvallis                0.3617848
## Moccasin-Corvallis                 0.9672588
## Richland-Corvallis                 0.8282749
## Sidney Dryland-Corvallis           0.4553083
## Sidney Irrigated-Corvallis         0.9997068
## Huntley Irrigated-Huntley Dryland  0.9999200
## Kalispell-Huntley Dryland          0.0740866
## Moccasin-Huntley Dryland           0.6062392
## Richland-Huntley Dryland           0.3429114
## Sidney Dryland-Huntley Dryland     0.1059547
## Sidney Irrigated-Huntley Dryland   0.9086105
## Kalispell-Huntley Irrigated        0.2059932
## Moccasin-Huntley Irrigated         0.8742599
## Richland-Huntley Irrigated         0.6405710
## Sidney Dryland-Huntley Irrigated   0.2746068
## Sidney Irrigated-Huntley Irrigated 0.9929035
## Moccasin-Kalispell                 0.9569325
## Richland-Kalispell                 0.9974455
## Sidney Dryland-Kalispell           1.0000000
## Sidney Irrigated-Kalispell         0.7183816
```

```
## Richland-Moccasin                  0.9999674
## Sidney Dryland-Moccasin            0.9814416
## Sidney Irrigated-Moccasin          0.9997048
## Sidney Dryland-Richland            0.9995028
## Sidney Irrigated-Richland          0.9856585
## Sidney Irrigated-Sidney Dryland    0.8069758
```

The results from the beta dispersion show that we have a significant difference in the heterogeneity of our sites due to each of the farm management factors. We see that there are sites that do not have significant differences and others that do. Beta dispersion cannot except models so we cannot detect if the differences are due to nestedness (prev_crop/Site).

Due to the design of the experiment it will be hard to determine if the farm management practices are responsible for the variation in bacterial population.

PERMANOVA (adoins)

We will test the significance of the farm management using the permutated-mulitvariate-ANOVA function in vegan called adonis. Adonis can test models and nestedness though the above beta dispersion test showed that most of the significance is due to the dispersion we cannot say much. But we can show we have the location effect.

```
adonis(distance(physeq_nifH_ord, method = "bray")
       ~Plot*prev_crop*Tillage*Pea_variety, data = meta2, permutations = 1000)
```

```
##
## Call:
## adonis(formula = distance(physeq_nifH_ord, method = "bray") ~      Plot * prev_crop * Tillage * Pea_
##
## Permutation: free
## Number of permutations: 1000
##
## Terms added sequentially (first to last)
##
##                          Df SumsOfSqs MeanSqs F.Model      R2   Pr(>F)
## Plot                      1    1.4093 1.40932  3.9327 0.07299 0.000999 ***
## prev_crop                 2    2.4983 1.24917  3.4858 0.12938 0.000999 ***
## Tillage                   2    3.1325 1.56624  4.3706 0.16222 0.000999 ***
## Pea_variety               6    1.3105 0.21841  0.6095 0.06787 1.000000
## Plot:Tillage              1    1.5306 1.53056  4.2711 0.07926 0.000999 ***
## Plot:Pea_variety          6    0.8231 0.13718  0.3828 0.04263 1.000000
## prev_crop:Pea_variety     9    1.6820 0.18689  0.5215 0.08711 1.000000
## Tillage:Pea_variety      10    1.9518 0.19518  0.5446 0.10108 1.000000
## Plot:Tillage:Pea_variety  4    0.6713 0.16782  0.4683 0.03476 1.000000
## Residuals                12    4.3003 0.35836         0.22270
## Total                    53   19.3096                 1.00000
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```r
adonis(distance(physeq_nifH_ord, method = "bray")
       ~(Plot*prev_crop*Tillage*Pea_variety)/Site, data = meta2, permutations = 1000)
```

```
##
## Call:
## adonis(formula = distance(physeq_nifH_ord, method = "bray") ~      (Plot * prev_crop * Tillage * Pea
##
## Permutation: free
## Number of permutations: 1000
##
## Terms added sequentially (first to last)
##
##                                          Df SumsOfSqs MeanSqs F.Model
## Plot                                      1    1.4093       1       0
## prev_crop                                 2    2.4983       1       0
## Tillage                                   2    3.1325       2       0
## Pea_variety                               6    1.3105       0       0
## Plot:Tillage                              1    1.5306       2       0
## Plot:Pea_variety                          6    0.8231       0       0
## prev_crop:Pea_variety                     9    1.6820       0       0
## Tillage:Pea_variety                      10    1.9518       0       0
## Plot:Tillage:Pea_variety                  4    0.6713       0       0
## Plot:prev_crop:Tillage:Pea_variety:Site  12    4.3003       0       0
## Residuals                                 0    0.0000    -Inf
## Total                                    53   19.3096
##                                              R2 Pr(>F)
## Plot                                    0.07299      1
## prev_crop                               0.12938      1
## Tillage                                 0.16222      1
## Pea_variety                             0.06787      1
## Plot:Tillage                            0.07926      1
## Plot:Pea_variety                        0.04263      1
## prev_crop:Pea_variety                   0.08711      1
## Tillage:Pea_variety                     0.10108      1
## Plot:Tillage:Pea_variety                0.03476      1
## Plot:prev_crop:Tillage:Pea_variety:Site 0.22270      1
## Residuals                               0.00000
## Total                                   1.00000
```

Data is nested within Site (Location effect) so the significance in the bray-curtis dissimilarity with respect to plot is not significant with the data nested due to the lack of reproduction of conditions at each plot.

Since there is issues with doing permutated anova over multivariate data lets try to fit the chemical and farm management data the NMDS orientation space using the envfit function in vegan

---

Model Selection

ENVFIT

Envfit does not like single variable values so we remove them

```
meta3<-meta2[,-c(3,4,10,11,27,29,35,38,42,46)]
```

Will remove Site categories like elevation, lat, long etc that do not differentiate between site we can call these all geographical factors as they do not change between sites.

```
meta3<-meta3[,-c(2,11:13)]
```

Model fitting will be biased by chemical outliers that are in some plots the best way to avoid this is to determine the outliers (See chemical_analysis.Rmd) and remove the whole variable since functions ENVFIT and BIOENV will remove it if there are any n/a values.

Removing Sulfate_Sulfur, Boron, Molybdenum, Potassium, Vanadium, Chromium and Sodium (From chemical_analysis.rmd)

```
meta3<-meta3[,-c(16,18,21,29,31)]
```

```
envfitnifH <- envfit(phynifH_ord_NMDS , meta3, na.rm = TRUE, permu= 10000)
envfitnifH
```

```
##
## ***VECTORS
##
##                     NMDS1    NMDS2     r2    Pr(>r)
## season_precip     0.43714 -0.89939 0.0400  0.354465
## irrgation         0.30011  0.95391 0.4424 9.999e-05 ***
## total_precip_irr  0.65883  0.75229 0.1486  0.016098 *
## grain_yield      -0.39481  0.91876 0.0227  0.562644
## Organic_Matter   -0.70859 -0.70562 0.4791 9.999e-05 ***
## Moisture_Content  0.58097 -0.81393 0.0484  0.287471
## Nitrate_Nitrite  -0.02415  0.99971 0.2201  0.001800 **
## Ammonia          -0.04719 -0.99889 0.0214  0.580542
## Av_Phosphorus     0.43085  0.90242 0.4094 9.999e-05 ***
## Av_Potassium      0.76331  0.64603 0.4202 9.999e-05 ***
## pH                0.02901 -0.99958 0.0218  0.582442
## Barium           -0.08073 -0.99674 0.4684 9.999e-05 ***
## Calcium           0.38027 -0.92488 0.0923  0.084192 .
## Cobalt            0.16341 -0.98656 0.0247  0.523448
## Copper            0.54680 -0.83726 0.0748  0.137786
## Iron              0.52535 -0.85088 0.0625  0.185881
## Magnesium         0.21991  0.97552 0.0438  0.321168
## Manganese         0.95991  0.28030 0.0048  0.886211
```

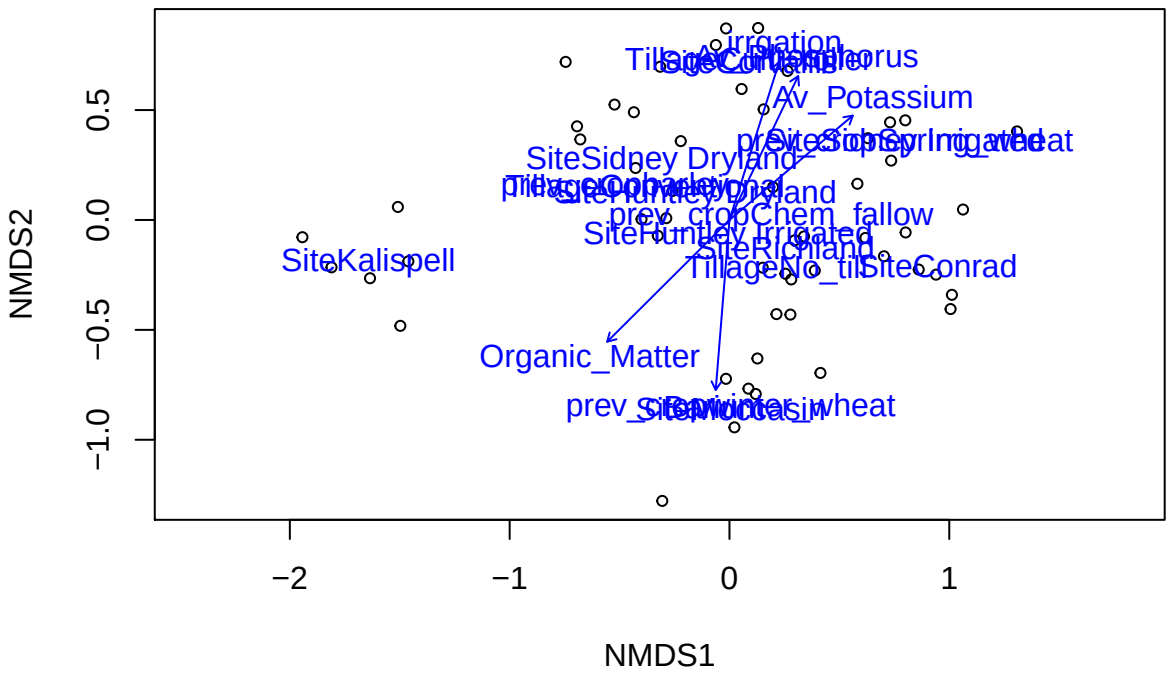
```
## Nickel            0.62941 -0.77707 0.1258  0.036296 *
## Phosphorus        0.80483  0.59350 0.0058  0.865413
## Sulfur            0.09856 -0.99513 0.1700  0.009399 **
## Zinc              0.56036 -0.82825 0.2387  0.001200 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## Permutation: free
## Number of permutations: 10000
##
## ***FACTORS:
##
## Centroids:
##                            NMDS1   NMDS2
## SiteConrad                0.9471 -0.2049
## SiteCorvallis             0.0880  0.7196
## SiteHuntley Dryland      -0.1353  0.1147
## SiteHuntley Irrigated    -0.0068 -0.0739
## SiteKalispell            -1.6425 -0.1949
## SiteMoccasin              0.0057 -0.8556
## SiteRichland              0.2534 -0.1251
## SiteSidney Dryland       -0.3073  0.2691
## SiteSidney Irrigated      0.7978  0.3512
## Pea_varietyAC Earlystar -0.1756  0.1583
## Pea_varietyCDC Saffron   0.0279 -0.1372
## Pea_varietyCDC Saffron   0.2077  0.5706
## Pea_varietyDelta          0.1676 -0.0482
## Pea_varietyDS Admiral     0.0456 -0.1395
## Pea_varietyMajoret       -0.1055  0.0036
## Pea_varietyNavarro        0.0000  0.0056
## PlotDryland               0.1527 -0.1604
## PlotIrrigated            -0.1909  0.2005
## TillageConventional      -0.3840  0.1418
## TillageCulti-roller       0.0880  0.7196
## TillageNo_till            0.2128 -0.2290
## prev_cropbarley          -0.5204  0.1502
## prev_cropChem_fallow      0.1895  0.0134
## prev_cropSpring_wheat     0.7978  0.3512
## prev_cropwinter_wheat     0.0057 -0.8556
##
## Goodness of fit:
##                  r2    Pr(>r)
## Site         0.8274 9.999e-05 ***
## Pea_variety  0.0449    0.9671
## Plot         0.0764    0.0223 *
## Tillage      0.2100    0.0002 ***
## prev_crop    0.3484 9.999e-05 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## Permutation: free
## Number of permutations: 10000
```

write to table

The envfit function allows us to see the correlation of our environmental vectors to the bray-curtis species dissimilarity matrix in NMDS space. This is a loose correlation to real linear correlation but it can tell us how the NMDS orientation is being driven.

Try a quick plot with base r and vegan for the vectors

