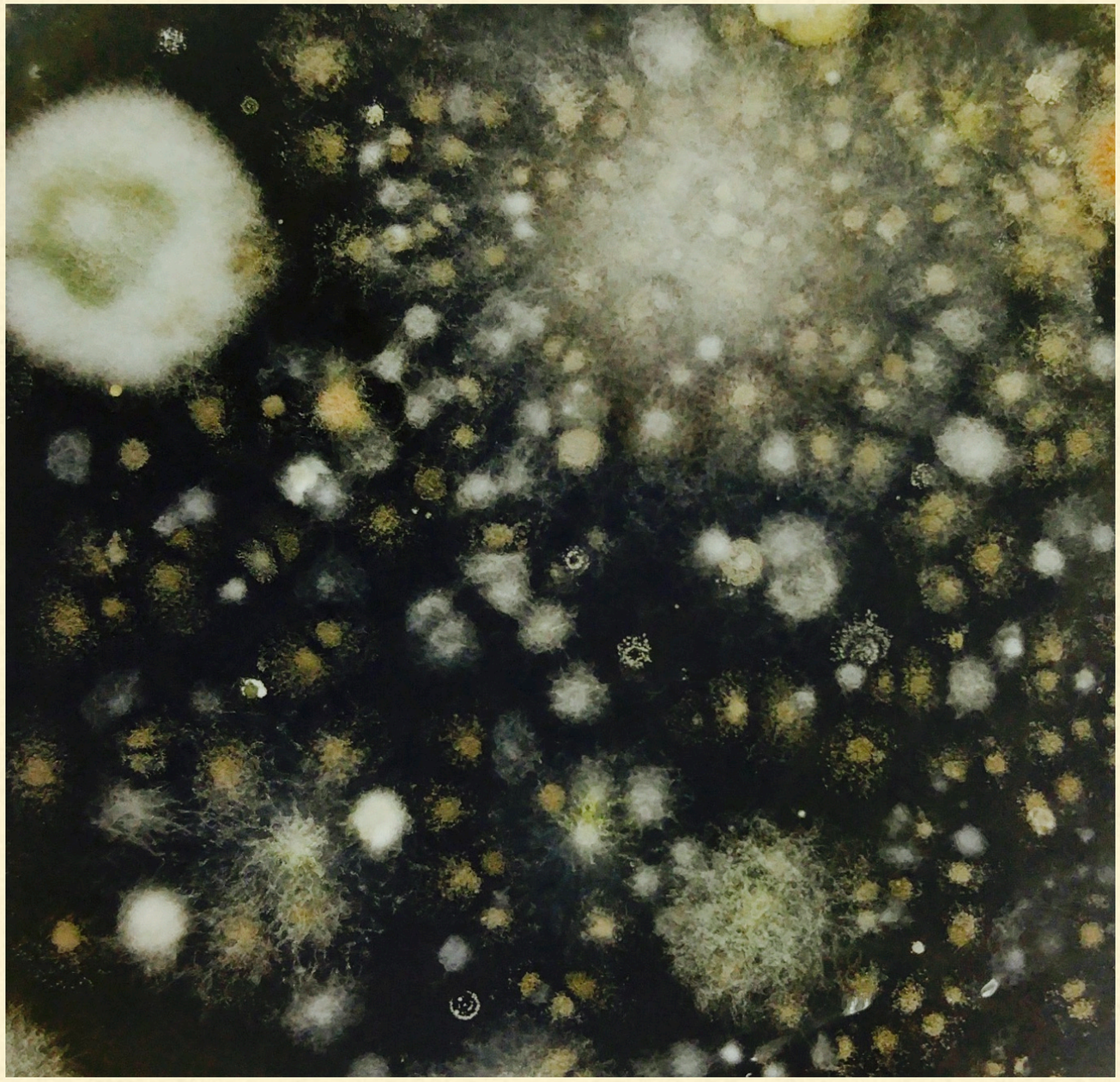
MODELING DIVERSITY



DIVERSITY

- Low dimensional summaries of entire communities
  - α-diversity: one community
    - e.g., species richness, Shannon diversity
  - β-diversity: multiple communities
    - e.g., UniFrac, Bray-Curtis, Jaccard
    - Usually based on distances
- Direct import from macroecology

DIVERSITY & PARAMETERS

- There are multiple choices to make when talking about diversity
  - Which taxonomic level? (strain/species/genus...)
  - Which diversity parameter?
  - Which estimate of the diversity parameter?

DIVERSITY & PARAMETERS

- There are multiple choices to make when talking about diversity
  - Which taxonomic level? (strain/species/genus...)
  - **Which diversity parameter?**
  - Which estimate of the diversity parameter?

ALPHA DIVERSITY

- Suppose we have C groups in our environment in proportions p_1, p_2, …, p_C

- Any function of
  - p_1, p_2, …, p_C OR
  - p_1, p_2, …, p_C and ~~some info about relationships amongst groups~~ phylogeny

