

Nitrapyrin effects on soil microbial community structure, composition, diversity & function

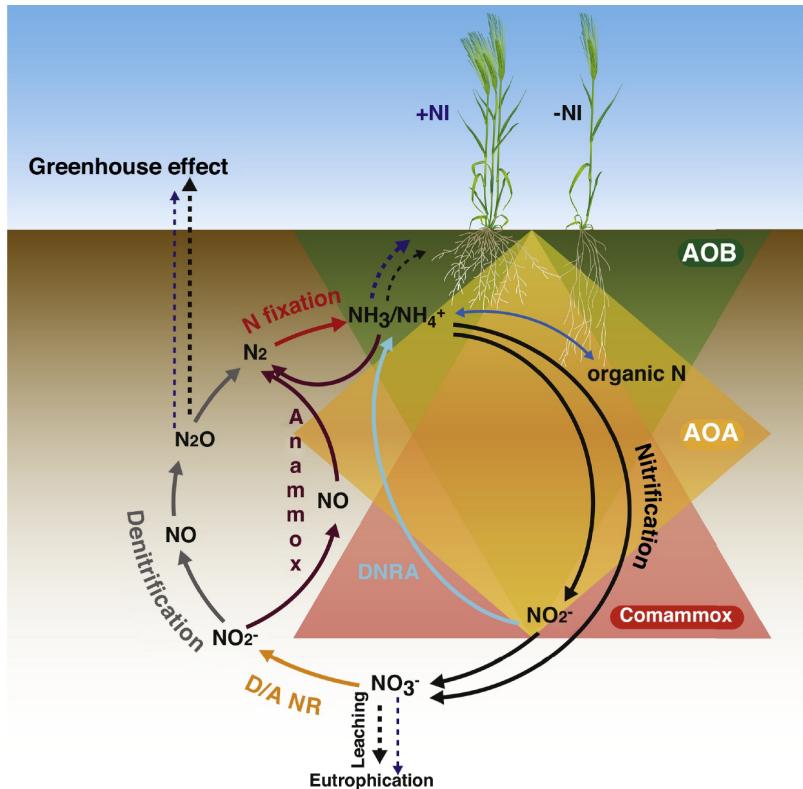
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N in agriculture & recap of N-cycle

- Nitrogen (N) most limiting nutrient for plant growth
- To counteract this problem, N-containing fertilizers are commonly applied
- Negative effects: atmospheric and groundwater pollution through nitrate leaching and volatilization
- Linked to soil microbial processes (nitrification and denitrification)

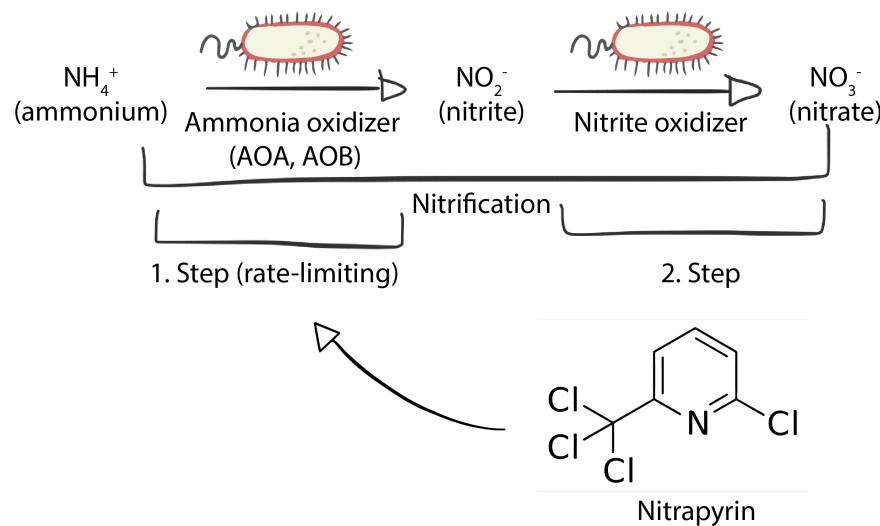


Current Opinion in Biotechnology

Figure source: Nitrification in agricultural soils: impact, actors and mitigation (Beeckmann et al., 2018)