```
plot(phynifH_ord_NMDS, display = "sites")
plot(envfitnifH, p.max = 0.001 )
```



Organic matter is resposible for 54% of the variance

Model Selection

Bioenv

Bioenv is an iterative procedure that links enviromental variables to community strucutre by seeking the best subset of enviromental variables that explains community strucutre

---

Info:

BIOENV

"The function calculates a community dissimilarity matrix using vegdist. Then it selects all possible subsets of environmental variables, scales the variables, and calculates Euclidean distances for this subset using dist.

The function finds the correlation between community dissimilarities and environmental distances, and for each size of subsets, saves the best result. There are 2^p-1 subsets of p variables, and an exhaustive search may take a very, very, very long time (parameter upto offers a partial relief)."

Mantel test

"Mantel statistic is simply a correlation between entries of two dissimilarity matrices (some use cross products, but these are linearly related). However, the significance cannot be directly assessed, because there are N(N-1)/2 entries for just N observations. Mantel developed asymptotic test, but here we use permutations of N rows and columns of dissimilarity matrix."

```
OTU_nifH_trim<- as(otu_table(physeq_nifH_ord ), "matrix")
```

```
#Transpose the data to have sample names on rows
abund_tablenifH<-t(OTU_nifH_trim)

nrow(abund_tablenifH)
```

```
## [1] 54
```

```
setdiff(rownames(meta3), rownames(abund_tablenifH))
```

```
## character(0)
```

Our meta data and sample data match with 0 diffrence in rownames

Will use parallel processing to speed up calculations

```
#First detect amount of cores avalible
detectCores()
```

```
## [1] 12
```

```
#get bray-curtis distance
abund_distnifH<-vegdist(abund_tablenifH, method = "bray")
#make bioenv model against whole meta table with ".", and use gower metric to measure distance in order
#nifH.bioenv <- bioenv(abund_distnifH ~ ., meta3, index = "bray", method = "pearson",
 #                  metric = "gower", upto = 7, parallel = 10)

#summary(nifH.bioenv)
```

```
#nifH.bioenvdist<-bioenvdist(nifH.bioenv, which = "best")
#mantel(nifH.bioenvdist, abund_distnifH)
```

```
#nifH.bioenv$whichbest
```

The best model from the inital BioEnv model selection shows that the Site explains 59% of the variation in the bray-curitis distance. But the best model explains 63% of the variation when Organic Matter and nitritie and nitrate are added in.

Try BioENV without site, this will favor farm managment variables since they are nested within.

Remove site from meta table

```
meta4<-meta3[,-c(1)]
```

Rerun BioEnv

```
#nifH.bioenv.site.na <- bioenv(abund_distnifH ~ ., meta4, index = "bray",
 #                             method = "pearson", metric = "gower", upto = 7, parallel = 10)

#summary(nifH.bioenv.site.na)
```

```
#nifH.bioenvdist.site.na<-bioenvdist(nifH.bioenv.site.na, which = "best")
#mantel(nifH.bioenvdist.site.na, abund_distnifH)
```

```
#nifH.bioenv.site.na$whichbest
```

When Site is removed from the model selection we can see that there is still a bias for the Farm managment variables because they are nested with the site factor. But we do see more come out to build a bigger model. With Tillage + prev_crop + Organic_Matter + Nitrate_Nitrite + Ammonia + Av_Potassium + Manganese + Zinc.

Bioenv with chem data only

```
#nifH.bioenv.chem <- bioenv(abund_tablenifH ~ Organic_Matter + Moisture_Content +
 #                            Nitrate_Nitrite + Ammonia + Av_Phosphorus + Av_Potassium +
  #                            pH + Barium + Calcium + Cobalt + Copper + Iron + Magnesium +
   #                            Manganese + Nickel + Phosphorus + Sulfur + Zinc, meta3,
    #                          index = "bray", method = "pearson", metric = "gower",
     #                         upto = 7, parallel = 10)

#summary(nifH.bioenv.chem)
```

```
#nifH.bioenvdist.chem<-bioenvdist(nifH.bioenv.chem, which = "best")
#mantel(nifH.bioenvdist.chem, abund_distnifH)
```

```
#nifH.bioenv.chem$whichbest
```

The fourth model Organic Matter, Nitrate, Phosphorus and Zinc is the best explaing 56% of the variance. Which is the same as before without the location variables.

CCA/ordistep model selection

CCA model selection uses a procedure to take the a constrained distance ordination with the complete model and compare it with a unconstrainted model. The model starts with no variables then adds variables that make the best model. These models can then be plotted against the same ordination space as vectors.

Ordistep use Akaike information criterion (AIC) which is a estimator of relative quality of the models. AIC is relative to models you are comparing when you compare two models the one with the lower AIC is favored.

```
m1_nifH <- cca(abund_tablenifH ~ ., meta3)
m0_nifH <- cca(abund_tablenifH ~ 1, meta3)
m1_nifH
```

```
## Call: cca(formula = abund_tablenifH ~ Site + Pea_variety + Plot +
## season_precip + irrgation + total_precip_irr + Tillage + prev_crop
## + grain_yield + Organic_Matter + Moisture_Content +
## Nitrate_Nitrite + Ammonia + Av_Phosphorus + Av_Potassium + pH +
## Barium + Calcium + Cobalt + Copper + Iron + Magnesium + Manganese
## + Nickel + Phosphorus + Sulfur + Zinc, data = meta3)
##
##               Inertia Proportion Rank
## Total           9.074      1.000
## Constrained     6.796      0.749   33
## Unconstrained   2.277      0.251   20
## Inertia is scaled Chi-square
## Some constraints were aliased because they were collinear (redundant)
##
## Eigenvalues for constrained axes:
##   CCA1   CCA2   CCA3   CCA4   CCA5   CCA6   CCA7   CCA8   CCA9  CCA10
## 0.8027 0.6026 0.5827 0.5207 0.4910 0.4632 0.3482 0.3227 0.2719 0.2619
##  CCA11  CCA12  CCA13  CCA14  CCA15  CCA16  CCA17  CCA18  CCA19  CCA20
## 0.2525 0.1977 0.1824 0.1742 0.1564 0.1370 0.1242 0.1143 0.1030 0.0979
##  CCA21  CCA22  CCA23  CCA24  CCA25  CCA26  CCA27  CCA28  CCA29  CCA30
## 0.0751 0.0699 0.0663 0.0567 0.0515 0.0488 0.0444 0.0370 0.0353 0.0326
##  CCA31  CCA32  CCA33
## 0.0283 0.0241 0.0193
##
## Eigenvalues for unconstrained axes:
##     CA1     CA2     CA3     CA4     CA5     CA6     CA7     CA8
## 0.27474 0.23444 0.19971 0.16799 0.14878 0.14661 0.14098 0.12503
## (Showing 8 of 20 unconstrained eigenvalues)
```

```
m0_nifH
```

```
## Call: cca(formula = abund_tablenifH ~ 1, data = meta3)
##
##               Inertia Rank
## Total           9.074
## Unconstrained   9.074   53
## Inertia is scaled Chi-square
```

```
##
## Eigenvalues for unconstrained axes:
##    CA1    CA2    CA3    CA4    CA5    CA6    CA7    CA8
## 0.8150 0.6423 0.6049 0.5542 0.5252 0.4870 0.4435 0.3923
## (Showing 8 of 53 unconstrained eigenvalues)
```

Ordistep

```
model_nifH <-ordistep(m0_nifH, scope=formula(m1_nifH))
```

```
model_nifH$anova
```

	Df	AIC	F	Pr(>F)
+ Site	8	856.3138	3.617355	0.005
+ Nitrate_Nitrite	1	855.3388	2.492073	0.005

Everytime a varaible is added the AIC is lowered favoring the new model. This goes till it stops.

cca without site

```
m1_nifH_site_na <- cca(abund_tablenifH ~ ., meta4)
m0_nifH_site_na <- cca(abund_tablenifH ~ 1, meta4)
m1_nifH_site_na
```

```
## Call: cca(formula = abund_tablenifH ~ Pea_variety + Plot +
## season_precip + irrgation + total_precip_irr + Tillage + prev_crop
## + grain_yield + Organic_Matter + Moisture_Content +
## Nitrate_Nitrite + Ammonia + Av_Phosphorus + Av_Potassium + pH +
## Barium + Calcium + Cobalt + Copper + Iron + Magnesium + Manganese
## + Nickel + Phosphorus + Sulfur + Zinc, data = meta4)
##
##               Inertia Proportion Rank
## Total          9.0738     1.0000
## Constrained    6.6182     0.7294   32
## Unconstrained  2.4557     0.2706   21
## Inertia is scaled Chi-square
## Some constraints were aliased because they were collinear (redundant)
##
## Eigenvalues for constrained axes:
##   CCA1   CCA2   CCA3   CCA4   CCA5   CCA6   CCA7   CCA8   CCA9  CCA10
## 0.8024 0.6003 0.5814 0.5066 0.4808 0.4506 0.3469 0.3226 0.2675 0.2609
##  CCA11  CCA12  CCA13  CCA14  CCA15  CCA16  CCA17  CCA18  CCA19  CCA20
## 0.2103 0.1972 0.1822 0.1609 0.1540 0.1356 0.1220 0.1142 0.1019 0.0810
##  CCA21  CCA22  CCA23  CCA24  CCA25  CCA26  CCA27  CCA28  CCA29  CCA30
## 0.0744 0.0663 0.0633 0.0522 0.0512 0.0468 0.0420 0.0355 0.0333 0.0283
##  CCA31  CCA32
## 0.0263 0.0193
##
```

```
## Eigenvalues for unconstrained axes:
##     CA1     CA2     CA3     CA4     CA5     CA6     CA7     CA8
## 0.28403 0.24996 0.21164 0.18851 0.16085 0.14877 0.14524 0.12522
## (Showing 8 of 21 unconstrained eigenvalues)
```

