WHERE WE LEFT OFF

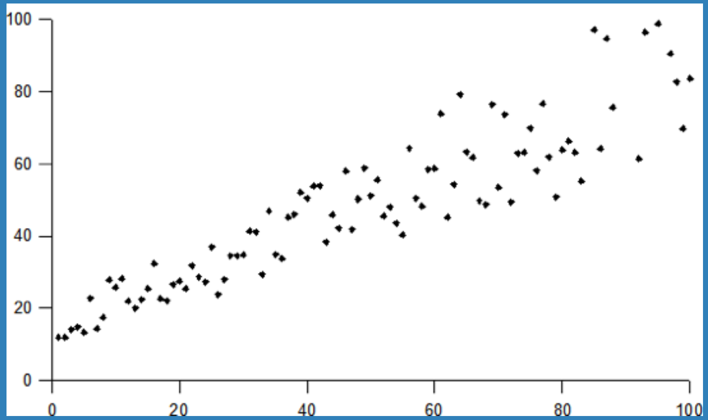
- 16S and ITS rRNA gene sequences (V4 region and ITS1, region respectively)
    - Joined paired ends
    - Filtered and trimmed low-quality base calls (hopefully)
    - Clustered into Amplicon Sequence Variants
    - Affiliated those sequences to their respective taxonomies
    - Predicted metabolic pathways/guilds
    - Merged the two kingdoms and produced the following:
        - A table file (without Chloroplasts/Mitochondria)
        - A normalized table file
        - A taxonomy file
        - A tree file

Qiime2 walkthrough for 16S, ITS and beyond

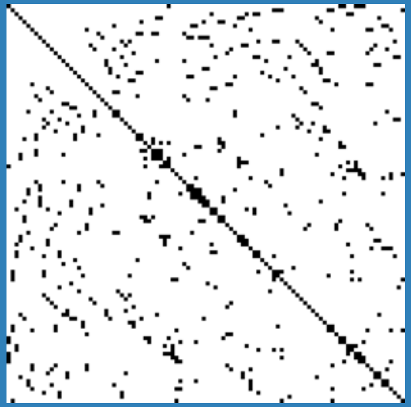
- Introduction
- Preliminary steps:
- 16S pipeline
    - 0_setup.sh
    - 1_import.sh
    - 2_dada2.sh
    - 3_feature_table.sh
    - 4_taxonomy.sh
    - 5_PICRUST.sh
- ITS pipeline
    - 0_setup.sh
    - 1_merge_and_trim.sh
    - 2_import.sh
    - 3_dada2_single.sh
    - 4_Taxonomy_UNITE.sh
    - 5_FUNGUILD.sh
- Merged kingdoms
    - 1_merge_kingdoms.sh
    - 2_to_biom_and_beyond.sh
    - 3_build_tree.sh