Nitrification & inhibitors

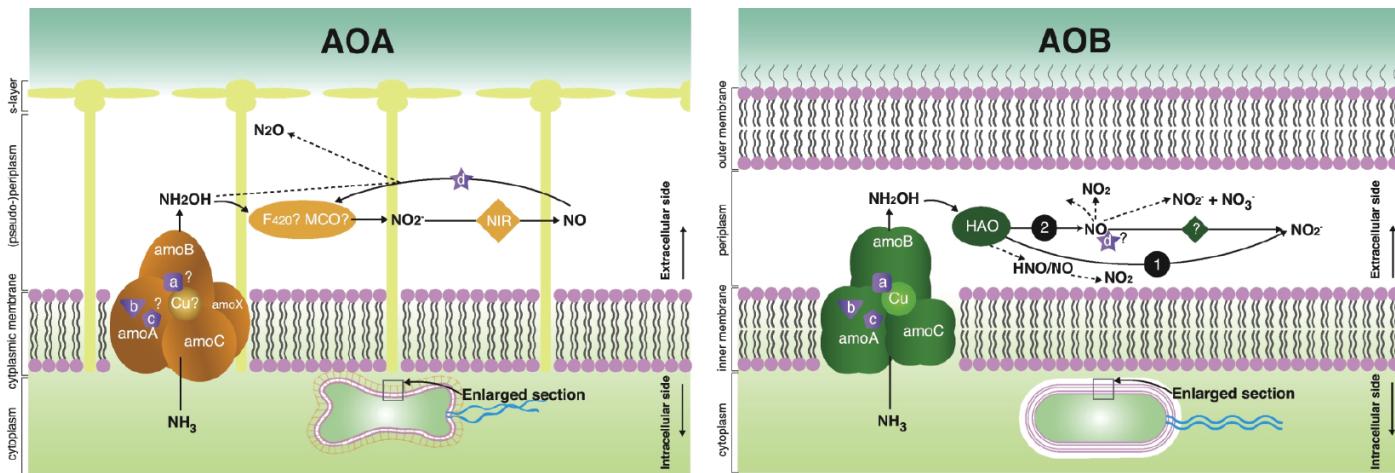
- First step of nitrification carried out by ammonia oxidizing bacteria and archaea (AOB & AOA)
- Keystone species* ubiquitously found in soil, AOA more abundant than AOB
- Nitrification inhibitors (e.g. Nitrapyrin) commonly used in combination with N-fertilizers to counteract adverse effects while increasing soil N retention and crop yields



*A keystone species is a species which has a disproportionately large effect on its natural environment relative to its abundance (source: Wikipedia)

Nitrapyrin mode of action

- One of the most common nitrification inhibitors
- Delays nitrification by temporarily deactivating of ammonia monooxygenase (AMO) - enzyme responsible for ammonia oxidation
- a subunit of AMO is encoded by the amoA gene, which is homologous in AOA and AOB
- Both AOA and AOB could be inhibited by nitrapyrin

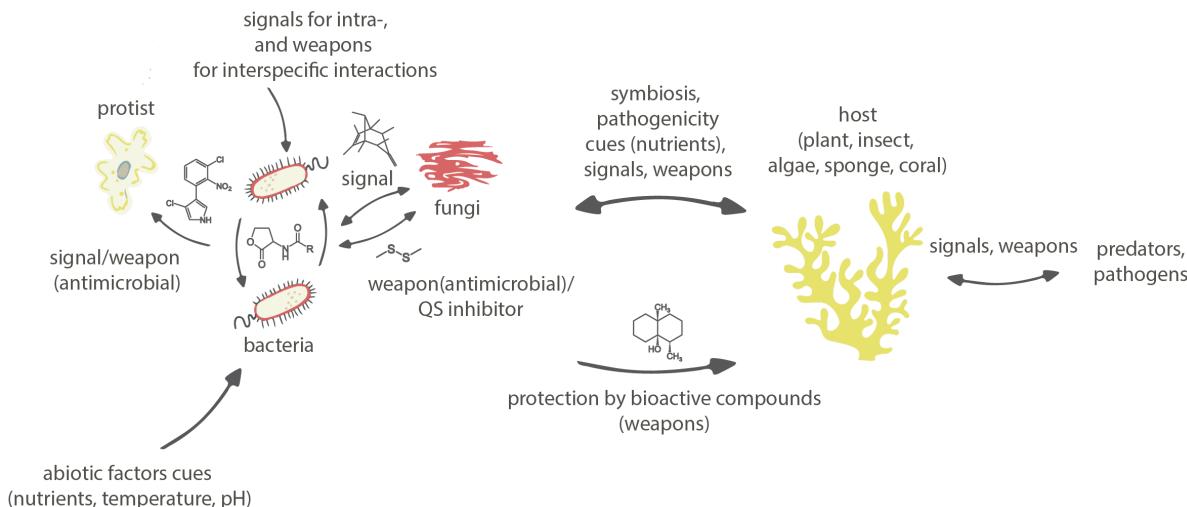


What are the effects of nitrapyrin on AOA and AOB?

- Studies found varying effects on AOA and AOB gene abundance (*amoA* gene), AOA:AOB ratio and nitrification rates
- Suggest that nitrapyrin shifts the AOA:AOB ratio, thus inhibiting nitrification at different rates depending on contribution of AOA and AOB to nitrification
- BUT: not known how nitrapyrin affects overall microbial community structure, composition, diversity and functions, and how this varies through the growing season

Nitrapyrin & volatile organic compounds (VOCs)

- VOCs are small compounds (up to C₂₀) with low molecular mass (100–500 Daltons), high vapour pressure, low boiling point and a lipophilic moiety ([Schmidt et al., 2015](#))
- Key metabolites in below-ground microbe and plant-microbe interactions & influence important biogeochemical processes (e.g. N-cycle) ([Schmidt et al., 2019](#), [Schulz-Bohm et al., 2017](#), [De la Porte et al., 2020](#))
- Some VOCs (especially monoterpenes) inhibit nitrification (target: AMO)



Hypothesis & Objectives

Hypothesis: Nitrappyrin-induced shifts in the abundance of the keystone groups AOA and AOB affect the overall microbial community structure, composition, diversity and functions