```
m0_nifH_site_na
```

```
## Call: cca(formula = abund_tablenifH ~ 1, data = meta4)
##
##               Inertia Rank
## Total           9.074
## Unconstrained   9.074   53
## Inertia is scaled Chi-square
##
## Eigenvalues for unconstrained axes:
##    CA1    CA2    CA3    CA4    CA5    CA6    CA7    CA8
## 0.8150 0.6423 0.6049 0.5542 0.5252 0.4870 0.4435 0.3923
## (Showing 8 of 53 unconstrained eigenvalues)
```

Ordistep

```
model_nifH_site_na <-ordistep(m0_nifH_site_na, scope=formula(m1_nifH_site_na))
```

```
model_nifH_site_na$anova
```

	Df	AIC	F	Pr(>F)
+ Tillage	2	864.3021	3.436408	0.005
+ prev_crop	3	860.8917	3.045976	0.005
+ irrgation	1	858.7091	3.785031	0.005
+ Ammonia	1	858.2541	2.139600	0.005
+ season_precip	1	857.5351	2.323828	0.005
+ Nitrate_Nitrite	1	856.6017	2.456277	0.005
+ grain_yield	1	856.1815	1.971028	0.005

```
m1_nifH_cca_chem <- cca(abund_tablenifH ~ Organic_Matter + Moisture_Content + Nitrate_Nitrite + Ammonia
m0_nifH_cca_chem <- cca(abund_tablenifH ~ 1, meta3)
m1_nifH_cca_chem
```

```
## Call: cca(formula = abund_tablenifH ~ Organic_Matter +
## Moisture_Content + Nitrate_Nitrite + Ammonia + Av_Phosphorus +
## Av_Potassium + pH + Barium + Calcium + Cobalt + Copper + Iron +
## Magnesium + Manganese + Nickel + Phosphorus + Sulfur + Zinc, data
## = meta3)
##
##               Inertia Proportion Rank
## Total          9.0738     1.0000
## Constrained    4.6226     0.5094   18
## Unconstrained  4.4513     0.4906   35
## Inertia is scaled Chi-square
```

```
##
## Eigenvalues for constrained axes:
##   CCA1   CCA2   CCA3   CCA4   CCA5   CCA6   CCA7   CCA8   CCA9  CCA10
## 0.7877 0.5566 0.5201 0.4856 0.4519 0.3928 0.3003 0.1899 0.1705 0.1467
##  CCA11  CCA12  CCA13  CCA14  CCA15  CCA16  CCA17  CCA18
## 0.1313 0.1064 0.0846 0.0741 0.0684 0.0621 0.0500 0.0436
##
## Eigenvalues for unconstrained axes:
##    CA1    CA2    CA3    CA4    CA5    CA6    CA7    CA8
## 0.4395 0.3474 0.3067 0.2537 0.2382 0.2336 0.2226 0.2018
## (Showing 8 of 35 unconstrained eigenvalues)
```

```
m0_nifH_cca_chem
```

```
## Call: cca(formula = abund_tablenifH ~ 1, data = meta3)
##
##               Inertia Rank
## Total           9.074
## Unconstrained   9.074   53
## Inertia is scaled Chi-square
##
## Eigenvalues for unconstrained axes:
##    CA1    CA2    CA3    CA4    CA5    CA6    CA7    CA8
## 0.8150 0.6423 0.6049 0.5542 0.5252 0.4870 0.4435 0.3923
## (Showing 8 of 53 unconstrained eigenvalues)
```

```
model_nifH_cca_chem <-ordistep(m0_nifH_cca_chem, scope=formula(m1_nifH_cca_chem))
```

```
model_nifH_cca_chem$anova
```

	Df	AIC	F	Pr(>F)
+ Organic_Matter	1	864.9180	4.217224	0.005
+ Nitrate_Nitrite	1	863.6253	3.206498	0.005
+ Av_Potassium	1	862.6412	2.840851	0.005
+ Av_Phosphorus	1	861.7903	2.656463	0.005
+ Manganese	1	861.1619	2.394125	0.005
+ Barium	1	860.3621	2.501186	0.005
+ Sulfur	1	859.9429	2.107586	0.005
+ Magnesium	1	858.8295	2.670746	0.005
+ Phosphorus	1	858.4552	1.977791	0.005

RDA is a linear cca

```
m1_nifH_rda <- rda(abund_tablenifH ~ ., meta3)
m0_nifH_rda <- rda(abund_tablenifH ~ 1, meta3)
m1_nifH_rda
```

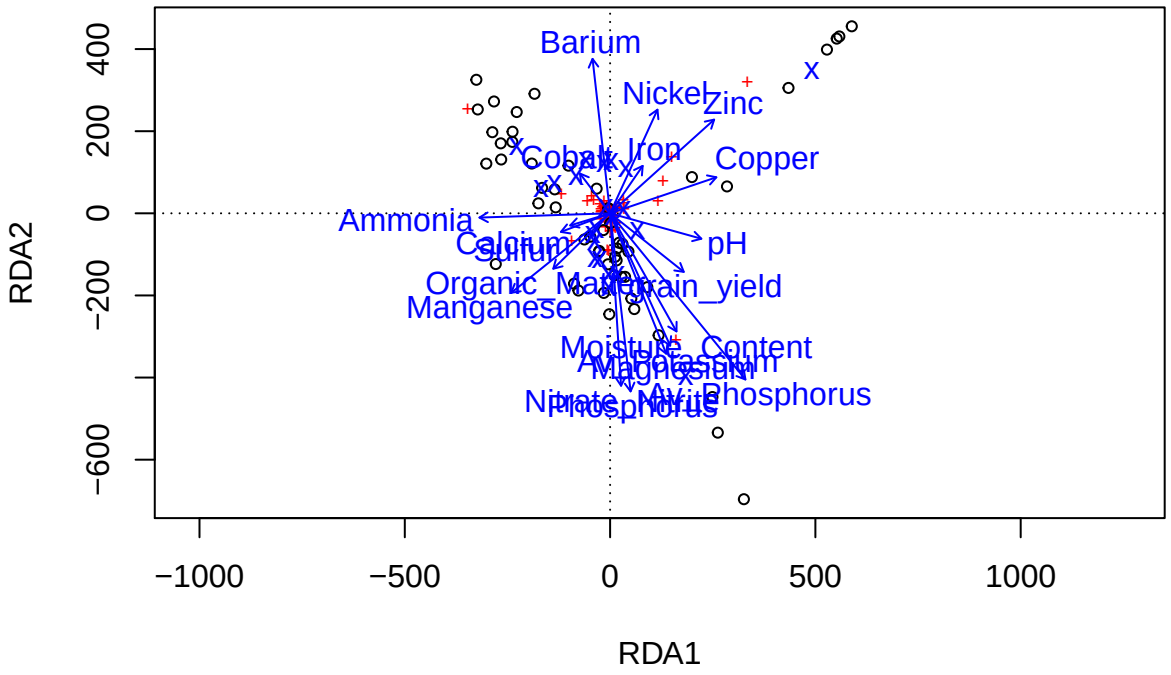
```
## Call: rda(formula = abund_tablenifH ~ Site + Pea_variety + Plot +
## season_precip + irrgation + total_precip_irr + Tillage + prev_crop
## + grain_yield + Organic_Matter + Moisture_Content +
```

```
## Nitrate_Nitrite + Ammonia + Av_Phosphorus + Av_Potassium + pH +
## Barium + Calcium + Cobalt + Copper + Iron + Magnesium + Manganese
## + Nickel + Phosphorus + Sulfur + Zinc, data = meta3)
##
##                 Inertia Proportion Rank
## Total         1.331e+11  1.000e+00
## Constrained   1.046e+11  7.862e-01   33
## Unconstrained 2.846e+10  2.138e-01   20
## Inertia is variance
## Some constraints were aliased because they were collinear (redundant)
##
## Eigenvalues for constrained axes:
##        RDA1        RDA2        RDA3        RDA4        RDA5        RDA6
## 17548667644 16499650004 12977757804 11589506943  7631119218  6761513528
##        RDA7        RDA8        RDA9       RDA10       RDA11       RDA12
##  5550551834  5004470215  4122249247  3318880699  2680128188  1968062864
##       RDA13       RDA14       RDA15       RDA16       RDA17       RDA18
##  1497032684  1192899104  1042107947   861647889   698124705   588564180
##       RDA19       RDA20       RDA21       RDA22       RDA23       RDA24
##   508094041   418009285   365033164   324702400   252472753   207429340
##       RDA25       RDA26       RDA27       RDA28       RDA29       RDA30
##   184825179   169758166   150199224   115815986   107173357    96024376
##       RDA31       RDA32       RDA33
##    84736502    60931011    58747462
##
## Eigenvalues for unconstrained axes:
##        PC1        PC2        PC3        PC4        PC5        PC6
## 5871852873 4402692918 4002911334 3724051954 2047287739 1519664997
##        PC7        PC8
## 1228123308  998854527
## (Showing 8 of 20 unconstrained eigenvalues)
```

```
m0_nifH_rda
```

```
## Call: rda(formula = abund_tablenifH ~ 1, data = meta3)
##
##                 Inertia Rank
## Total         1.331e+11
## Unconstrained 1.331e+11   53
## Inertia is variance
##
## Eigenvalues for unconstrained axes:
##         PC1         PC2         PC3         PC4         PC5         PC6
## 19425920664 18485720261 15308306751 13963841933 10802018954  8105770060
##         PC7         PC8
##  7008929496  6473486029
## (Showing 8 of 53 unconstrained eigenvalues)
```

```
plot(m1_nifH_rda)
```




```
model_rda_nifH <-ordiR2step(m0_nifH_rda, scope=formula(m1_nifH_rda))
```

```
model_rda_nifH$anova
```

	R2.adj	Df	AIC	F	Pr(>F)
+ Site	0.4096331	8	1362.869	5.596835	0.002
	0.4334037	NA	NA	NA	NA

Only site appears in the complete RDA model. This is a liner based method and might not work well with our data.

Chemistry only RDA model

```
m1_nifH_rda_chem <- rda(abund_tablenifH ~ Organic_Matter + Moisture_Content + Nitrate_Nitrite + Ammonia
m0_nifH_rda_chem <- rda(abund_tablenifH ~ 1, meta3)
m1_nifH_rda_chem
```

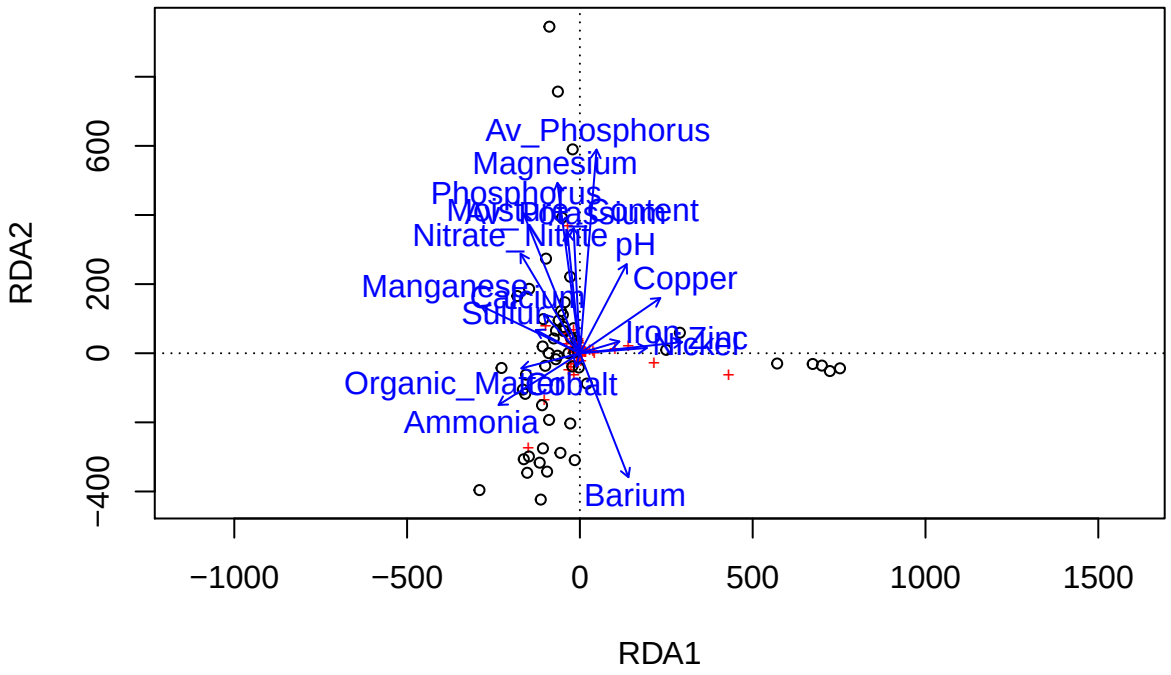
```
## Call: rda(formula = abund_tablenifH ~ Organic_Matter +
## Moisture_Content + Nitrate_Nitrite + Ammonia + Av_Phosphorus +
## Av_Potassium + pH + Barium + Calcium + Cobalt + Copper + Iron +
## Magnesium + Manganese + Nickel + Phosphorus + Sulfur + Zinc, data
## = meta3)
##
##                 Inertia Proportion Rank
## Total         1.331e+11  1.000e+00
```