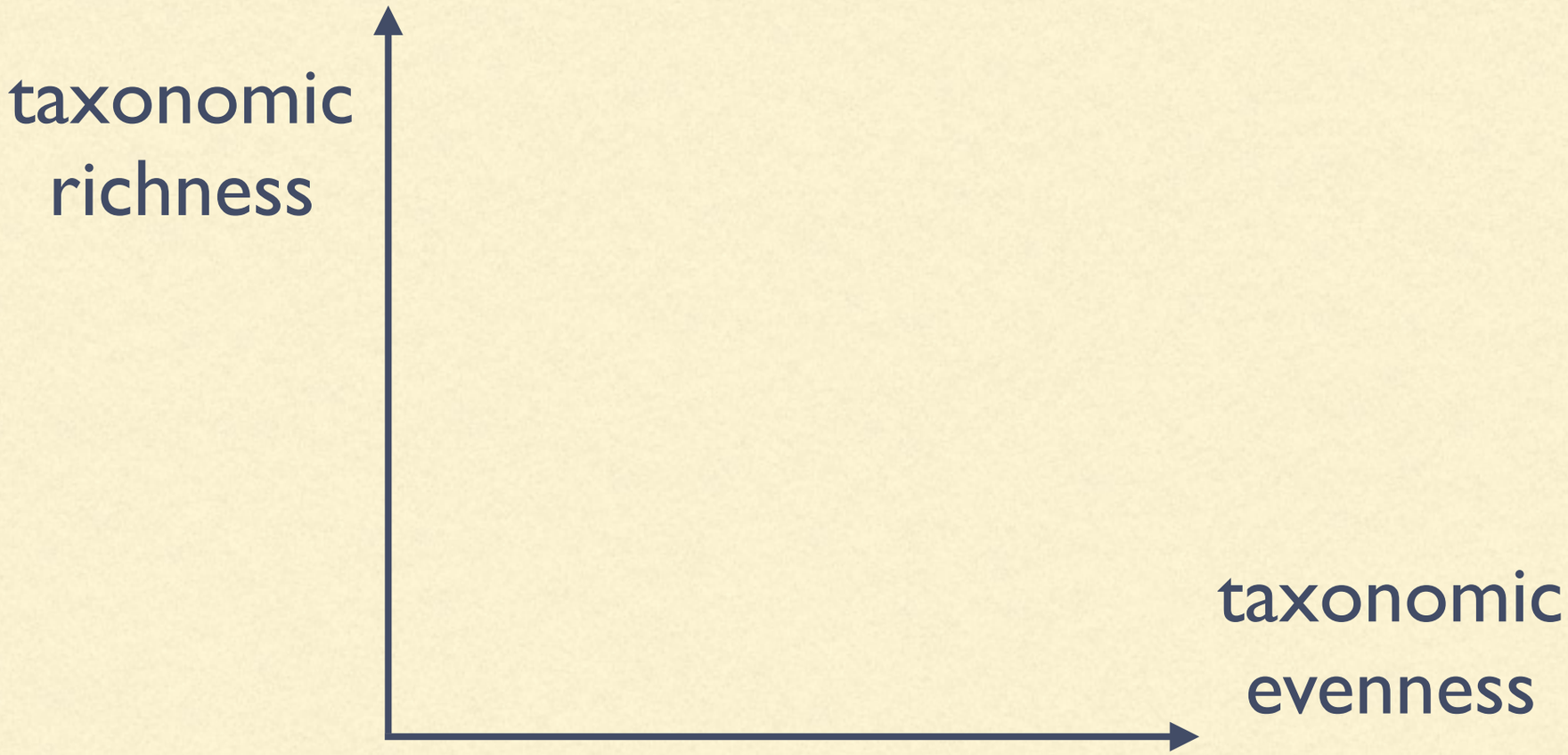
is a valid α-diversity parameter

ALPHA DIVERSITY

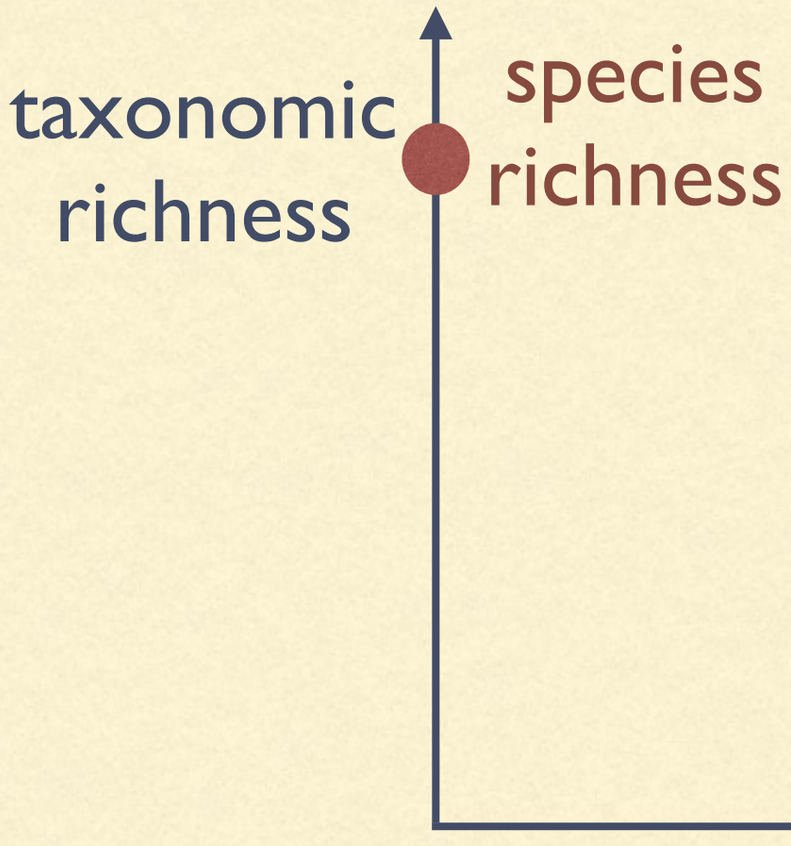
- Some examples of α-diversity measures include
  - Species richness: C
  - Simpson's index: $\sum_{i=1}^{C} p_i^2$
  - Shannon diversity: $-\sum_{i=1}^{C} p_i \ln p_i$
  - Shannon's E: $\frac{-\sum_{i=1}^{C} p_i \ln p_i}{\ln C}$

YOUR CHOICE

- Think: What difference do you want to highlight?