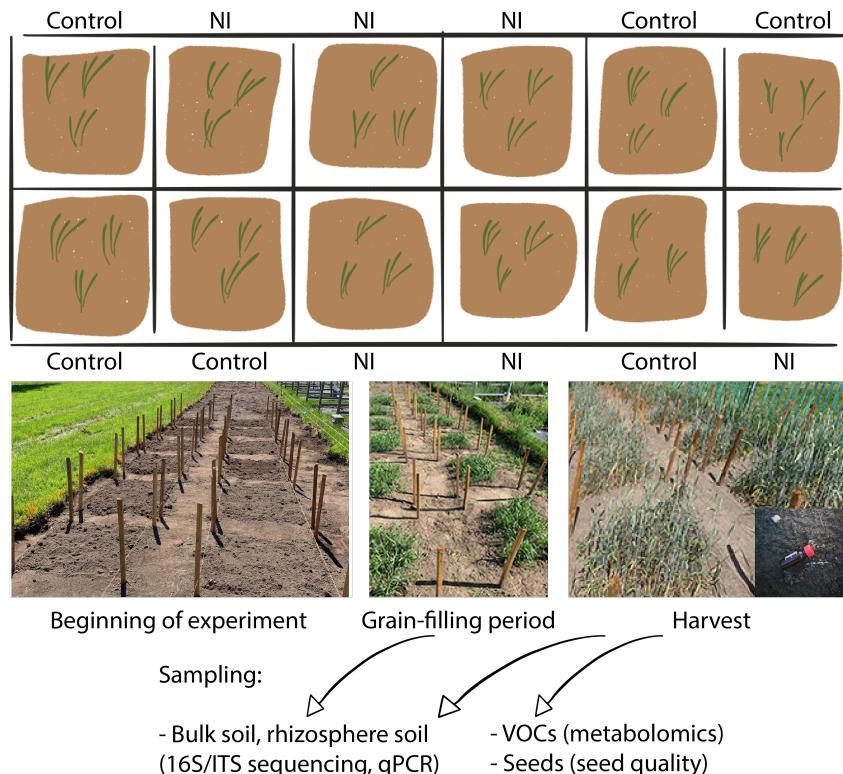
Objectives: Explore the effects of nitrappyrin applied on field-grown wheat on

- overall microbial community structure, composition and diversity
- abundance of genes encoding for enzymes involved in ammonia oxidation, N-fixation, and denitrification
- microbial VOC emission in the rhizopshere
- the effect of nitrappyrin on the wheat yield and grain quality (tbd)

A field study

Design & setup: Random block design (6 replicates), wheat seeds (AC Walton) sown in 4 rows on each plot

Fertilizer liquid ammonium nitrate (NH_4NO_3) & nitrification inhibitor nitrpyrin (NI) applied twice during the growing season



Data overview

Treatment:

- Control (without application of fertilizer + NI)
- NI (wit application of fertilizer + NI)

Sampling date:

- 2019-07-23 = grain-filling period
- 2019-09-05 = harvest

Compartment:

- Bulk soil
- Rhizosphere soil

Diversity, composition and structure of soil microbial communities

Alpha diversity not affected by NI treatment, but by Date & Compartment

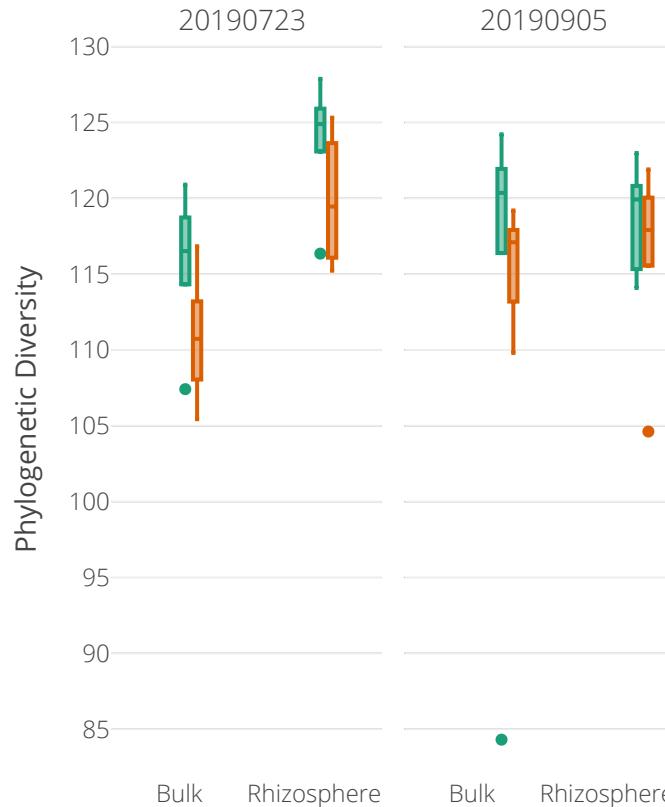
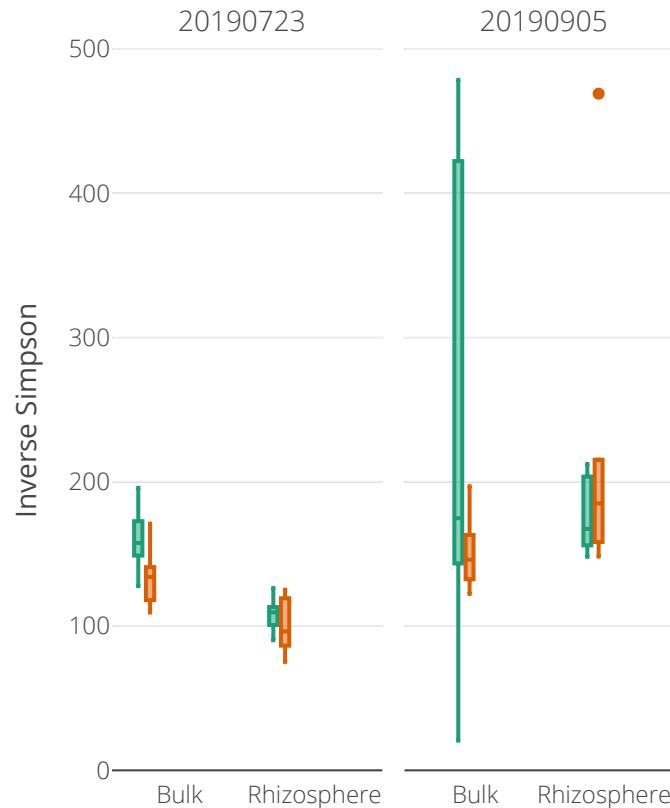
Three-way repeated measures ANOVA of bacterial, archaeal (16S) and fungal (ITS) alpha diversity examined by Shannon index, Inverse Simpson and Faith's PD