NEW CHALLENGES

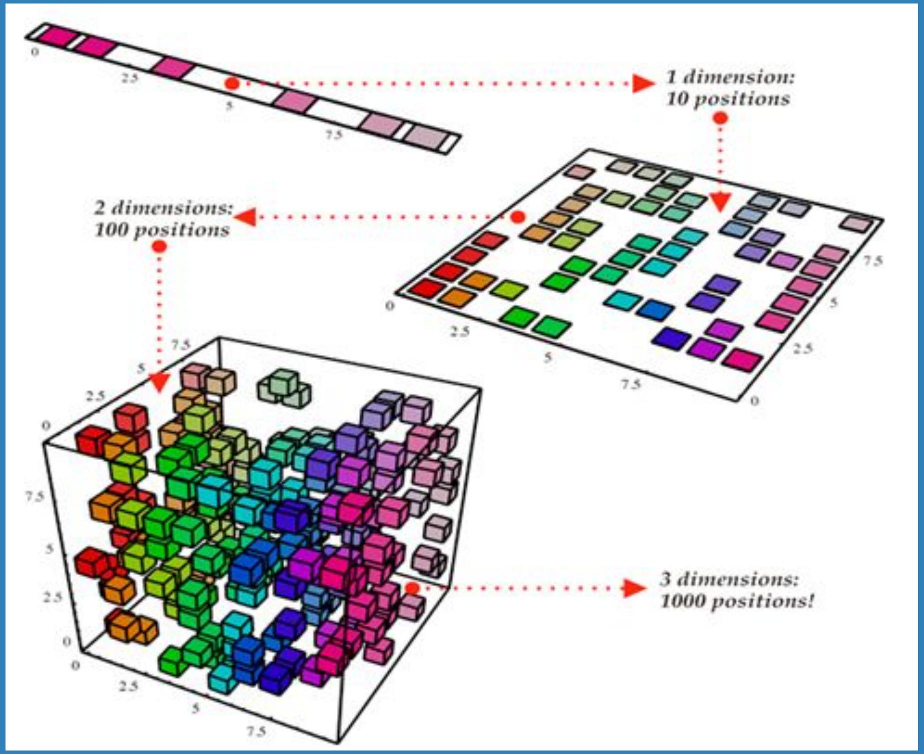
Heteroscedasticity



Sparsity

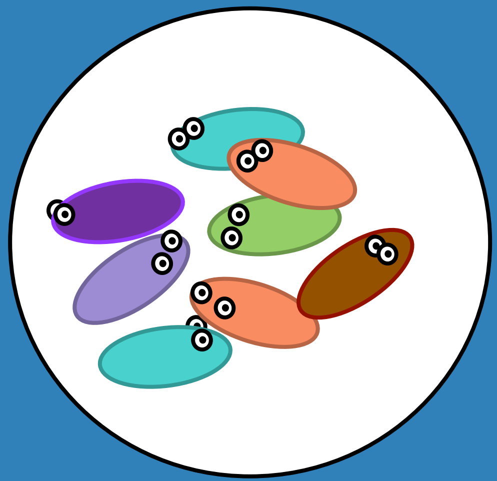


Dimensionality



DIVERSITY ANALYSIS

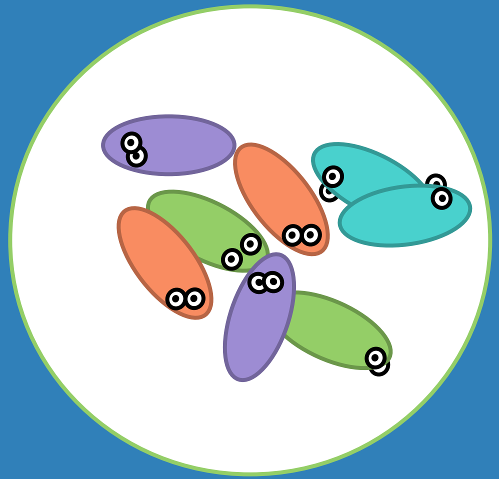
Sample 1



α_1

Alpha diversity of sample 1: 6 species with different abundances

Sample 2



α_2

Alpha diversity of sample 1: 4 species with similar abundance

$\beta_{1\text{-}2}$

Beta diversity: differences between the two samples (i.e. distances)