YOUR CHOICE



YOUR CHOICE

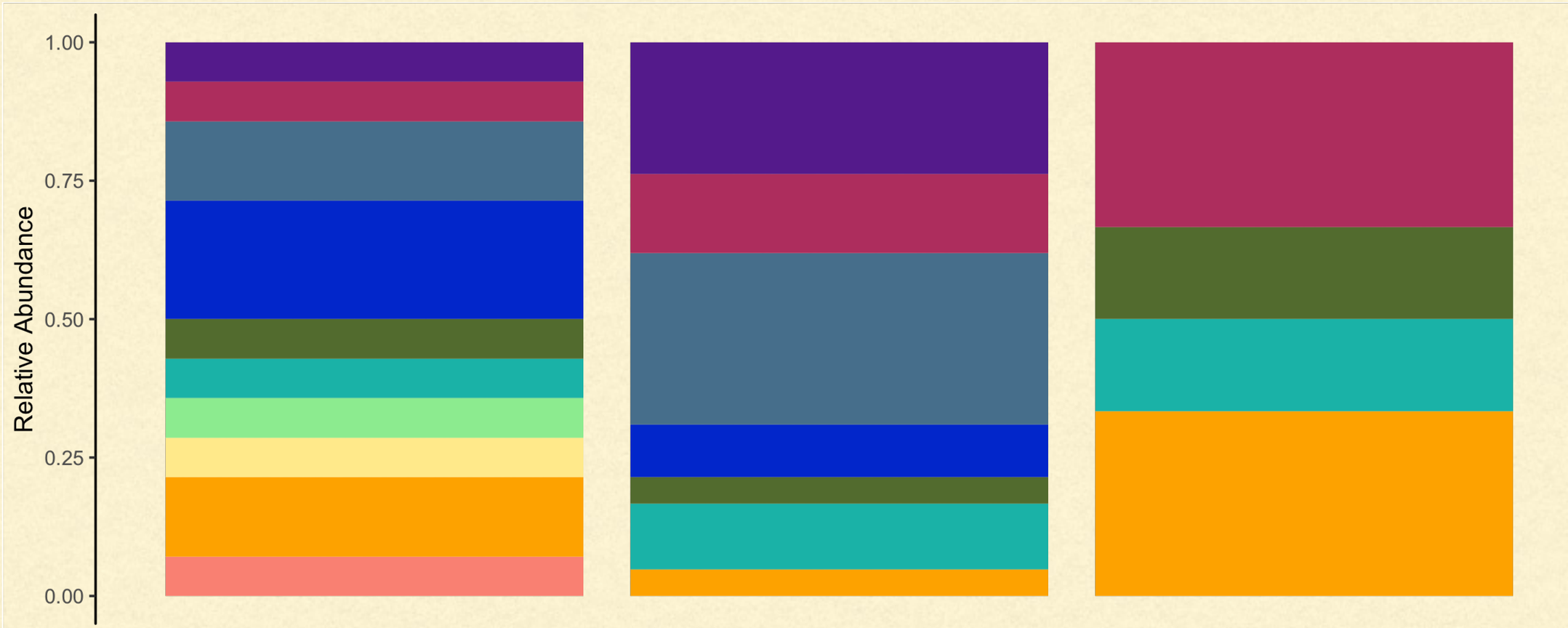

taxonomic
richness
species
richness
Shannon's E
taxonomic
evenness

YOUR CHOICE


taxonomic
richness
species
richness
Shannon
taxonomic
evenness
Shannon's E

YOUR CHOICE

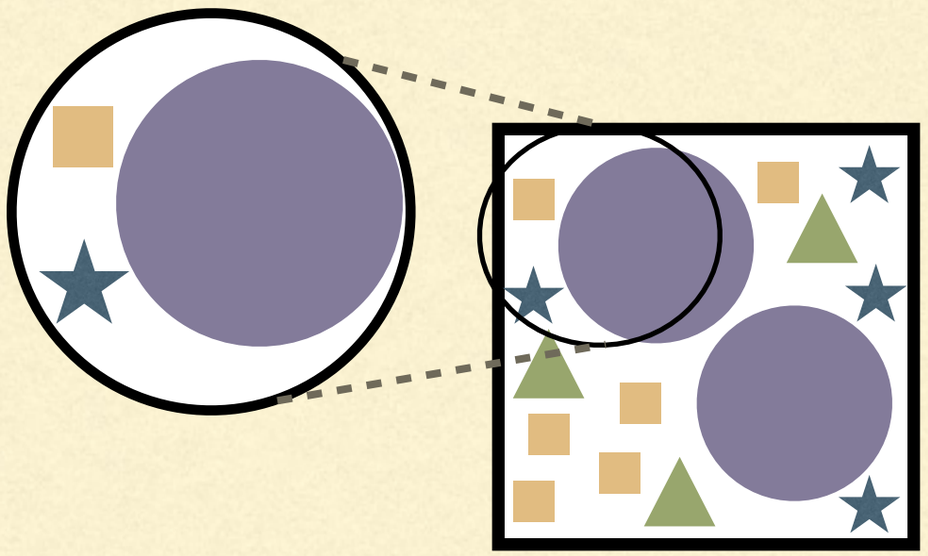
This is a question of *parameter choice:*
Which parameter highlights the differences I care about?



Richness	10	7	4
Shannon	2.21	1.75	1.33
Evenness	0.96	0.90	0.96
Simpson's	0.88	0.80	0.72

THE PROBLEM

- In practice, we don't observe the entire community, just a sample from it
  - we don't know C or $p_1, p_2, \ldots, p_C$