	16S								ITS							
	Shannon		Inverse Simpson		PD		Shannon		Inverse Simpson		PD					
	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P
Treatment	0.001	0.971	0.555	0.461	1.741	0.195	0.588	0.448	0.658	0.422	0.924	0.342				
Compartment	0.281	0.599	0.612	0.439	7.977	0.007	9.523	0.004	17.086	<0.001	0.308	0.582				
Date	1.175	0.285	0.016	0.003	0.378	0.542	49.577	<0.001	53.389	<0.001	26.062	<0.001				
Treatment × Compartment	0.02	0.888	2.838	0.1	0.121	0.73	1.314	0.258	2.237	0.143	0.74	0.395				
Treatment × Date	0.978	0.329	0	0.997	0.923	0.342	1.492	0.229	0.905	0.347	0.21	0.649				
Compartment × Date	1.552	0.22	1.273	0.266	2.485	0.123	0	0.989	2.586	0.116	1.434	0.238				
Treatment × Compartment × Date	0.082	0.777	1.714	0.198	0.345	0.560	0.119	0.732	1.948	0.171	0.416	0.522				

Time for some interactive
graphs!

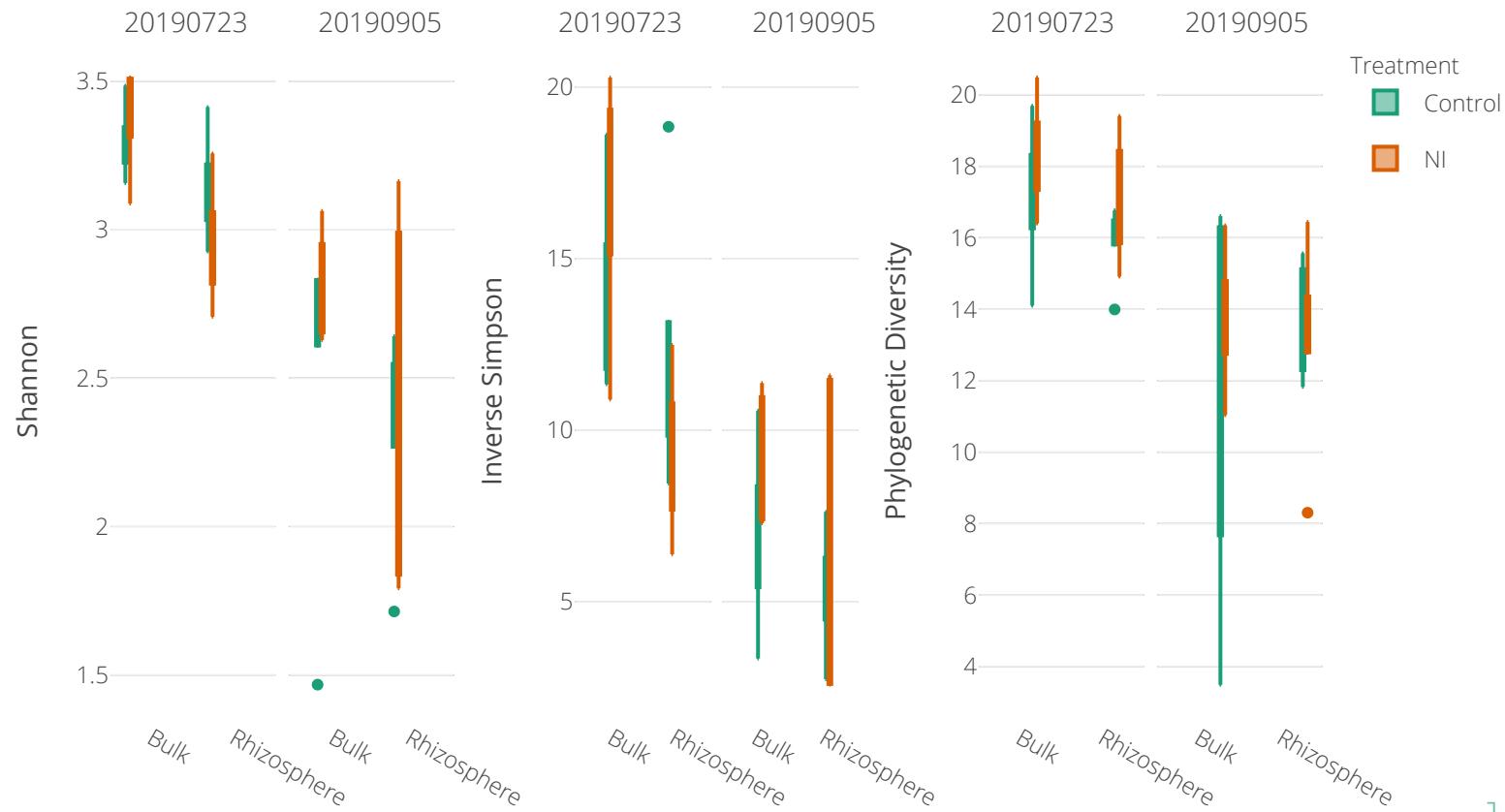
Increased diversity at the second date (Inv Simpson) & lower diversity in bulk soil (PD)