ALPHA DIVERSITY

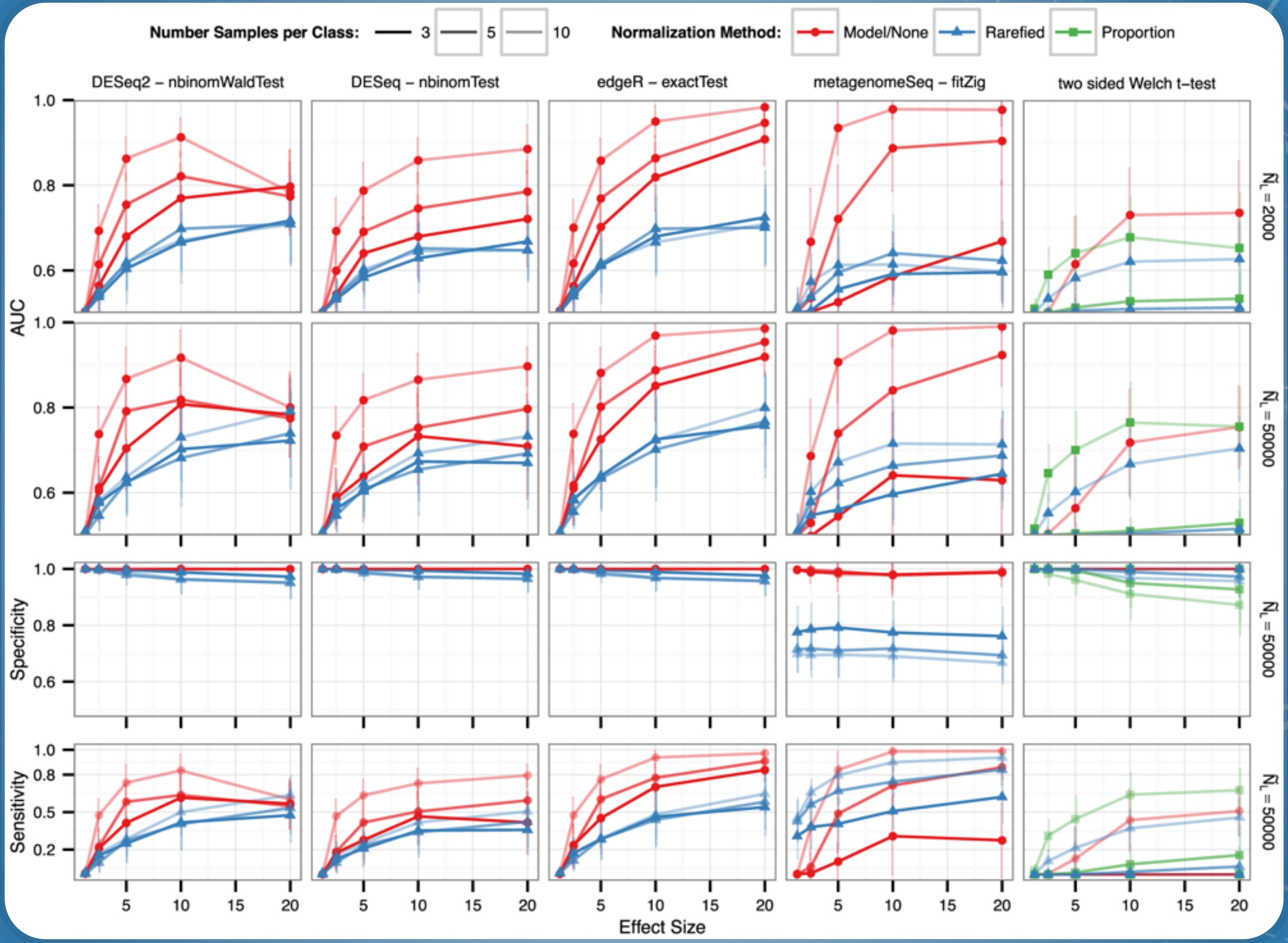
- DOES NOT NEED distribution assumptions:
  - DO NOT normalize your data
  - DO NOT filter your data
- Several indexes are possible (and often different names mean the same index), but they do different things, with different power, and measure different aspects:
  - Shannon, chao1, observed: measures *Richness*
  - Simpson: measures *Dominance*
  - Inverse Simpson: measures *Evenness*
- Rarefaction curves are a good proxy for data quality check!
  - Split the dataset into bins and plot the cumulative sums of the selected α diversity index
  - Look for plateau

BETA DIVERSITY

- Due to the nature of the data, making comparisons between samples is pretty difficult
- To compare samples diversities, you NEED to make them comparable, which means:
  - Normalize (i.e. fitting counts in a Gaussian curve within same range for all samples)
  - Reduce number of outliers
  - Etc.
- Introducing DISTANCES:
  - Indexes representing “dissimilarity” between samples
  - Make lots of assumptions
- Several indexes existing, including Bray-Curtis (works better on log- or root-transformed data), Jaccard (intersection over union), Manhattan (Euclidean distances), UniFrac (includes phylogeny)
- Overall, when plotting beta diversity, samples close together are more similar with each other