- **We need to *estimate* them using the data we collected**

naive

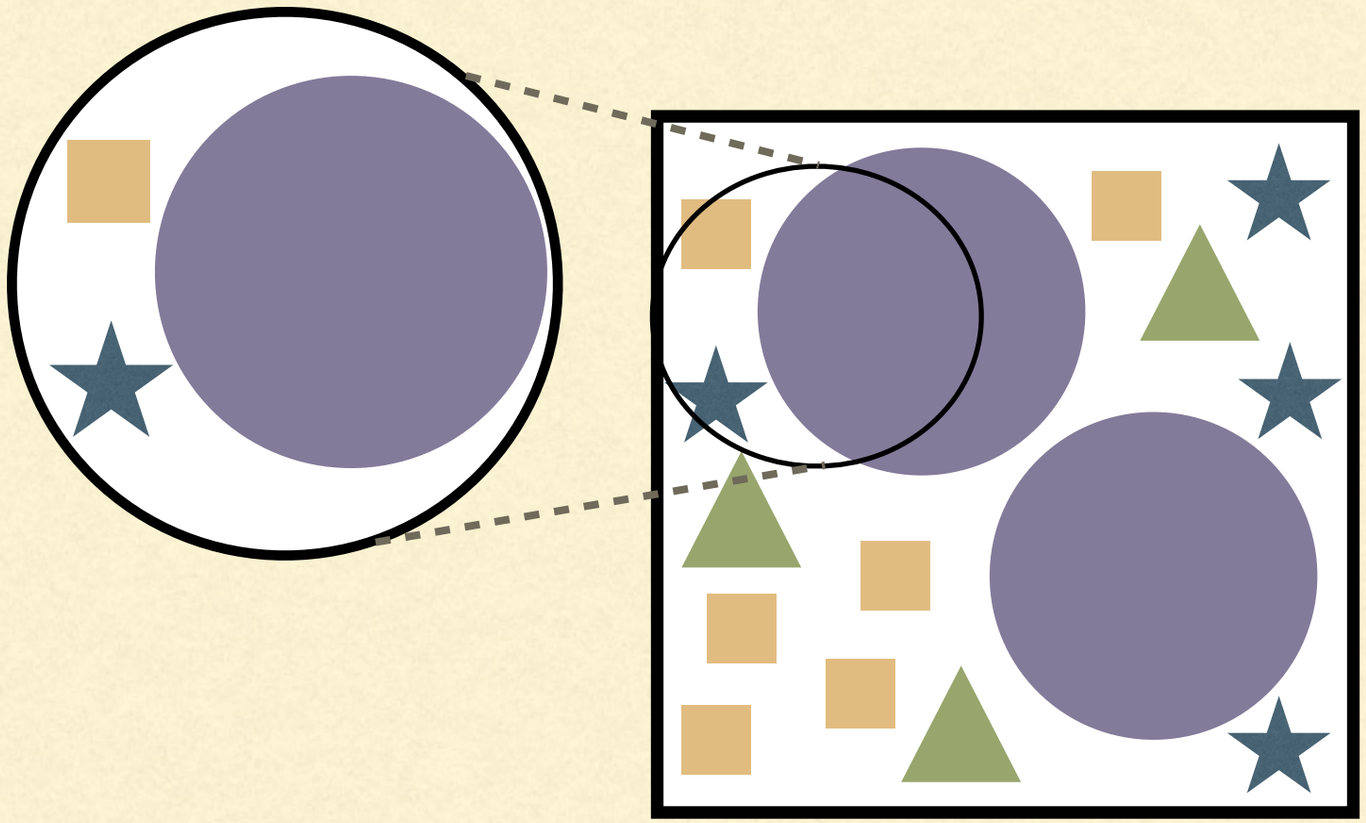
THE "~~CLASSICAL~~" APPROACH

- Substitute the observed abundances $\hat{p}_1, \ldots, \hat{p}_c$ for the unknown, true abundances $p_1, p_2, \ldots, p_c$ and pretend nothing happened
  - e.g. Estimate the richness with: $c = \#\{i : \hat{p}_i \neq 0\}$
  - e.g. Estimate the Simpsons index: $\sum_{i=1}^{c} \hat{p}_i^2$

ONE PROBLEM (OF MANY)

- Species richness: plug-in estimate *underestimates*
- Simpson: estimate *overestimates*

- ~~Need new indices~~
- Need new estimators



HOW TO FIX

- Two things are wrong here:
  - bias (under/overestimation)
  - variance (how big are the error bars — you'll never be exactly right)

SPECIES RICHNESS

- The "species problem": how many species were missing from the sample

- Idea

  - If many rare species in sample, likely there are many missing species

  - If few rare species in sample, likely there are few missing species

  - Use data on rare species to predict # missing species

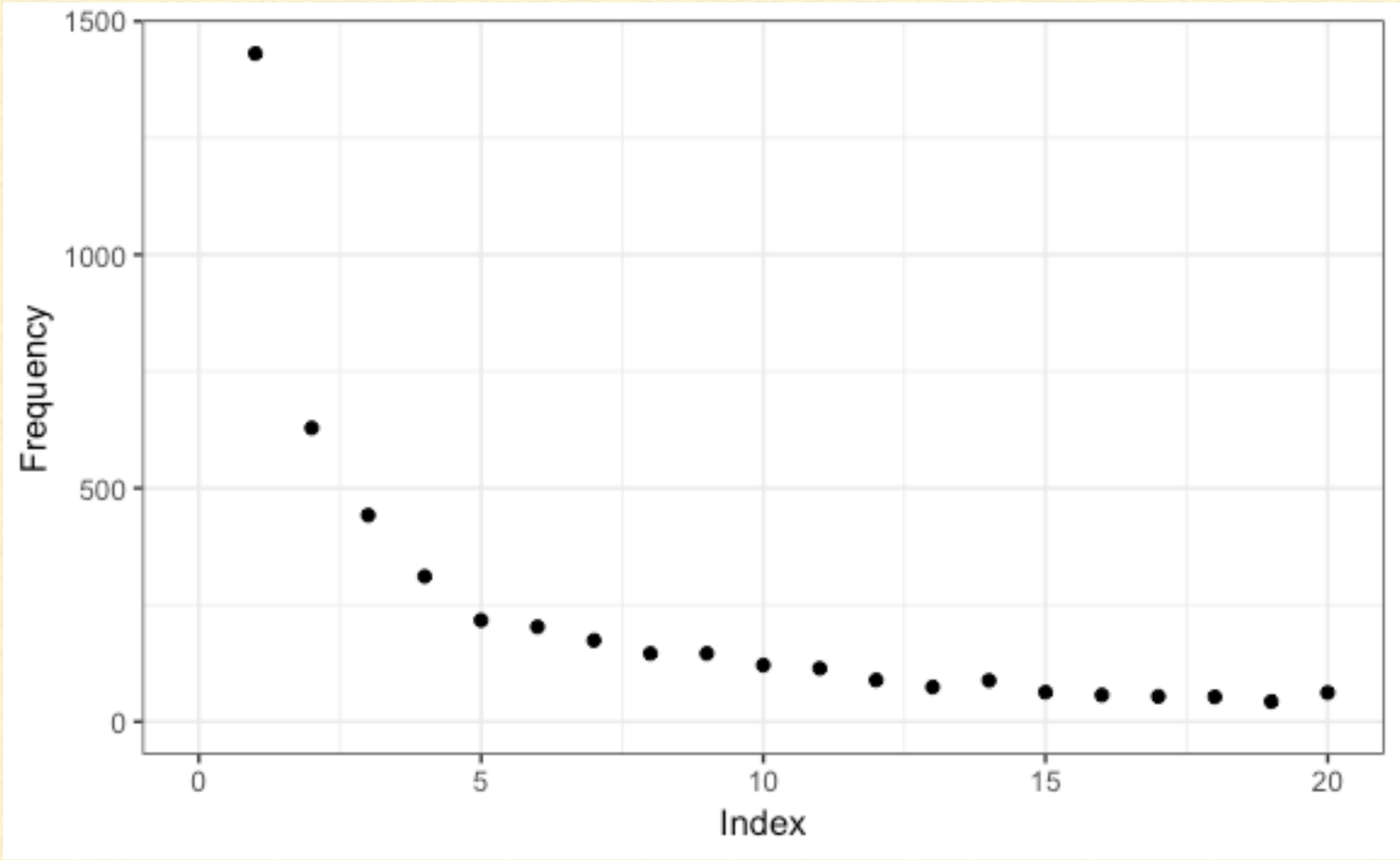
SPECIES RICHNESS ESTIMATION

- The necessary data for richness is the **frequency counts**
- f_j = number of species observed j times
- f_1 = singletons,
- f_2 = doubletons, …
- *e.g. 1431 strains observed once*

```
> library(phyloseq)
> library(magrittr)
> library(breakaway)
> data("GlobalPatterns")
> GlobalPatterns %>%
+   otu_table %>%
+   build_frequency_count_tables %>%
+   head(1)
$CL3
      Index Frequency
 [1,]     1      1431
 [2,]     2       629
 [3,]     3       442
 [4,]     4       311
 [5,]     5       217
 [6,]     6       203
 [7,]     7       174
 [8,]     8       146
 [9,]     9       146
[10,]    10       121
[11,]    11       114
[12,]    12        89
[13,]    13        74
[14,]    14        88
```