Bacterial & archaeal alpha diversity



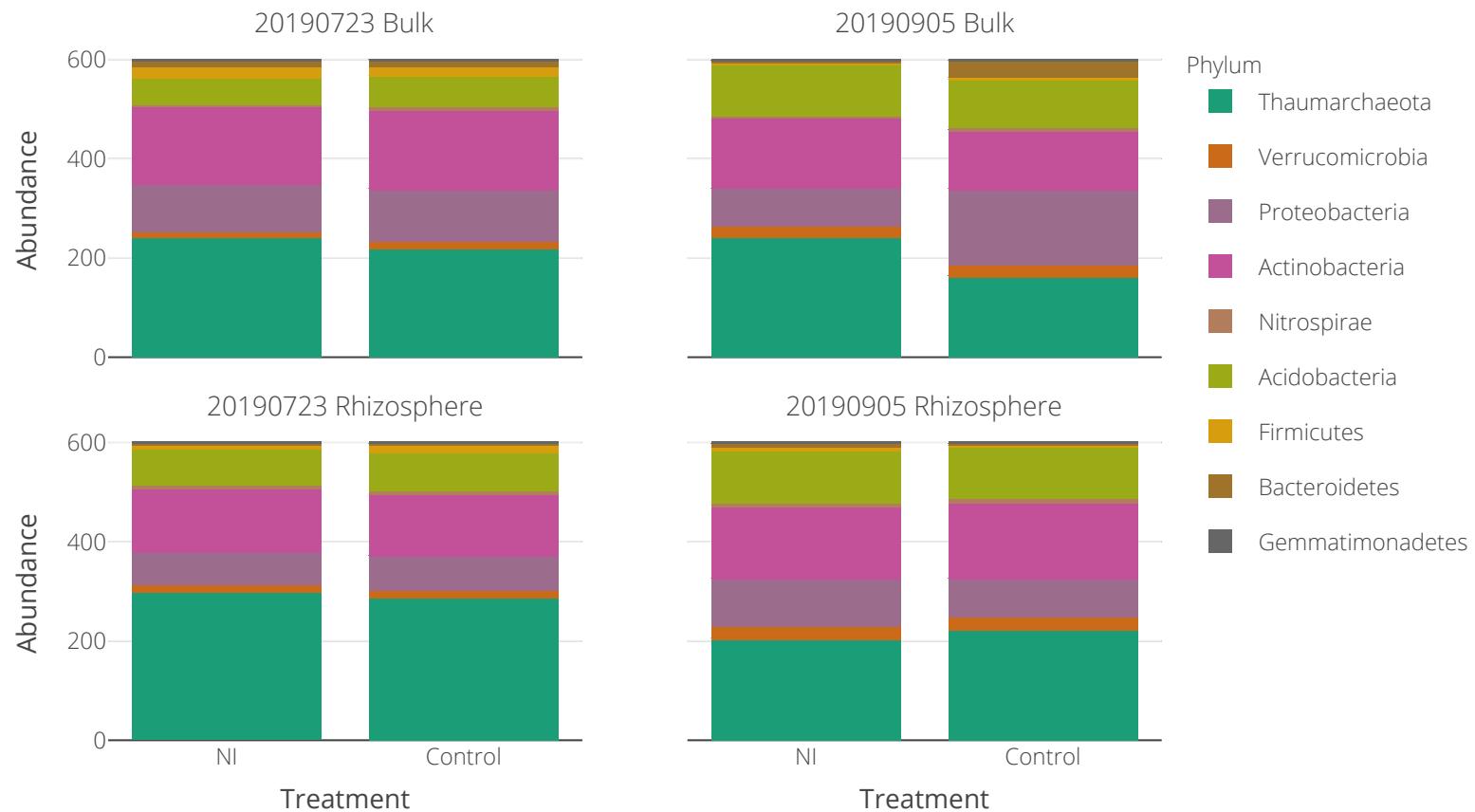
Lower diversity in rhizosphere (Shannon & Inv Simpson), and second date (Shannon, Inv Simpson & PD)