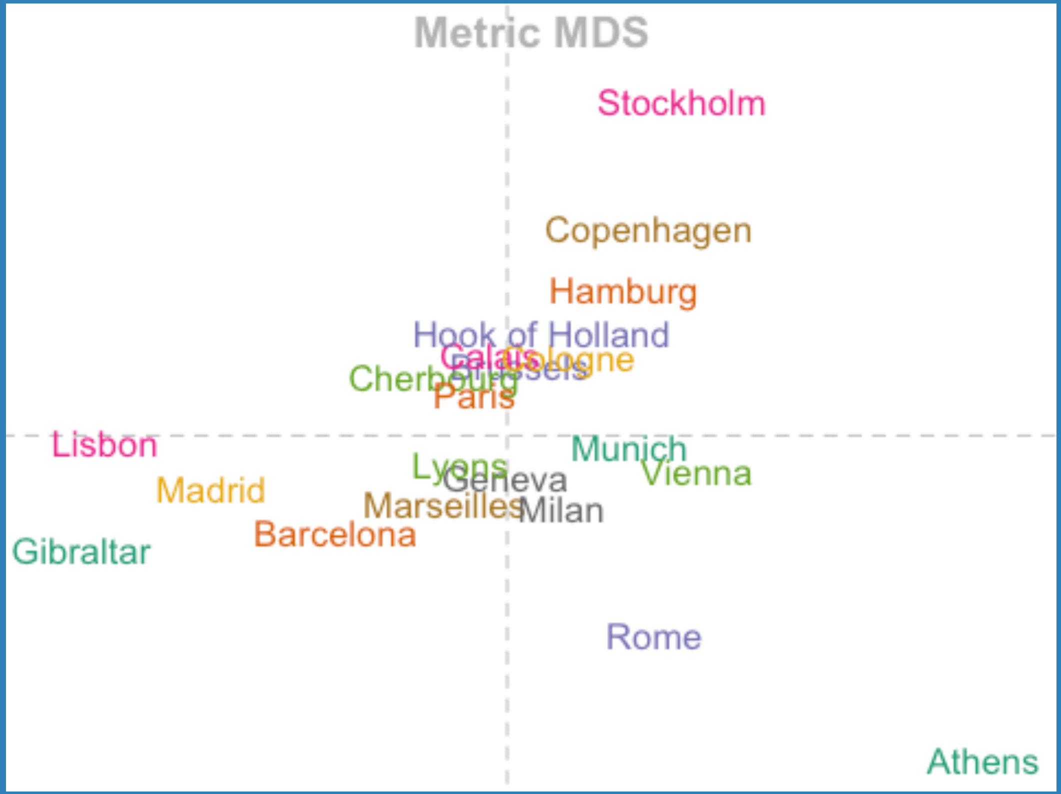
WASTE NOT WANT NOT: DO NOT RAREFY!

- Rarefying = resampling
    - Equalize number of counts/sample
    - With or without replacement
- Microbes are NEVER normally distributed:
    - Negative binomial distribution (mostly)
- Rarefying kills!
    - Does not solve heteroscedasticity
    - Loss of Accuracy, Sensitivity
    - Tendency to false positive
    - Increase of Type II errors (FN)
    - Arbitrary
- Flaws especially true for differential analyses on small number of samples



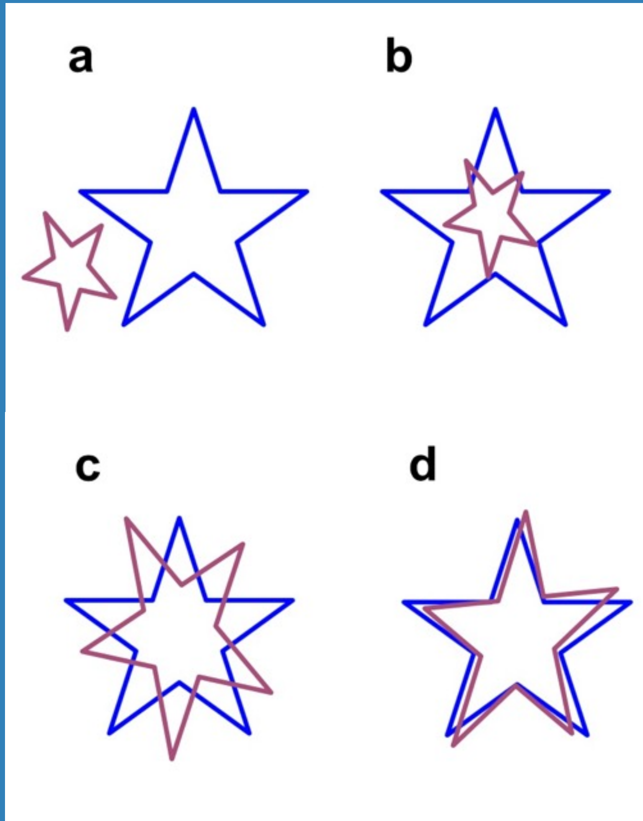
PCoA: PRINCIPAL COORDINATE ANALYSIS (or MDS)

- NOT PCA
- If I know distance between cities, but not their coordinates, how can I draw a map?
- Your count table is converted into a matrix of dissimilarity (using the diversity index chosen)
- May be impacted by high variance (so, you need to normalize)
- If some results in the dissimilarity matrices are negative, it leads to imaginary numbers in the eigenvectors
- The eigenvectors are not your variables, but are correlated with different percentages