SPECIES RICHNESS ESTIMATION

- Idea: extend the pattern in $f_1, f_2, f_3 \ldots$ to f_0



- Rare taxa are most informative for missing taxa

SPECIES RICHNESS ESTIMATION

- Good options
  - breakaway::breakaway() - Kemp models
  - breakaway::chao_bunge() - Negative binomial model
  - breakaway::objective_bayes_*() - mixed Poisson
  - CatchAll - mixed Poisson

- Bad options
  - anything involving rarefaction
  - QIIME2: chao1; scikitbio…
  - R:vegan::…

SPECIES RICHNESS ESTIMATION

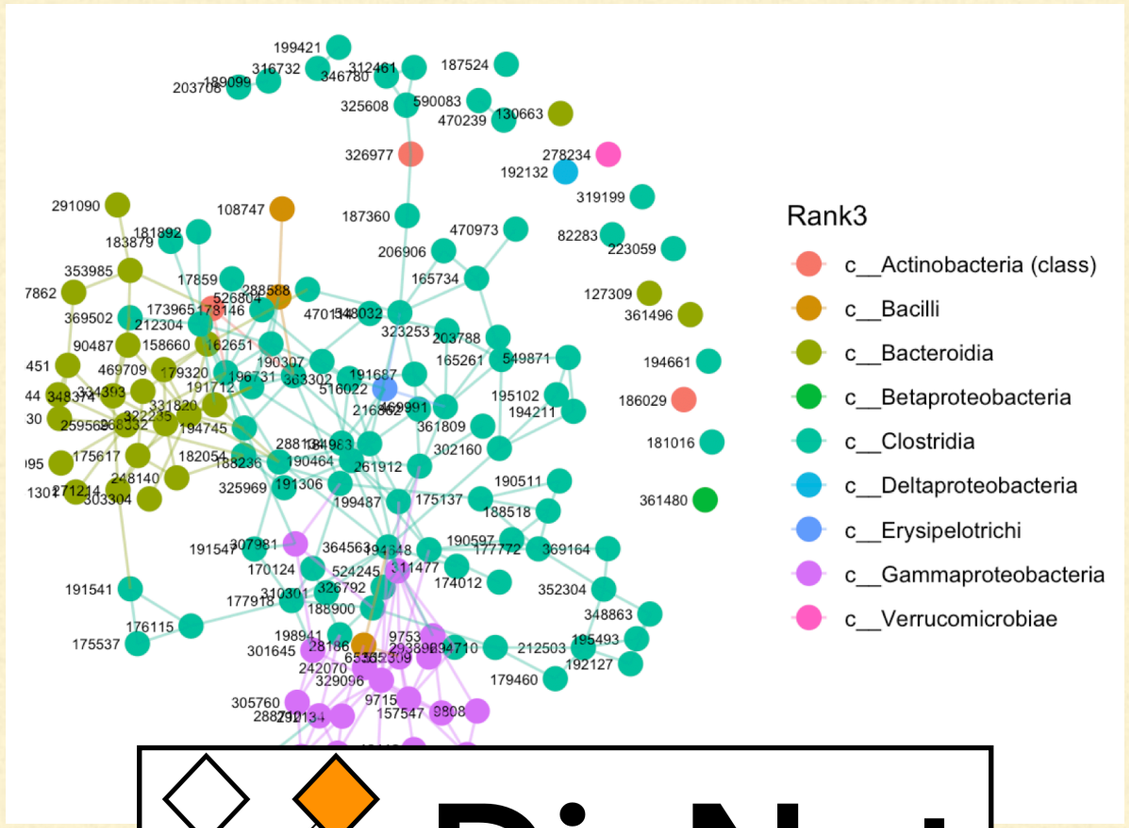
- "Chao1 diversity index" is not an index — it's an estimate of species richness, and it's based on the questionable assumption that

all species have the same abundance

- Large negative bias; very high variance
- Should not be used

ALPHA DIVERSITY: SHANNON & SIMPSON

- Slightly different approach:
  - Share strength across multiple samples to estimate C and $p_1, p_2, \ldots, p_C$, then use network models to get variance



github.com/adw96/DivNet

DivNet

BIAS AND DIVERSITY

- Alternative approach that I loathe: rarefaction
- Idea:
  - Discover more diversity with more sequencing
  - Can't directly compare samples with different depths
  - Randomly throw away reads until all samples have same depth

BIAS AND DIVERSITY

- Alternative approach that I loathe: rarefaction
- Idea:
  - Discover more diversity with more sequencing
  - Can't directly compare samples with different depths
  - Randomly throw away reads until all samples have same depth
- Better idea: **Statistical estimation that accounts for different sequencing depths!**

BIAS AND DIVERSITY

- Alternative approach that I loathe: rarefaction

PLOS | COMPUTATIONAL BIOLOGY

BROWSE PUBLISH ABOUT

OPEN ACCESS PEER-REVIEWED

RESEARCH ARTICLE

Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible

Paul J. McMurdie, Susan Holmes

Published: April 3, 2014 • https://doi.org/10.1371/journal.pcbi.1003531

epth

- Better idea: **Statistical estimation that accounts for different sequencing depths!**

BIAS AND DIVERSITY

- Alternative approa

PLOS | COMPUTATIO BIOLOGY

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Normalization and microbial differential abundance strategies depend upon data characteristics

Sophie Weiss, Zhenjiang Zech Xu, Shyamal Peddada, Amnon Amir, Kyle Bittinger, Antonio Gonzalez, Catherine Lozupone, Jesse R. Zaneveld, Yoshiki Vázquez-Baeza, Amanda Birmingham, Embriette R. Hyde and Rob Knight

Microbiome 2017 **5**:27

https://doi.org/10.1186/s40168-017-0237-y | © The Author(s). 2017

Received: 9 October 2015 | **Accepted:** 27 January 2017 | **Published:** 3 March 2017

- Better idea: **Statis**
  different seque

BIAS AND DIVERSITY

- Alternative approa

DIVERSITY

- Useful summary of (high-dimensional) compositional data… in many settings!