```
## Constrained   7.463e+10  5.607e-01   18
## Unconstrained 5.846e+10  4.393e-01   35
## Inertia is variance
##
## Eigenvalues for constrained axes:
##        RDA1        RDA2        RDA3        RDA4        RDA5        RDA6
## 16122227703 13523241448 11094526423  9005789057  5921938961  5359338168
##        RDA7        RDA8        RDA9       RDA10       RDA11       RDA12
##  3780506525  3080678651  1894819093  1508918418   940183925   624720502
##       RDA13       RDA14       RDA15       RDA16       RDA17       RDA18
##   471289405   382996377   289104096   239329825   227754724   162233619
##
## Eigenvalues for unconstrained axes:
##         PC1         PC2         PC3         PC4         PC5         PC6
## 11949328777  7624704552  6257652081  5543387518  4129403632  3255191916
##         PC7         PC8
##  3002483572  2695244220
## (Showing 8 of 35 unconstrained eigenvalues)
```

```
m0_nifH_rda_chem
```

```
## Call: rda(formula = abund_tablenifH ~ 1, data = meta3)
##
##                 Inertia Rank
## Total         1.331e+11
## Unconstrained 1.331e+11   53
## Inertia is variance
##
## Eigenvalues for unconstrained axes:
##         PC1         PC2         PC3         PC4         PC5         PC6
## 19425920664 18485720261 15308306751 13963841933 10802018954  8105770060
##         PC7         PC8
##  7008929496  6473486029
## (Showing 8 of 53 unconstrained eigenvalues)
```

```
plot(m1_nifH_rda_chem)
```




```
model_rda_chem_nifH <-ordiR2step(m0_nifH_rda_chem, scope=formula(m1_nifH_rda_chem))
```

```
model_rda_chem_nifH$anova
```

	R2.adj	Df	AIC	F	Pr(>F)
+ Av_Phosphorus	0.0580227	1	1381.907	4.264624	0.002
+ Av_Potassium	0.1012829	1	1380.320	3.503049	0.002
+ Organic_Matter	0.1424550	1	1378.719	3.448590	0.002
+ Barium	0.1852360	1	1376.864	3.625360	0.002
+ Manganese	0.2506562	1	1373.231	5.277867	0.002
+ Iron	0.2865280	1	1371.445	3.413331	0.002
+ Sulfur	0.3191470	1	1369.757	3.251722	0.002
+ Magnesium	0.3325579	1	1369.496	1.924280	0.028
	0.3348198	NA	NA	NA	NA

Our RDA chem model can explain 30% of the variance in the bacterial community.

CAP Ordination model building

canonical analysis of principal coordinates (CAP) is similar to RDA but allows for non-euclidian dissimilarity like Bray-Curtis which we have been using.

https://esajournals.onlinelibrary.wiley.com/doi/epdf/10.1890/0012-9658%282003%29084%5B0511%3ACAOPCA%5D2.0.CO%3B2

Guide for the CAP Ordination

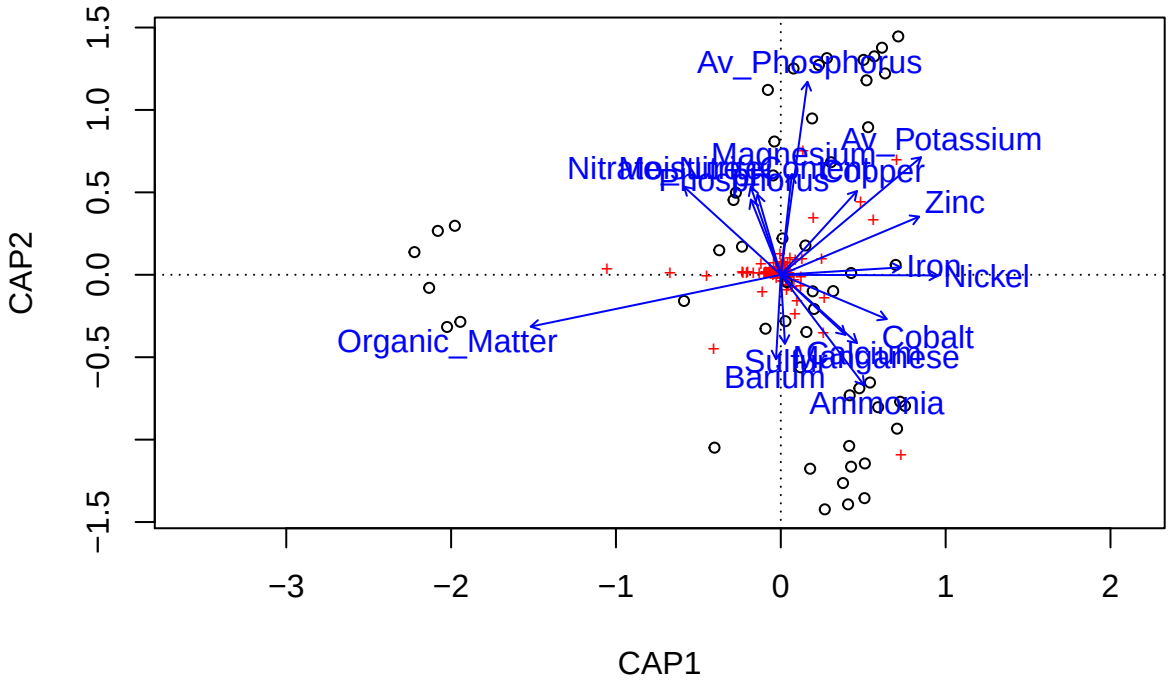
https://quantpalaeo.wordpress.com/2014/04/14/variance-inflation-factors-and-ordination-model-selection/

If the VIF of a predictor is high, it indicates that that predictor is highly correlated with other predictors, it contains little or no unique information, and there is redundancy in the set of predictors.

STEPS

```
1)Generate a constrained ordination with all available predictors.
2)Calculate the VIF of each variable.
3)If any variable has a VIF over a threshold (typically 10), drop the variable with the highest VIF
4)Repeat until all remaining variables have a VIF below the threshold.
```

```
m1_nifH_cap_chem<- capscale(abund_tablenifH ~ Organic_Matter + Moisture_Content + Nitrate_Nitrite + Amm
m0_nifH_cap_chem<- capscale(abund_distnifH ~ 1, meta3)
plot(m1_nifH_cap_chem)
```



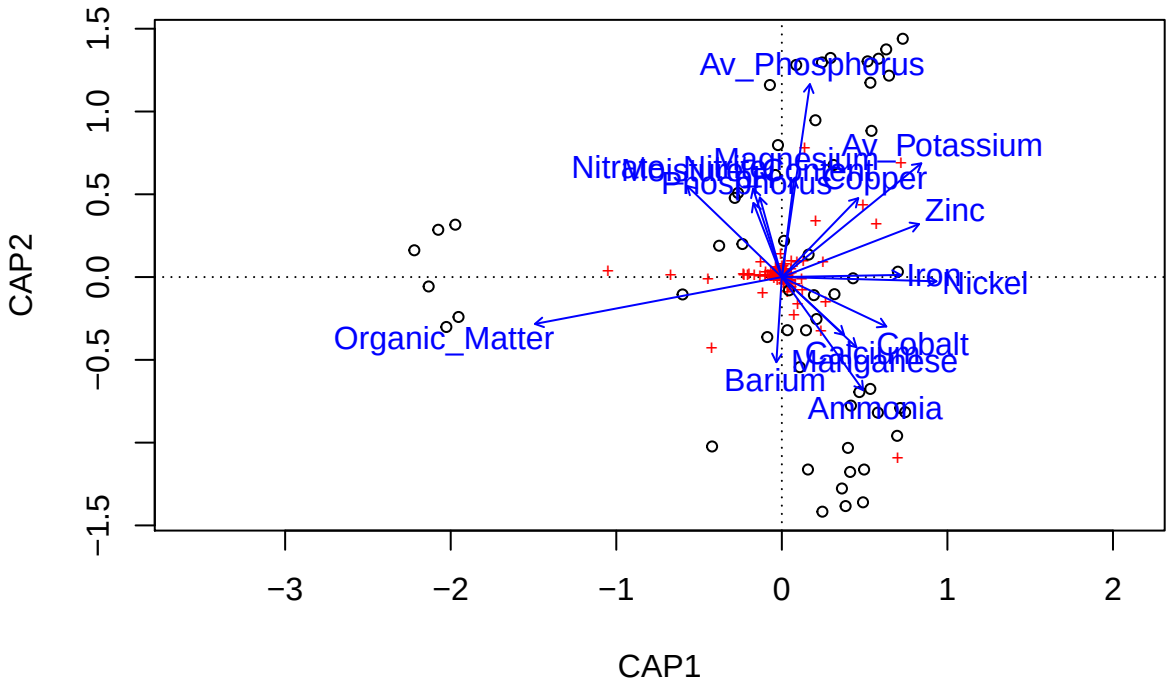

```
vif.cca(m1_nifH_cap_chem)
```

```
##   Organic_Matter Moisture_Content  Nitrate_Nitrite          Ammonia
##        15.457094         8.111272         4.360780         4.369015
##    Av_Phosphorus     Av_Potassium               pH          Barium
##        11.821766        13.816708        19.007192        34.110255
##          Calcium           Cobalt           Copper             Iron
##        69.959837        20.726173        37.675483        55.160314
##        Magnesium        Manganese           Nickel       Phosphorus
```

```
##        38.155344        25.416138        39.209635        37.072036
##           Sulfur             Zinc
##       126.793579        83.408013
```

Removing Sulfur