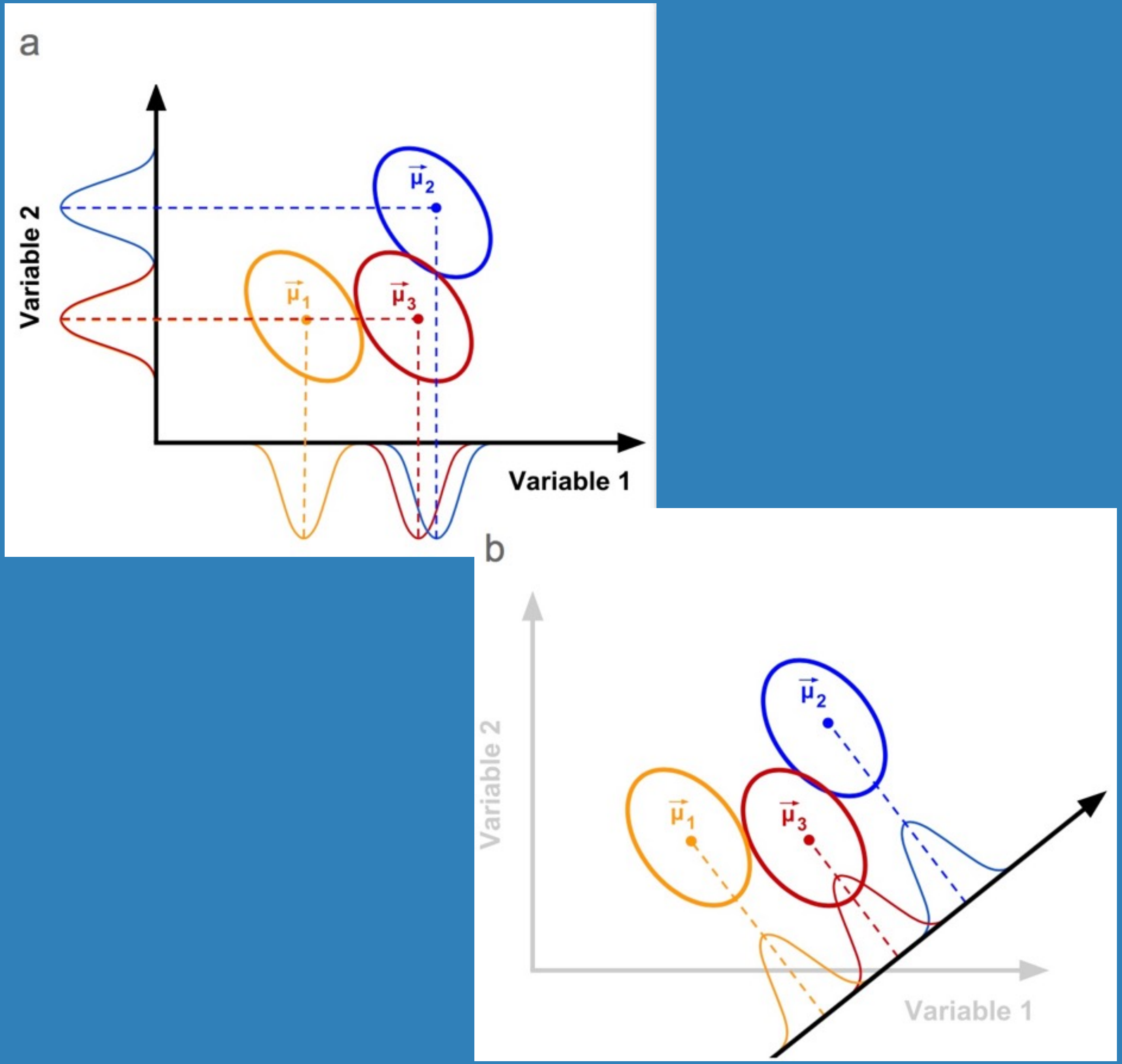
NMDS: NON-METRIC MULTIDIMENSIONAL SCALING

- Starts from a dissimilarity matrix
    - Ranking
- More robust than PCoA
- Applies Procrustes analysis to modify the matrix so that the eigenvectors are close to the original dimensions
    - Generate a ”stress value”
        - <0.1 good model
        - 0.1 < stress < 0.2 meh model
        - 0.2 < stress < 0.3 bad model
        - >0.3 random
- Eingenvectors are still NOT the original dimensions