- A change in diversity: a useful *first question*

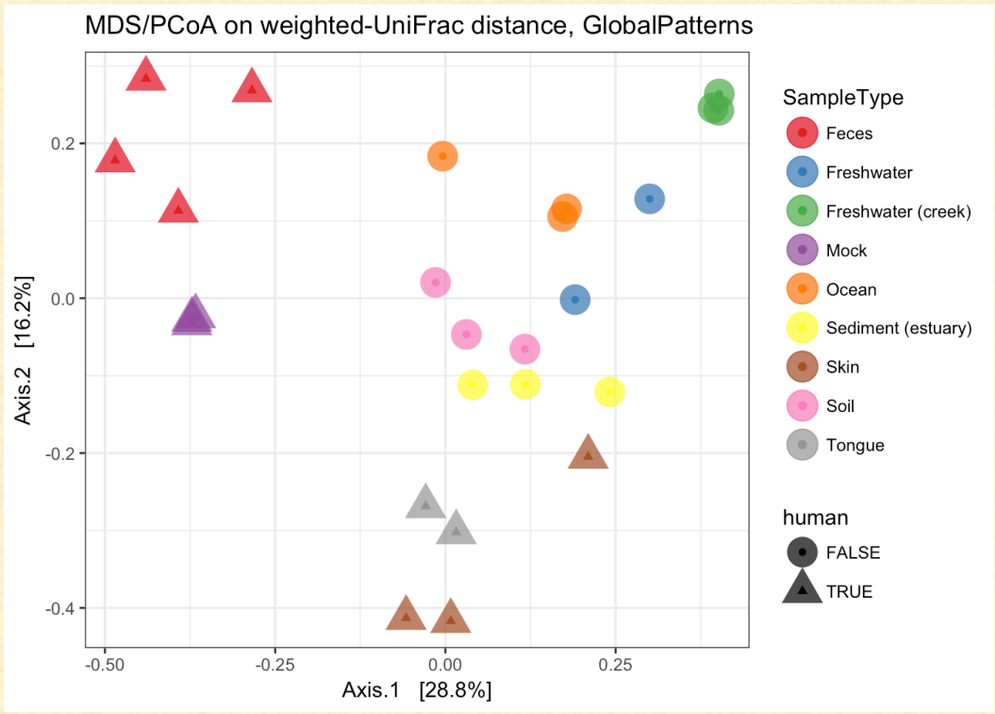
THOUGHTS ON BETA DIVERSITY

BETA DIVERSITY

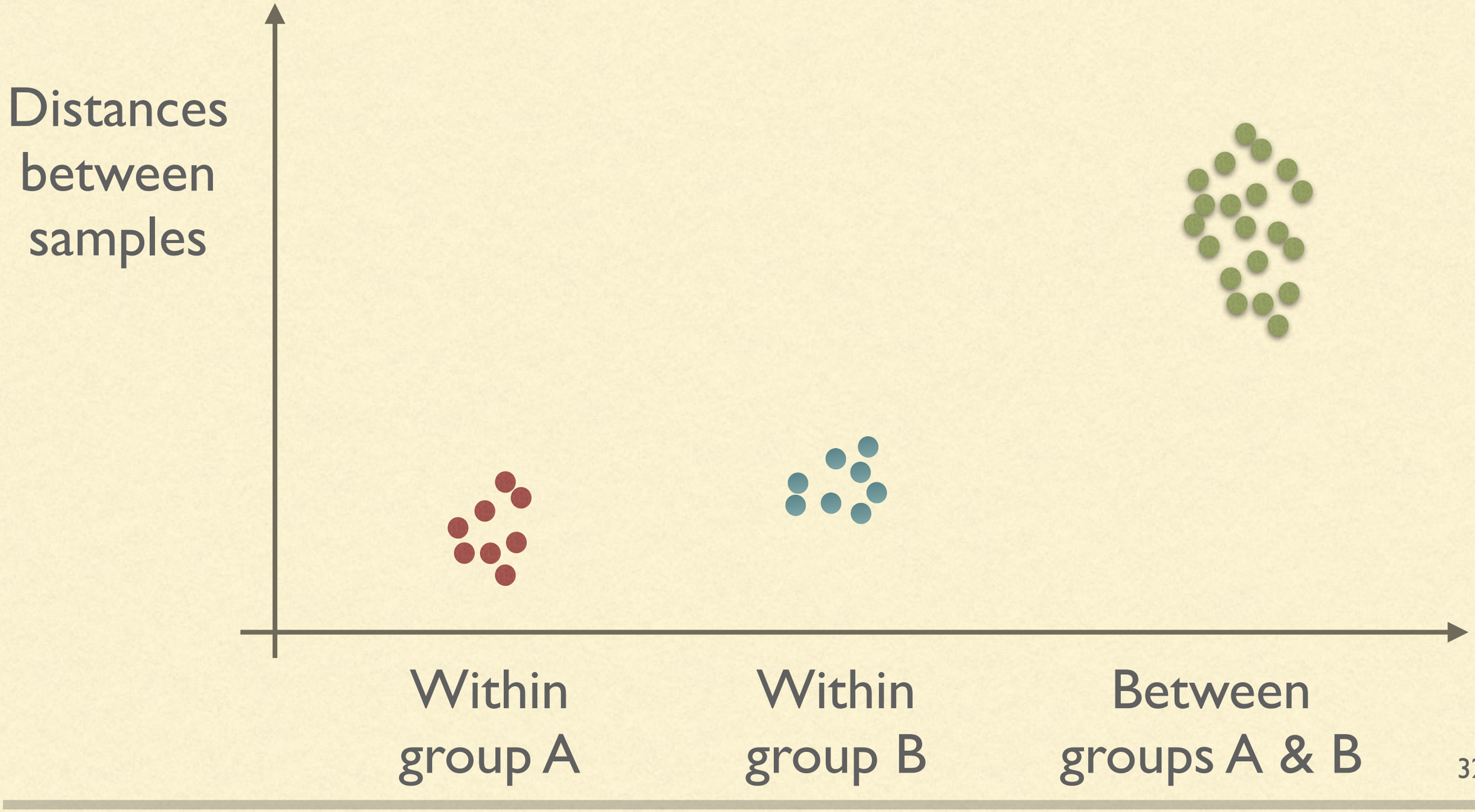
- Community 1: $p_1^{(1)}, p_2^{(1)}, \ldots, p_C^{(1)}$; Community 2: $p_1^{(2)}, p_2^{(2)}, \ldots, p_C^{(2)}$
  - β-diversity parameters are usually distances between compositional vectors
- Bray-Curtis: $\beta_{BC} = 1 - \sum_{i=1}^{C} \min(p_i^{(1)}, p_i^{(2)})$
- Jaccard: $\beta_J =$ % taxa not shared
- UniFrac: Weights phylogeny

DIVERSITY: EXPLORATORY

- Sometimes diversity is analysed as an exploratory tool
  - e.g., ordination
  - *However:* consider not doing an ordination
    - Difficult to interpret (& often over-interpreted)
    - Is there a more informative way to plot your data?