```
m1_nifH_cap_chem_1<- capscale(abund_tablenifH ~ Organic_Matter + Moisture_Content + Nitrate_Nitrite + Am
m0_nifH_cap_chem<- capscale(abund_distnifH ~ 1, meta3)
plot(m1_nifH_cap_chem_1)
```

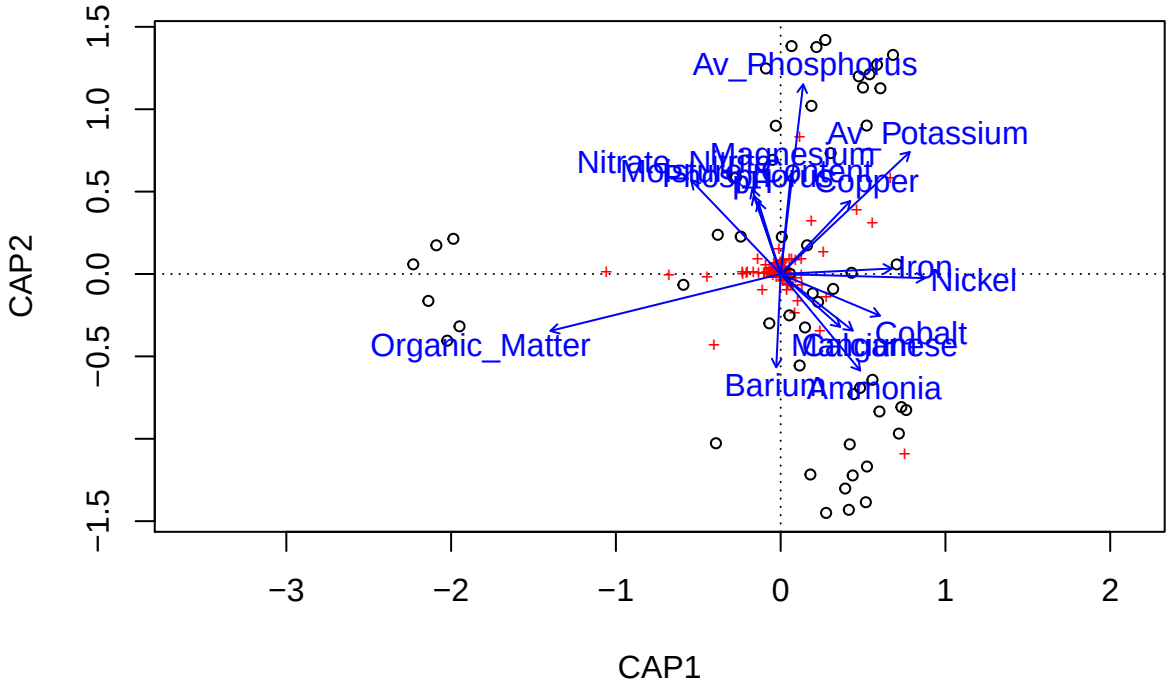



```
vif.cca(m1_nifH_cap_chem_1)
```

```
##   Organic_Matter Moisture_Content  Nitrate_Nitrite          Ammonia
##        14.498699         6.988162         3.713414         4.309993
##    Av_Phosphorus     Av_Potassium               pH          Barium
##        11.350534        12.916393        16.155227        31.533526
##          Calcium           Cobalt           Copper             Iron
##         9.761153        15.971211        35.302426        53.661315
##        Magnesium        Manganese           Nickel       Phosphorus
##        31.551213        17.840002        36.191208        28.682249
##             Zinc
##        80.556370
```

Removing Zinc

```
m1_nifH_cap_chem_2<- capscale(abund_tablenifH ~ Organic_Matter + Moisture_Content + Nitrate_Nitrite + Am
m0_nifH_cap_chem<- capscale(abund_distnifH ~ 1, meta3)
plot(m1_nifH_cap_chem_2)
```

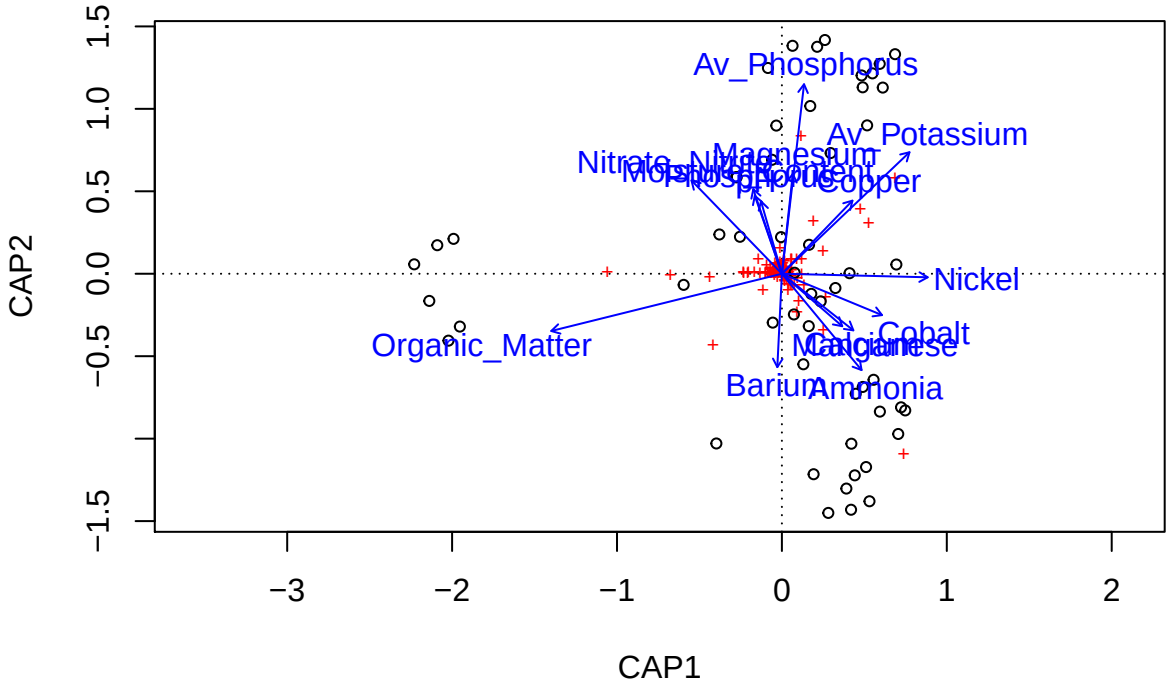



```
vif.cca(m1_nifH_cap_chem_2)
```

```
##   Organic_Matter Moisture_Content  Nitrate_Nitrite          Ammonia
##        12.646738         5.847957         3.672388         4.309848
##    Av_Phosphorus     Av_Potassium               pH          Barium
##        11.225914        10.884874        15.913142        18.081149
##          Calcium           Cobalt           Copper             Iron
##         9.388663        14.920297        31.854122        41.065111
##        Magnesium        Manganese           Nickel       Phosphorus
##        31.536509        16.206354        29.523059        28.553042
```

Removing Iron

```
m1_nifH_cap_chem_3<- capscale(abund_tablenifH ~ Organic_Matter + Moisture_Content + Nitrate_Nitrite + Am
m0_nifH_cap_chem<- capscale(abund_distnifH ~ 1, meta3)
plot(m1_nifH_cap_chem_3)
```

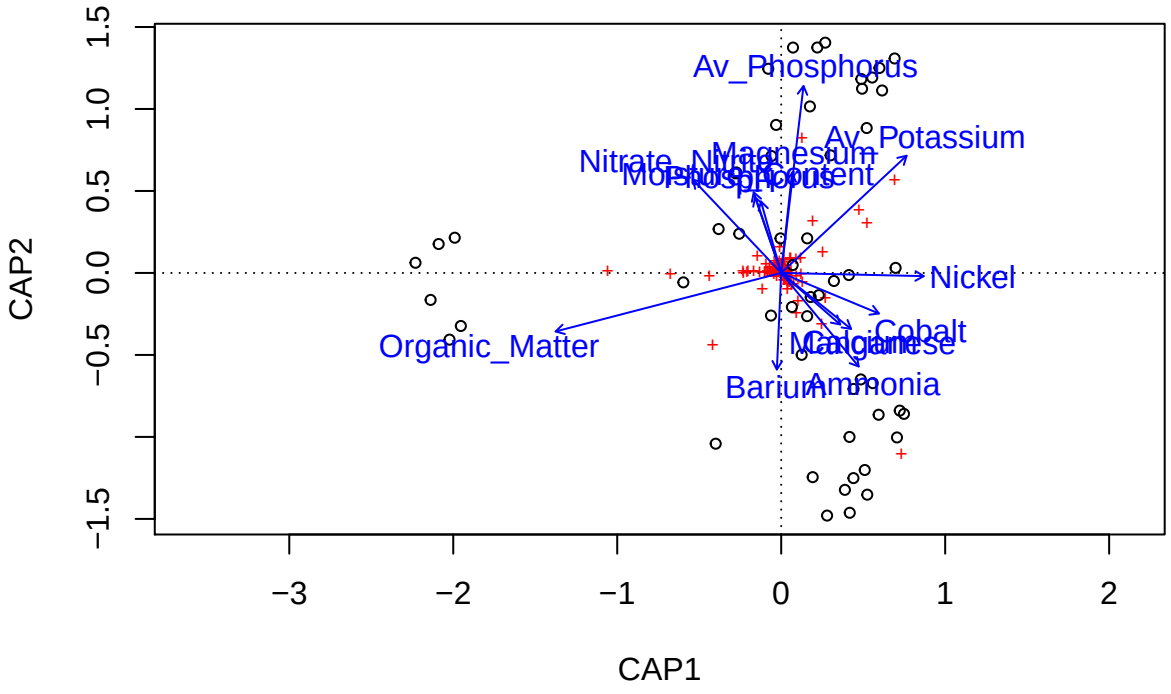



```
vif.cca(m1_nifH_cap_chem_3)
```

```
##   Organic_Matter Moisture_Content  Nitrate_Nitrite          Ammonia
##        10.243305         5.564319         3.533075         4.304512
##    Av_Phosphorus     Av_Potassium               pH          Barium
##        10.829226        10.743500        14.653678        17.693848
##          Calcium           Cobalt           Copper        Magnesium
##         6.990169        13.202442        30.558543        29.869859
##        Manganese           Nickel       Phosphorus
##        14.958783        24.569283        17.904712
```

Removing Copper