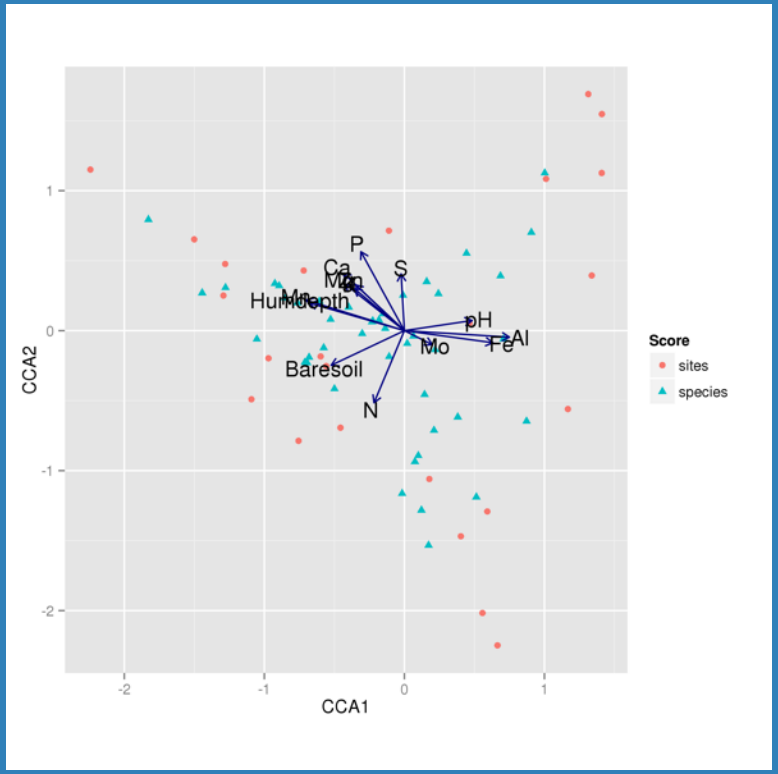
ANOSIM & PERMANOVA

- Both similar to ANOVA
- ANOSIM uses a dissimilarity matrix instead of the raw data
    - Finds differences between groups
    - Highly sensitive to dispersity
- PERMANOVA is a
    - Multivariate ANOVA (i.e. multiple factors influence multiple responses)
    - With PERmutations (solves the problem of limited number of samples)
    - Sensitive to dispersity



CCA: CANONICAL CORRESPONDENCE ANALYSIS

- Analyzes correspondences between a matrix of frequencies and a matrix of variables
  - How much the frequencies deviate from random per each variable?
- Correlate counts to variables (finally!)
- Does not try to maximize coverage of variance (unlike PCA)
- You can use ANOVA on CCA
- BEWARE of using only significant variables



MULTICOLLINEARITY

Why is it a problem?

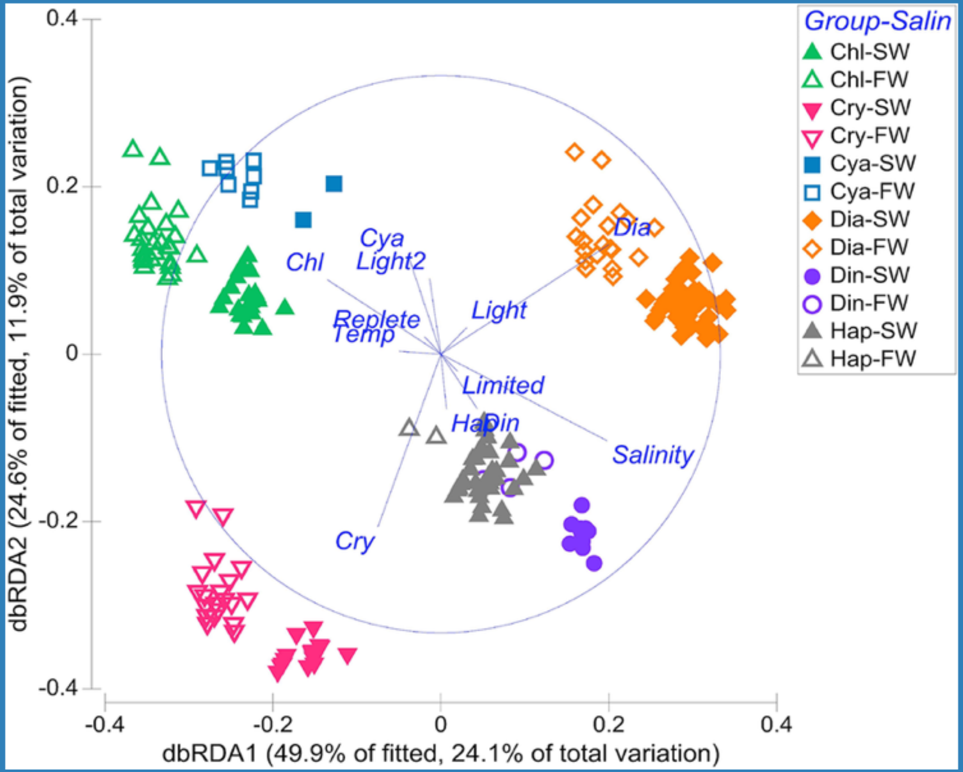
- Variables are highly correlated
- Thus, data are redundant
- Impacts of variables are wrongly estimated
- Small change of inputs -> high change of outputs
- Overfitting and huge errors

How can I solve it?