ALTERNATIVE VIZ



A COMPROMISE

- Supplementary Figure 1, not Figure 1?

DIVERSITY: HYPOTHESIS TESTING

- Other times you want to do inference on beta diversities
  - e.g., H_0: dissimilarity within two communities is same as dissimilarity across
  - e.g., H_0: communities A & B have same dissimilarity as communities A & C
- But (yet again): *before running a test, ask "am I sure testing this hypothesis answers a meaningful scientific question?"*

HYPOTHESIS TESTING FOR DIVERSITY

- Common approach: PERMANOVA
- Best solution = ask *"do I really want to do this test?"*
- Better solution = use error bars
  - breakaway::betta(); DivNet::testDiversity
- (Bad solution = rarefy)

ACCESSING `DIVERSITY` LAB

1. Go to schedule on Wiki to Thursday morning, click on "Labs"

2. Copy the command under the lab we're working on

diversity lab:

```
download.file("https://raw.githubusercontent.com/statdivlab/stamps2023/main/labs/diversity-lab/diversity-lab
```

3. Run this command in your RStudio Server console

```
> download.file("https://raw.githubusercontent.com/statdivlab/stamps2023/main/labs
  /diversity-lab/diversity-lab.R", "diversity-lab.R")
```

EXPERIMENTAL DESIGN

The first rule of experimental design is there are no rules.
The second rule of experimental design is that you probably want more data.

~~PRINCIPLES OF EXPERIMENTAL DESIGN~~

- A random smattering of topics in experimental design
  - Randomization
  - Power
  - Observational studies
  - Control data
- Happy to discuss!

POWER CALCULATION

The power calculation to rule them all…

A StatDivLab exclusive…

POWER CALCULATION

$$\#\ \text{samples} = \frac{\text{budget (\$)}}{\text{cost per sample (\$ / sample)}}$$

SHOTGUN METAGENOMICS

- A recent shotgun quote (150 bp PE reads):
  - Extraction: $35 per sample
  - Library prep: $36 per sample
  - Sequencing
    - 20M reads: $250 per sample
    - 80M reads: $730 per sample
    - 100M reads: $904 per sample
  - Annotation: $25 per sample
- ~$10k for 30 samples at 20M reads

POWER

- Recall that failing to reject a null hypothesis (large p-value) may be because
  - the null is true, or
  - the null is false, but because we didn't have enough evidence to reject

POWER

- Power = real
  - Probability of rejecting a false null
    - “Finding a true signal”
- Only meaningful for tests that control Type I error

POWER

- Depends on..
  - True effect size (e.g., β_1)
  - Level of significance α
  - Sample size
    - Number of samples
  - Strong assumptions about the data generating process
    - Depending on setting, possibly also sequencing depth
  - The estimator and test you're using
  - …

POWER CURVES

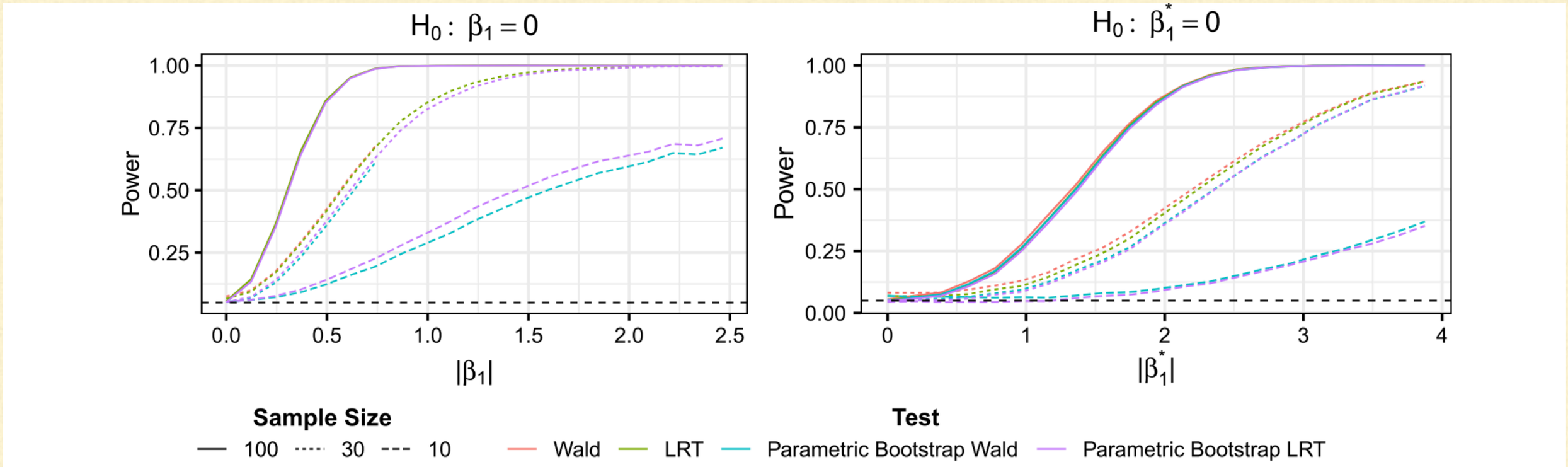


FIG. 3. *Power curves of p-values obtained from the power simulations. Setting* 4 (*left*) *tests* $H_0 : \beta_1 = 0$. *Setting* 5 (*right*) *tests* $H_0 : \beta_1^* = 0$. *A horizontal dashed line is shown at* 0.05. *p-values were obtained using Wald* (*red*), *likelihood ratio* (*green*), *parametric bootstrap Wald* (*blue*) *and parametric bootstrap likelihood ratio* (*purple*) *tests. Sample sizes used were* 10 (*dashed*), 30 (*dotted*) *and* 100 (*solid*).

POWER CALCULATIONS

- Power calculations = fiction
- Often expected in grant applications
- In general, meaningless in ‘omics studies
  - Unless you have a single parameter of interest, power calculations are a furfy*

*A furphy is Australian slang for an erroneous or improbable story that is claimed to be factual.