```
m1_nifH_cap_chem_4<- capscale(abund_tablenifH ~ Organic_Matter + Moisture_Content + Nitrate_Nitrite + Am
m0_nifH_cap_chem<- capscale(abund_distnifH ~ 1, meta3)
plot(m1_nifH_cap_chem_4)
```

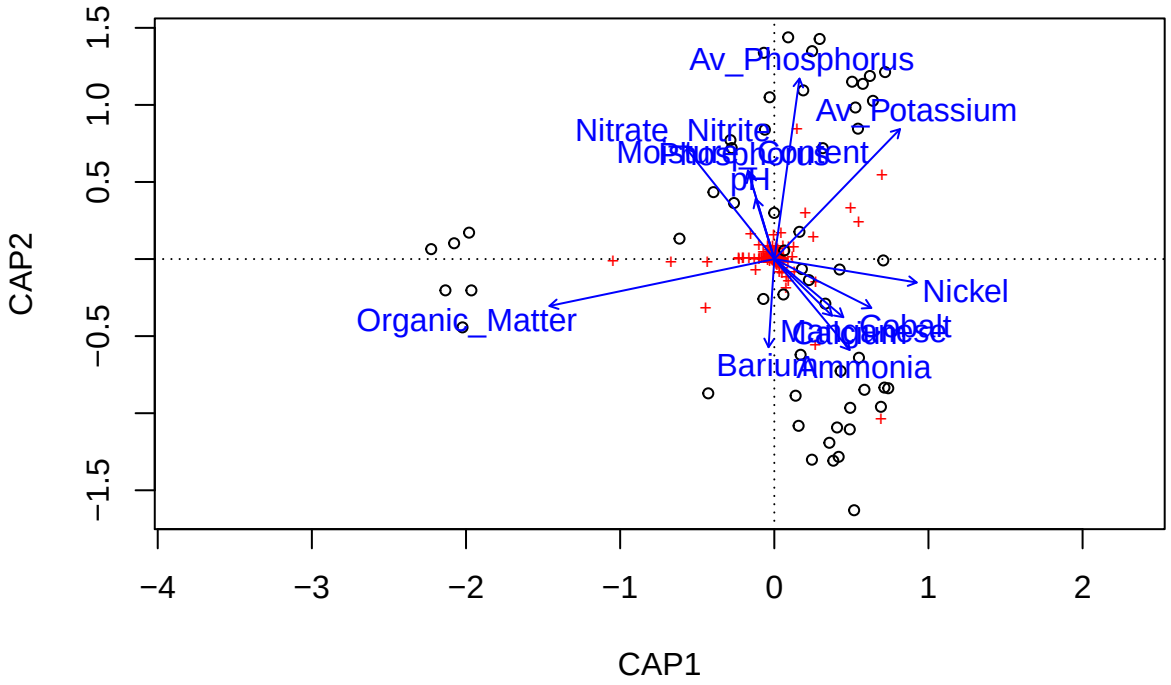



```
vif.cca(m1_nifH_cap_chem_4)
```

```
##   Organic_Matter Moisture_Content  Nitrate_Nitrite          Ammonia
##         9.382412         5.403654         3.279764         4.290460
##    Av_Phosphorus     Av_Potassium               pH          Barium
##         9.817094        10.719638        14.623835         9.620284
##          Calcium           Cobalt        Magnesium        Manganese
##         6.023507        11.675665        22.563802        10.794157
##           Nickel       Phosphorus
##        21.922046        16.432692
```

Removing Magnesium

```
m1_nifH_cap_chem_5<- capscale(abund_tablenifH ~ Organic_Matter + Moisture_Content + Nitrate_Nitrite + Am
m0_nifH_cap_chem<- capscale(abund_distnifH ~ 1, meta3)
plot(m1_nifH_cap_chem_5)
```

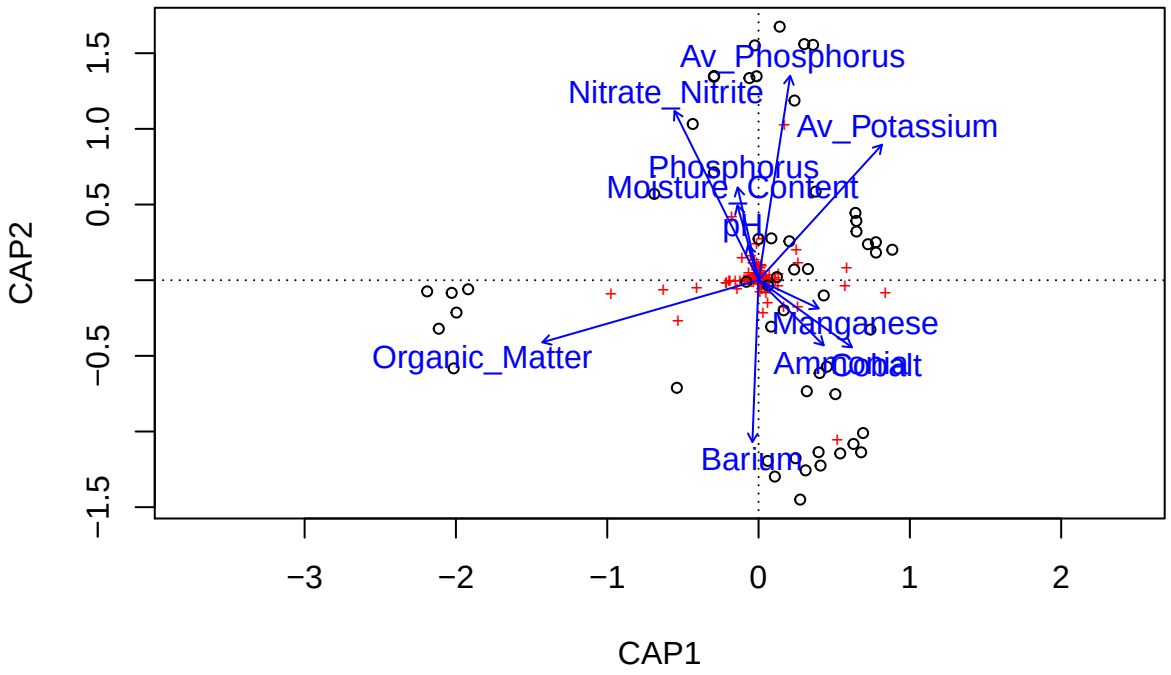



```
vif.cca(m1_nifH_cap_chem_5)
```

```
##   Organic_Matter Moisture_Content  Nitrate_Nitrite          Ammonia
##         9.280442         5.335735         3.115965         4.278840
##    Av_Phosphorus     Av_Potassium               pH          Barium
##         9.280456         9.657791        12.581736         5.989495
##          Calcium           Cobalt        Manganese           Nickel
##         4.196095        11.661999         7.354337        18.320162
##       Phosphorus
##        10.931904
```

Removing Nickel

```
m1_nifH_cap_chem_6<- capscale(abund_tablenifH ~ Organic_Matter + Moisture_Content + Nitrate_Nitrite + Am
m0_nifH_cap_chem<- capscale(abund_distnifH ~ 1, meta3)
plot(m1_nifH_cap_chem_6)
```

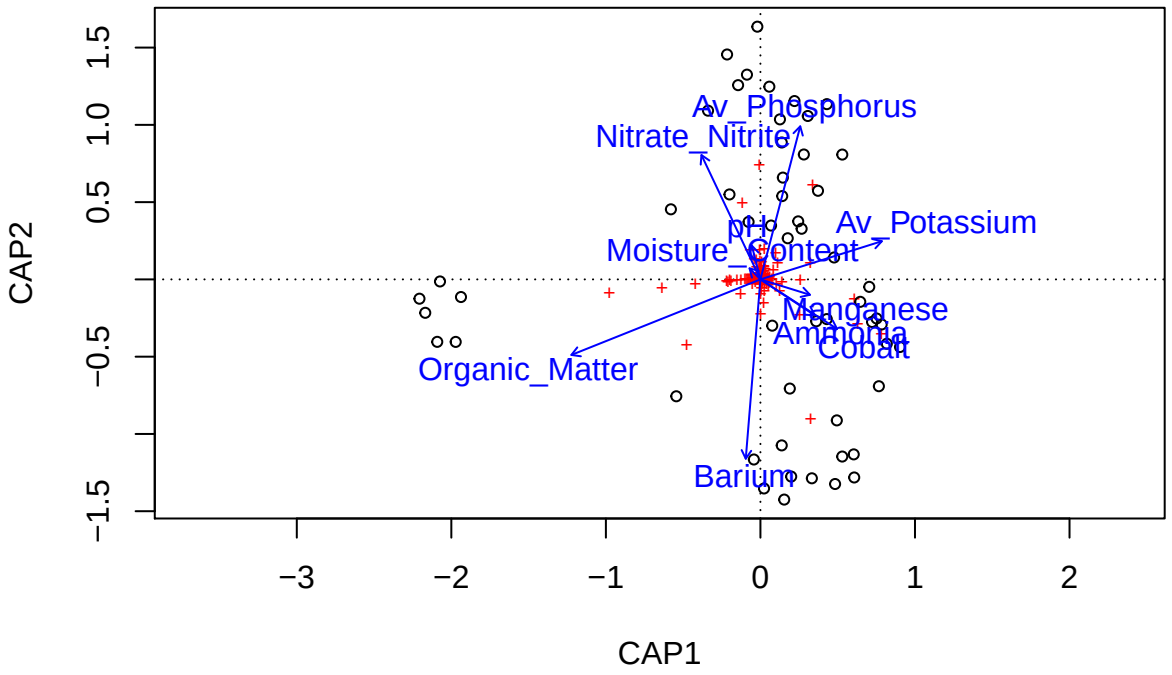



```
vif.cca(m1_nifH_cap_chem_6)
```

```
##   Organic_Matter Moisture_Content  Nitrate_Nitrite          Ammonia
##         4.217221          5.071432         3.036605         4.024085
##    Av_Phosphorus     Av_Potassium                pH          Barium
##         6.845426          6.772406         9.848316         3.102538
##           Cobalt        Manganese       Phosphorus
##         6.612077          5.670644        10.227267
```

Removing Phosphorus as final corvariant

```
m1_nifH_cap_chem_7<- capscale(abund_tablenifH ~ Organic_Matter + Moisture_Content + Nitrate_Nitrite + Am
m0_nifH_cap_chem<- capscale(abund_distnifH ~ 1, meta3)
plot(m1_nifH_cap_chem_7)
```




```
vif.cca(m1_nifH_cap_chem_7)
```

```
##   Organic_Matter Moisture_Content  Nitrate_Nitrite          Ammonia
##         3.289053         4.848379         2.385440         3.637139
##    Av_Phosphorus     Av_Potassium               pH          Barium
##         6.842583         5.484280         7.125107         3.102513
##           Cobalt        Manganese
##         6.161856         5.169878
```

Every variable is under 10 will proceed to model building

```
model_cap_chem_nifH <-ordiR2step(m0_nifH_cap_chem, scope=formula(m1_nifH_cap_chem_7))
```

```
aov_model_cap_chem_nifH<-model_cap_chem_nifH$anova
aov_model_cap_chem_nifH
```

	R2.adj	Df	AIC	F	Pr(>F)
+ Organic_Matter	0.0902795	1	158.5307	6.053807	0.002
+ Barium	0.1477390	1	156.1156	4.345045	0.002
+ Av_Potassium	0.2066181	1	153.3646	4.598390	0.002
+ Manganese	0.2657762	1	150.3047	4.813408	0.002
+ Av_Phosphorus	0.3155079	1	147.6230	4.347257	0.002
+ Cobalt	0.3584885	1	145.2098	4.002428	0.002
+ Nitrate_Nitrite	0.3794547	1	144.4078	2.449920	0.002
	0.3935511	NA	NA	NA	NA

make table