- Detect collinearity with VIF (variance inflation factor)
- Check dummy variable trap (encoding)
- Increase data
- Decrease number of variables

CAP (or db-RDA): CONSTRAINED ANALYSIS OF PRINCIPAL COORDINATES

- Basically a PCA where you constrain your components to your variables
  - Maximizes explanation of variance
- Similar (but different) to CCA
  - Still sensitive to collinearity
- BEWARE of using only significant variables



NETWORK ANALYSES IN THE MICROBIOME

- Borrows from non-bio sciences (graph theory, social networks)
- Reveal hidden patterns
- Biological networks are usually:
  - Scale-free networks (i.e. follow Poissonian distributions)
  - Small-world networks (every node is accessible with a relatively short path)
- Can be obtained via
  - Pairwise dissimilarities
  - Correlations
  - Regressions
  - Probabilistic Graphs Models (i.e. Bayes, Markov, etc.)
- Cluster detection
- Hub-species detection
  - i.e. keystone species
- Microbiome dynamics

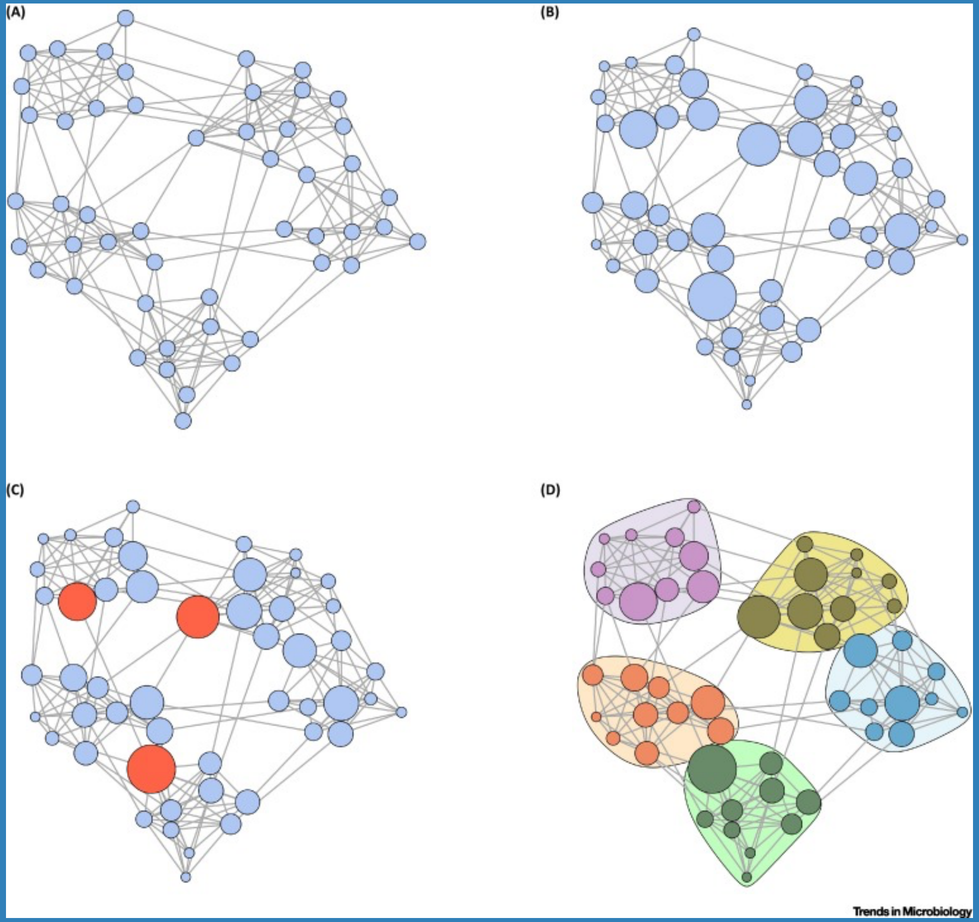
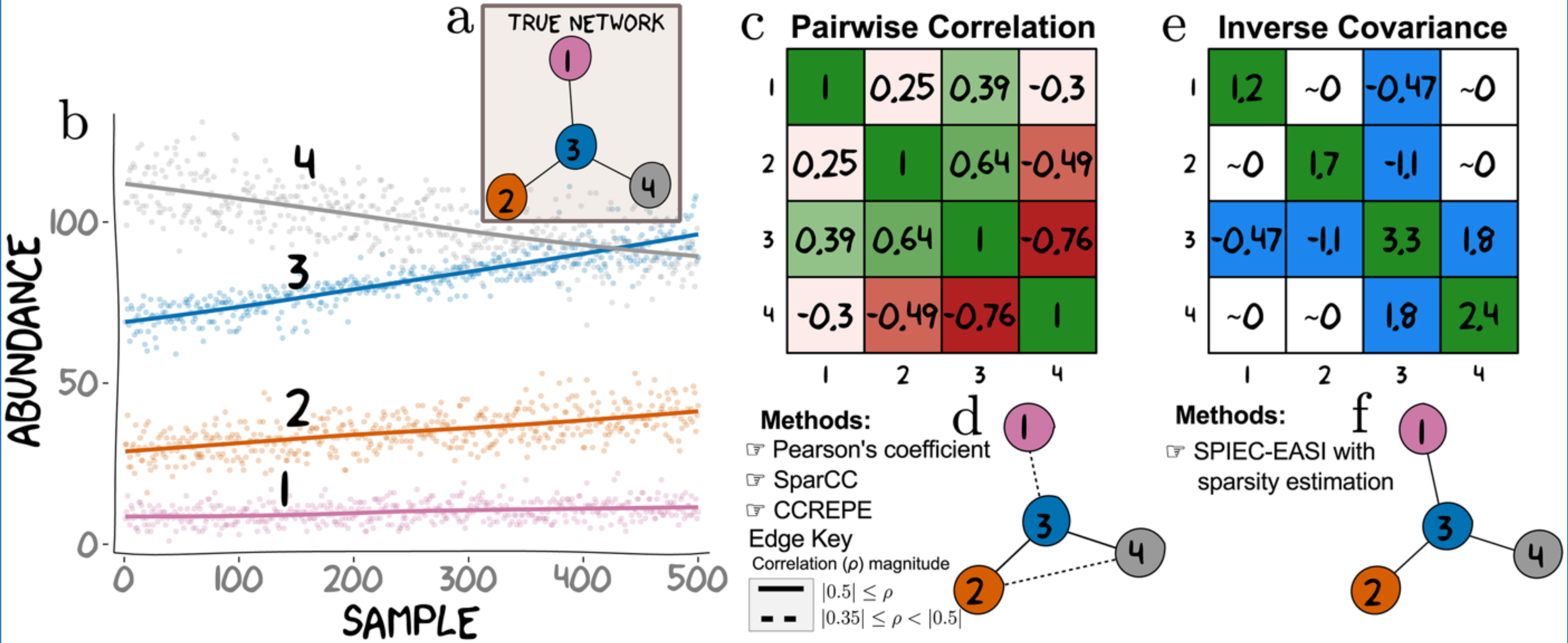


Image from Layeghifard, M. et al. (2016). Trend. Micro., 25, 3, 217-228


13 WILT CHAMBERLAIN

NETWORK ANALYSES WITH SPIEC-EASI

- Assume interdependencies of OTUs
- Draws samples from negative binomial distributions
- Sparse data → inverse covariance matrix depends on the conditional states of all available nodes
- Avoids weak or false positive associations



*Kurtz, Z.D. et al. (2015). PLoS Comput. Biol., 11, 1–25

HOW ARE WE DOING?

A

B

C

RECAP OF MULTIVARIATE ANALYSES

- You want to find out how the WHOLE microbiome is related to your Conditions or plant data
  - Possibly, which part of the microbiome is the most important
- α diversity: single index for each sample
- β diversity: distance index between samples
- DO NOT RAREFY

PCoA
- NOT PCA
- Useful to visualize dissimilarity matrix

NMDS
- More powerful than PCoA
- Shows how your samples can be clustered together
- NMDS axes are NOT your variables
- PERMANOVA and ANOSIM to check group differences

CCA
- Correlate counts to your variables
- Sensitive to multicollinearity
- ANOVA to check group differences

CAP
- Forces a PCA to explain variance of your samples with your variables
- Sensitive to multicollinearity
- ANOVA to check group differences

CODE