Fungal alpha diversity



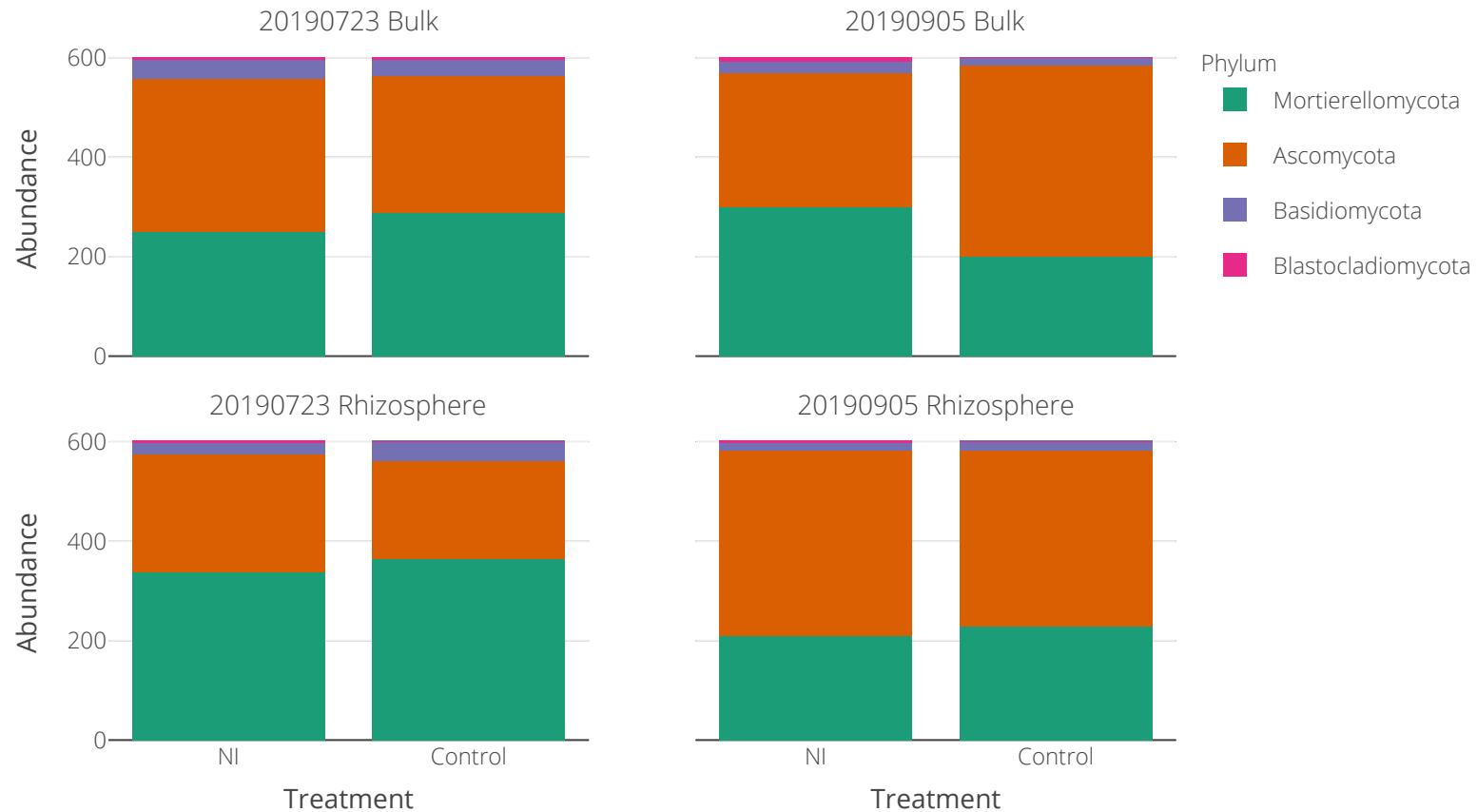
Stacked bar charts are not that boring after all (let's have a look at Thaumarchaeota)

Bacterial and archaeal communities (mean relative abundance >1%)



Most abundant fungal phyum: Mortierellomycota & Ascomycota (have a look at Basidiomycota)

Fungal communities (mean relative abundance >1%)