DESIGNS

- Yesterday predictor of interest
- Ideal: randomize your predictor of interest to your ‘biomes
  - Breaks any possible association between outcome and other covariates
  - Confounding is impossible
- Not always possible
  - e.g., observational studies, practical impossibilities…

OBSERVATIONAL STUDIES

- Cross-sectional
  - Allows you to make comparisons *across groups*
    - outcome ~ group
- Longitudinal
  - Allows you to make comparisons within individuals *across groups*
  - outcome ~ group | subject (with structured errors)

OBSERVATIONAL STUDIES

- There exists unhelpful rhetoric in the field about "longitudinal being better"
  - Effect sizes *within* individuals may be larger than effect sizes *across* groups…
  - … but these are *different* parameters 🍎🍊◼️🧀
- Cost also a consideration

TANK, CAGE EFFECTS

- If working with animal models, want to deconvolve housing & treatment as much as possible
  - Some Group A mice & some Group B mice in all cages is vastly preferable to all treatment mice in the same cage & all control mice in the same cage
  - Lots of cages & fewer mice per cage >> lots of mice in a cage
  - Sometimes this is impossible… ideally seek multiple cages per treatment group (to understand inter-cage variation)

BATCH EFFECTS

- If you are sequencing/sampling in batches, try to *balance* your batches across your other covariates
  - If you can't balance, *randomize*

IF YOU HAVE A CHOICE…

- If you have control over your design (not everyone does!)
- Take the time to simulate data under various designs and see which have the greater power
- *Much* easier to do this in R than IRL!

MOCKS

- If you contract a core for your sequencing…
  - Encourage them to sequence mock communities alongside all samples
- If you work at a core…
  - Please consider adding in system-specific mock communities in every run, and *sharing this data with your clients*

BLANKS

- Useful for detecting contamination
  - Ben says: 4+ useful for *decontam*
  - Cost: 4 additional extractions

REPLICATES

- Historically, variance prioritized over accuracy
- Good to know that your data generating process doesn't *just* produce noise
- If I can add technical replicates for free, yes!
- If I have to decide between biological replicates and technical replicates, *I will choose biological replicates* over technical replicates *almost always*
- BUT there may be settings where technical replicates are helpful
  - e.g., protocol optimization…

QUESTIONS ON EXPERIMENTAL DESIGN

CLOSING THOUGHTS

DIVERSITY

- If you really care about diversity, I recommend using
  - breakaway for species richness
  - DivNet for Shannon/Simpson diversity
  - ~~DivNet for weighted UniFrac/Bray-Curtis/Aitchison asking yourself why you care about β-diversity~~ Thinking creatively about how to make the comparisons that you care about

WELL-PLACING YOUR WORRY

- I discourage you from worrying about…
  - Is data compositional?
  - Where to rarefy to?
  - Which beta diversity metric?
- I encourage you to worry about…
  - Am I analyzing something I care about?
  - Can I estimate what I care about?
  - Is sequencing the technology that I need to answer my question?

AMY'S RECAP ON STATISTICAL PRACTICE

- Many current conventions for statistical analysis in science are inherently unscientific (yes, this is unfair!)
- I encourage you to
  - know what your hypothesis tests are testing: what is the parameter, model, null hypothesis…
  - not consider "p < [favourite number]" a benchmark for publication!

WHAT ELSE CAN WE DO?

- Be scientific
  - "What else could have driven this result?"
  - Corroborate your findings using multiple approaches
    - e.g. multiple HTS technologies, qPCR, explore literature with consideration of their methods, *in silico* modelling…

WHAT ELSE CAN WE DO?

- Report estimates, confidence intervals, and p-/q-values
- Understand your methods
- 🐤 test
- Plot your data & publish your figures, not (just) p-values
- Ask the developers!

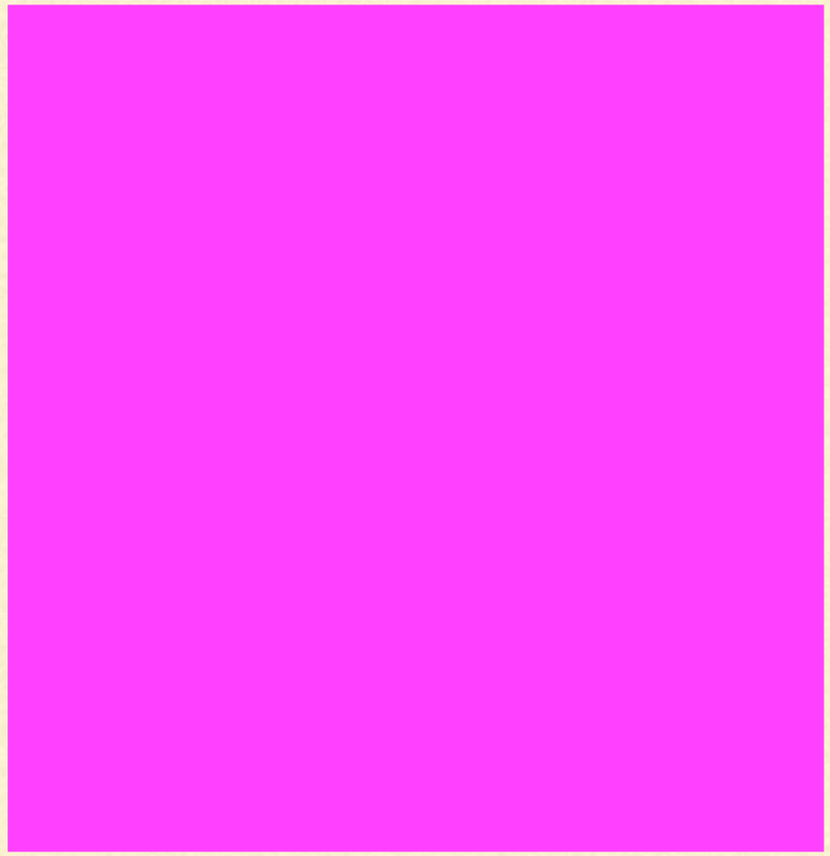
WHAT ELSE CAN WE DO?

- Be honest
  - Keep all analyses that you ran, not just the final one
- Write down all of the hypotheses that you care about
  - Before doing the experiment, before doing the analysis
- Your university might house a statistician; try to involve them...
  - ...in the entire process!

CLOSING THOUGHTS

- Methods for modeling microbiome data is a fast-moving field, and new methods are constantly emerging
- Talk to lots of people
  - "What's the biggest limitation of this?"
- Stay critical but open-minded

A COLLECTION OF KNOWLEDGE… OPINIONS… INSTINCTS… FROM



Statistical Diversity Lab @ University of Washington

Amy Willis — @AmyDWillis — Associate Professor

Sarah Teichman — @sarah_teichman — PhD Candidate

Photo credit: T.D. Berry, Whitman lab, UW Madison

OPEN LAB TIME

- LM
- ABUNDANCE
- DIVERSITY