```
capture.output(aov_model_cap_chem_nifH,file="aov_model_cap_chem_nifH.txt")
```

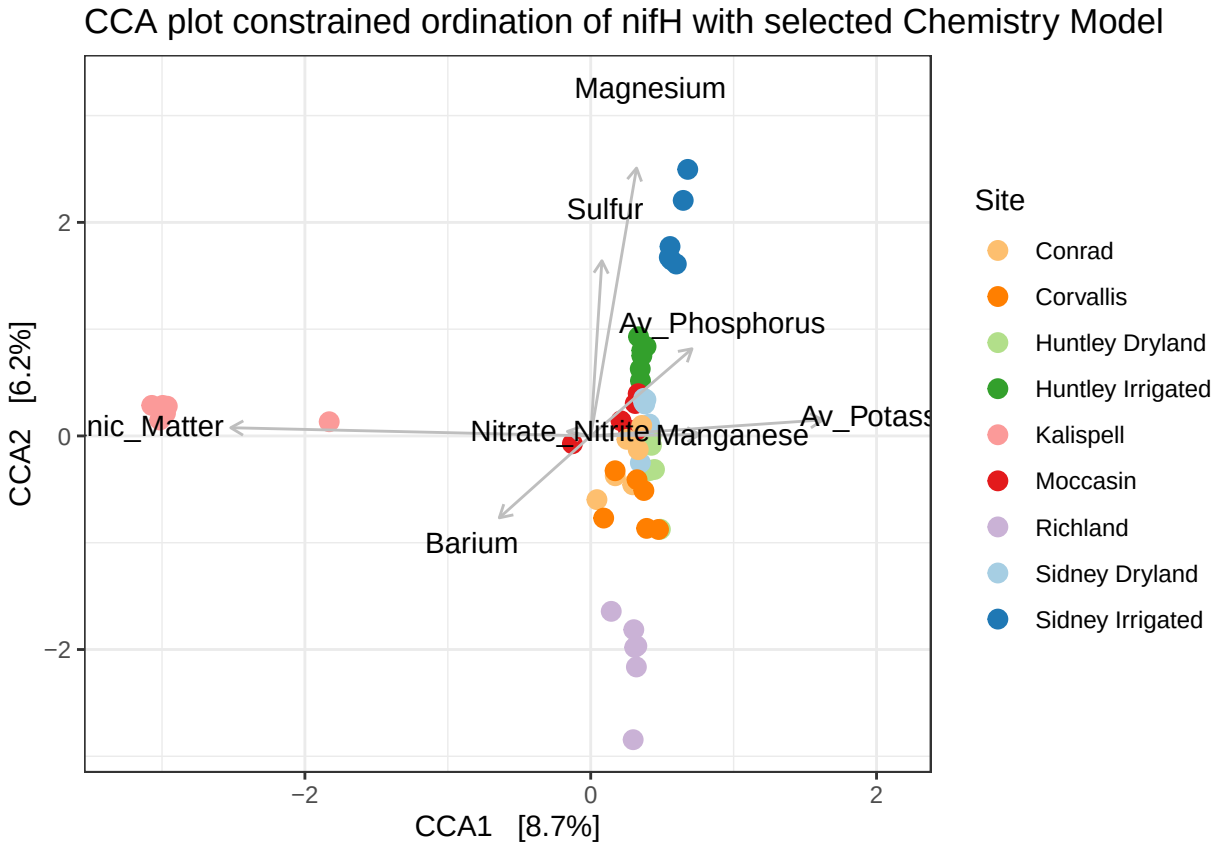
Constrained Ordination

"http://deneflab.github.io/MicrobeMiseq/demos/mothur_2_phyloseq.html#constrained_ordinations"

CCA with selected model from Chemical CCA model

```
# CCA ordinate
cca_ord_nifH <- ordinate(
    physeq = physeq_nifH_ord,
    method = "CCA",
    distance = abund_distnifH,
    formula = ~  Organic_Matter + Nitrate_Nitrite + Av_Potassium +
      Av_Phosphorus + Manganese + Barium + Sulfur + Magnesium  )

# CCA plot
cca_plot_nifH <- plot_ordination(
  physeq = physeq_nifH_ord,
  ordination = cca_ord_nifH,
    color = "Site",
    axes = c(1,2)) +
    geom_point(aes(colour = Site), size = 3) +
    scale_color_manual(values =  farm_col_paired)

# Now add the environmental variables as arrows
arrowmat_nifH_cca <- vegan::scores(cca_ord_nifH, display = "bp")

#Get appropiate scalling multipler
mul<-vegan::ordiArrowMul(arrowmat_nifH_cca)

#Multiply biplot by scaling multiplier
arrowmat_nifH_cca_scale<-arrowmat_nifH_cca*3

# Add labels, make a data.frame
arrowdf_nifH_cca <- data.frame(labels = rownames(arrowmat_nifH_cca_scale), arrowmat_nifH_cca_scale)

# Define the arrow aesthetic mapping
arrow_map <- aes(xend = CCA1,
    yend = CCA2,
    x = 0,
    y = 0,
    shape = NULL,
    color = NULL,
    label = labels)

label_map <- aes(x = 1.3* CCA1,
    y = 1.3 * CCA2,
    shape = NULL,
    color = NULL,
```

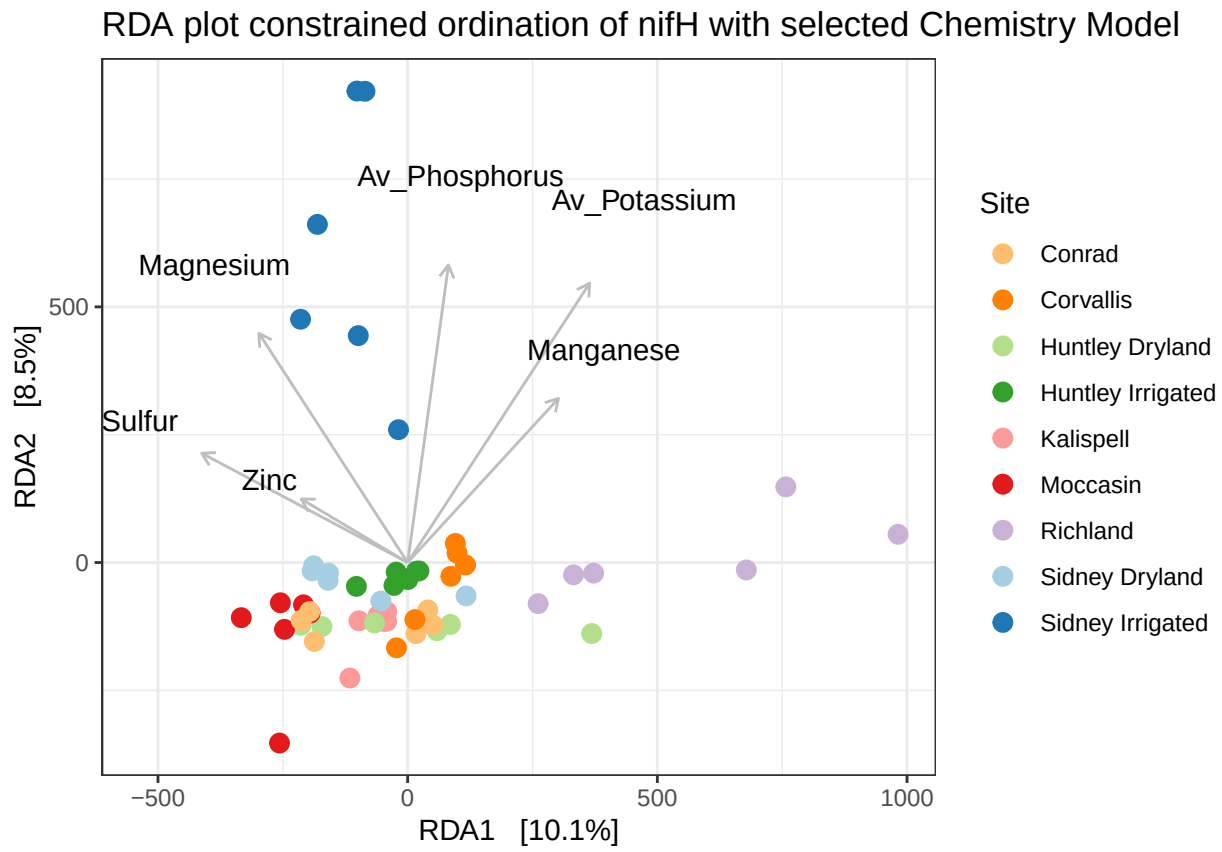
```
    label = labels)

arrowhead = arrow(length = unit(0.02, "npc"))

# Make a new graphic
cca_plot_nifH +
  geom_segment(
    mapping = arrow_map,
    size = .5,
    data = arrowdf_nifH_cca,
    color = "gray",
    arrow = arrowhead
  ) +
  geom_text(
    mapping = label_map,
    size = 4,
    data = arrowdf_nifH_cca,
    show.legend = FALSE
  )+
  ggtitle("CCA plot constrained ordination of nifH with selected Chemistry Model")+
  theme_bw()
```



RDA with selected model from Chemical RDA model

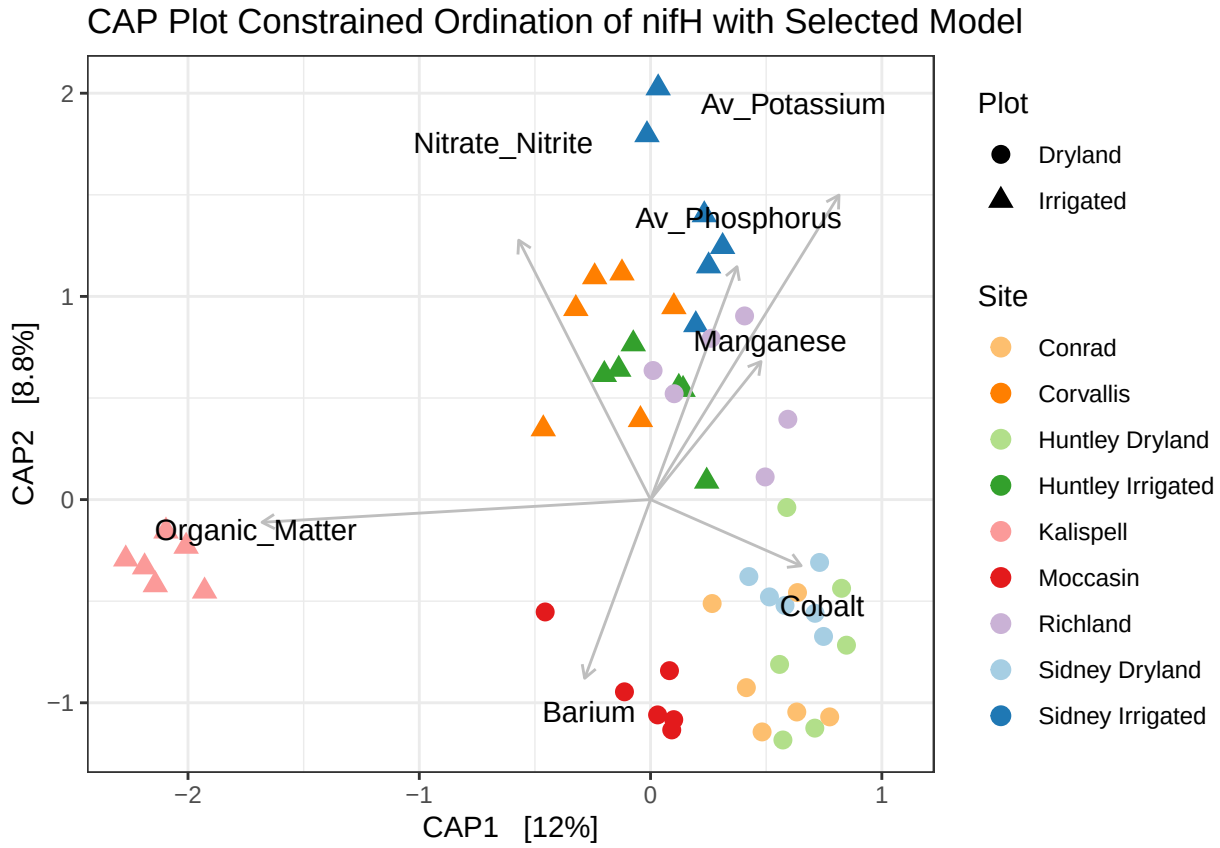
```
# RDA ordinate
rda_ord_nifH <- ordinate(
    physeq = physeq_nifH_ord,
    method = "RDA",
    distance = abund_distnifH,
    formula = ~   Av_Phosphorus + Sulfur + Magnesium + Zinc + Av_Potassium + Manganese )