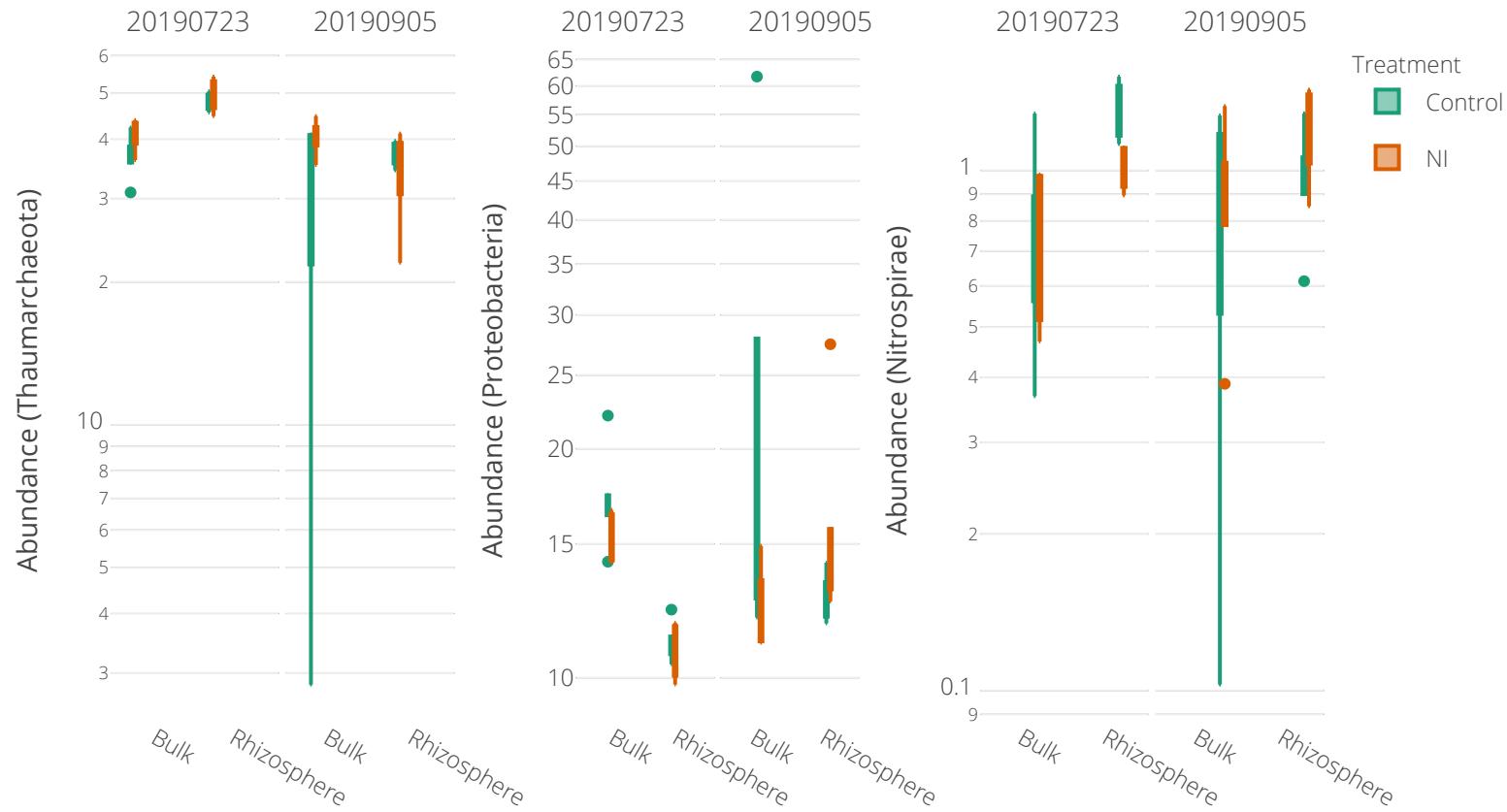
NI treatment affected relative abundance of archaeal phylum Thaumarchaeota, Date & Compartment affected several Phyla

Three-way repeated measures ANOVA of relative abundance of dominant phyla of bacterial & archaeal (16S) communities

16S Phyla	T		C		D		TxC		TxD		CxD		TxCxD	
	F	P	F	P	F	P	F	P	F	P	F	P	F	P
Thaumarchaeota	4.463	0.041	11.899	0.001	23.796	<0.001	5.845	0.02	0.282	0.598	5.301	0.027	3.873	0.056
Verrucomicrobia	0.216	0.645	3.353	0.075	42.408	<0.001	1.456	0.235	2.848	0.099	0.341	0.563	1.353	0.252
Proteobacteria	2.233	0.143	6.288	0.016	2.218	0.144	4.912	0.032	0.917	0.344	0.047	0.829	3.643	0.064
Actinobacteria	0.571	0.454	1.162	0.288	0.019	0.89	0.581	0.45	0.292	0.592	17.476	<0.001	2.934	0.094
Nitrospirae	0.041	0.841	17.094	<0.001	0.067	0.797	0.353	0.556	4.91	0.032	1.626	0.21	0.751	0.391
Acidobacteria	0.314	0.578	4.703	0.036	50.902	<0.001	0.095	0.759	0.838	0.366	1.952	0.17	0.001	0.979
Firmicutes	0.828	0.368	10.89	0.002	75.806	<0.001	2.044	0.161	1.378	0.247	19.477	<0.001	1.766	0.191
Bacteroidetes	1.346	0.253	2.812	0.101	0.548	0.463	2.205	0.145	1.075	0.306	0.127	0.723	1.889	0.177
Gemmatimonadetes	1.605	0.213	0.897	0.349	10.954	0.002	0.323	0.573	0.386	0.538	1.292	0.262	0.817	0.371

NI treatment increased Thaumarchaeota abundance on first sampling date

Relative abundance of Thaumarchaeota, Proteobacteria & Nitrospirae



NI treatment affected relative abundance of archaeal genus *Nitrososphaera*

Three-way repeated measures ANOVA of relative abundance of dominant genera of bacterial & archaeal (16S) communities