# RDA plot
rda_plot_nifH <- plot_ordination(
  physeq = physeq_nifH_ord,
  ordination = rda_ord_nifH,
    color = "Site",
    axes = c(1,2)) +
    geom_point(aes(colour = Site), size = 3) +
    scale_color_manual(values =  farm_col_paired)

# Now add the environmental variables as arrows
arrowmat_nifH_rda <- vegan::scores(rda_ord_nifH, display = "bp")

#Get appropiate scalling multipler
mul<-vegan::ordiArrowMul(arrowmat_nifH_rda)

#Multiply biplot by scaling multiplier
arrowmat_nifH_rda_scale<-arrowmat_nifH_rda*700

# Add labels, make a data.frame
arrowdf_nifH_rda <- data.frame(labels = rownames(arrowmat_nifH_rda_scale), arrowmat_nifH_rda_scale)

# Define the arrow aesthetic mapping
arrow_map <- aes(xend = RDA1,
    yend = RDA2,
    x = 0,
    y = 0,
    shape = NULL,
    color = NULL,
    label = labels)

label_map <- aes(x = 1.3* RDA1,
    y = 1.3 * RDA2,
    shape = NULL,
    color = NULL,
    label = labels)

arrowhead = arrow(length = unit(0.02, "npc"))

# Make a new graphic
rda_plot_nifH +
  geom_segment(
    mapping = arrow_map,
    size = .5,
    data = arrowdf_nifH_rda,
    color = "gray",
    arrow = arrowhead
```

```
  ) +
  geom_text(
    mapping = label_map,
    size = 4,
    data = arrowdf_nifH_rda,
    show.legend = FALSE
  )+
  ggtitle("RDA plot constrained ordination of nifH with selected Chemistry Model")+
  theme_bw()
```




```
# CAP ordinate
cap_ord_nifH <- ordinate(
    physeq = physeq_nifH_ord,
    method = "CAP",
    distance = abund_distnifH,
    formula = ~ Organic_Matter + Barium + Av_Potassium +
      Manganese + Av_Phosphorus + Cobalt + Nitrate_Nitrite)

# CAP plot
cap_plot_nifH <- plot_ordination(
  physeq = physeq_nifH_ord,
  ordination = cap_ord_nifH,
    color = "Site",
    shape = "Plot",
    axes = c(1,2)) +
    geom_point(aes(colour = Site), size = 3) +
```

```
    scale_color_manual(values =  farm_col_paired)

# Now add the environmental variables as arrows
arrowmat_nifH_cap <- vegan::scores(cap_ord_nifH, display = "bp")

#Get appropiate scalling multipler
mul<-vegan::ordiArrowMul(arrowmat_nifH_cap)

#Multiply biplot by scaling multiplier
arrowmat_nifH_cap_scale<-arrowmat_nifH_cap*1.9

# Add labels, make a data.frame
arrowdf_nifH_cap <- data.frame(labels = rownames(arrowmat_nifH_cap_scale), arrowmat_nifH_cap_scale)

# Define the arrow aesthetic mapping
arrow_map <- aes(xend = CAP1,
    yend = CAP2,
    x = 0,
    y = 0,
    shape = NULL,
    color = NULL,
    label = labels)

label_map <- aes(x = 1.3 * CAP1,
    y = 1.3 * CAP2,
    shape = NULL,
    color = NULL,
    label = labels)

arrowhead = arrow(length = unit(0.02, "npc"))

# Make a new graphic
cap_plot_nifH +
  geom_segment(
    mapping = arrow_map,
    size = .5,
    data = arrowdf_nifH_cap,
    color = "gray",
    arrow = arrowhead
  ) +
  geom_text_repel(
    mapping = label_map,
    size = 4,
    data = arrowdf_nifH_cap,
    show.legend = FALSE
  )+
  ggtitle("CAP Plot Constrained Ordination of nifH with Selected Model")+
  theme_bw()
```




```
## Warning: Ignoring unknown aesthetics: label
```

```
## pdf
##   2
```

Fitting species to cap plot

```
m1_nifH_cap_chem_species<- capscale(abund_tablenifH~ Organic_Matter + Barium + Av_Potassium +
      Manganese + Av_Phosphorus + Cobalt + Nitrate_Nitrite, data = meta3, distance = "bray")
dims=c(1,2)
site=scores(m1_nifH_cap_chem_species,display="wa",choices=dims)
cor.min=0.9 #below this threshold, arrows will be not plotted
#because orrelation is considered too much week
cor_sp=as.data.frame(scores(m1_nifH_cap_chem_species, dis="sp", scaling=1,choices=dims))
cor_sp$cor=with(cor_sp,sqrt(CAP1^2+CAP2^2))
cor_sp$sup=FALSE;cor_sp$sup[cor_sp$cor>=cor.min]<-TRUE
cor_sp$labels=row.names(cor_sp)
cor_sp=cor_sp[cor_sp$sup==TRUE,]
cor_sp_s1=cor_sp
```

```
tax_nifH_cap<- read.delim("~/Alex Alleman/Statewide Microbiome Analysis/Statewide analysis/nifH_OTU_ids
head(tax_nifH_cap)[,1:8]
```

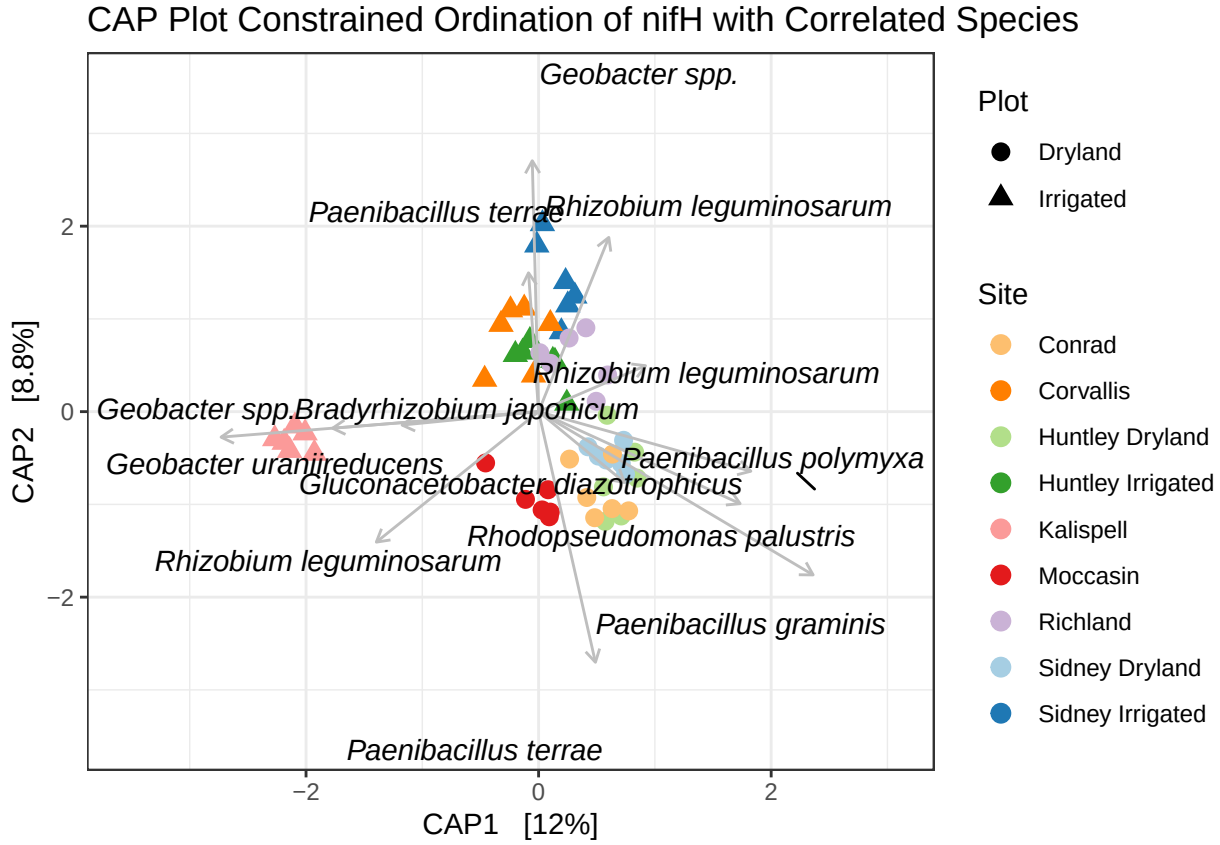
	kingdom	phylum	class	order	family	genus
OTU1	k___bacteria	p___firmicutes	c___bacilli	o___bacillales	f___paenibacillaceae	g___pae
OTU2	k___bacteria	p___firmicutes	c___bacilli	o___bacillales	f___paenibacillaceae	g___pae
OTU3	k___bacteria	p___proteobacteria	c___alphaproteobacteria	o___rhizobiales	f___rhizobiaceae	g___rhiz
OTU4	k___bacteria	p___firmicutes	c___bacilli	o___bacillales	f___paenibacillaceae	g___pae
OTU5	k___bacteria	p___proteobacteria	c___alphaproteobacteria	o___rhizobiales	f___bradyrhizobiaceae	g___bra
OTU6	k___bacteria	p___proteobacteria	c___alphaproteobacteria	o___rhizobiales	f___bradyrhizobiaceae	g___rho

```
#use perl scirpt to remove teh g_
tax_nifH_cap$species<- sub(".*_", "", tax_nifH_cap$species)
#use the function captilize to captilize first letter
tax_nifH_cap$species<- capitalize(tax_nifH_cap$species)

species_nifH<-merge(cor_sp_s1, tax_nifH_cap, by="row.names")
```

Replot with species

```
# CAP ordinate
cap_ord_nifH <- ordinate(
    physeq = physeq_nifH_ord,
    method = "CAP",
    distance = abund_distnifH,
    formula = ~ Organic_Matter + Barium + Av_Potassium +
      Manganese + Av_Phosphorus + Cobalt + Nitrate_Nitrite)

# CAP plot
cap_plot_nifH <- plot_ordination(
  physeq = physeq_nifH_ord,
  ordination = cap_ord_nifH,
    color = "Site",
    shape = "Plot",
    axes = c(1,2)) +
    geom_point(aes(colour = Site), size = 3) +
    scale_color_manual(values =  farm_col_paired)


# Define the arrow aesthetic mapping
arrow_map <- aes(xend = CAP1,
    yend = CAP2,
    x = 0,
    y = 0,
    shape = NULL,
    color = NULL,
    label = species)

label_map <- aes(x = 1.3 * CAP1,
    y = 1.3 * CAP2,
    shape = NULL,
    color = NULL,
    label = species)

arrowhead = arrow(length = unit(0.02, "npc"))
```

```
# Make a new graphic
cap_plot_nifH +
  geom_segment(
    mapping = arrow_map,
    size = .5,
    data = species_nifH,
    color = "gray",
    arrow = arrowhead
  ) +
  geom_text_repel(
    mapping = label_map,
    size = 4,
    data = species_nifH,
    show.legend = FALSE,
    fontface="italic"
  )+
  ggtitle("CAP Plot Constrained Ordination of nifH with Correlated Species")+
  theme_bw()
```



Publish to tiff

```
## Warning: Ignoring unknown aesthetics: label
```

```
## pdf
##   2
```