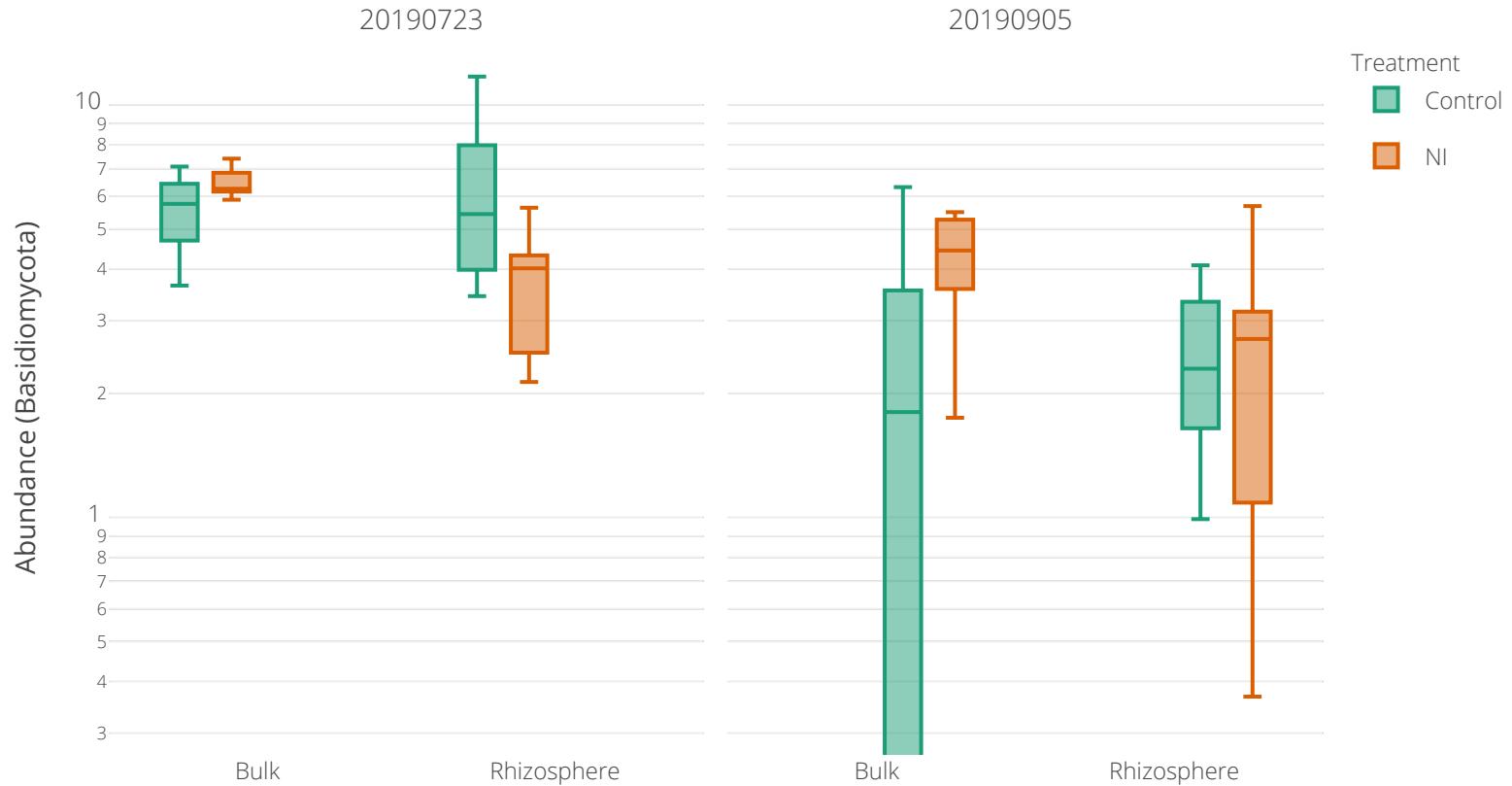
16S Genera	T		C		D		TxC		TxD		CxD		TxCxD	
	F	P	F	P	F	P	F	P	F	P	F	P	F	P
<i>Nitrososphaera</i>	4.463	0.041	11.899	0.001	23.796	<0.001	5.845	0.02	0.282	0.598	5.301	0.027	3.873	0.056
<i>Gaiella</i>	0.46	0.501	0.022	0.883	2.026	0.162	4.697	0.036	0.757	0.39	3.386	0.073	8.954	0.005
<i>Gp6</i>	0.345	0.561	6.855	0.012	40.88	<0.001	0.004	0.95	2.253	0.141	2.912	0.096	0.131	0.719
<i>Solirubrobacter</i>	0.468	0.498	2.183	0.147	0.016	0.899	0.863	0.358	0.202	0.656	20.462	<0.001	4.491	0.04
<i>Gp16</i>	0.067	0.797	2.407	0.129	26.357	<0.001	3.064	0.088	0.067	0.797	0.017	0.897	3.673	0.062
<i>Hyphomicrobium</i>	0.113	0.738	43.124	<0.001	23.043	<0.001	1.307	0.26	3.194	0.081	40.601	<0.001	5.7	0.022
<i>Rhodoplanes</i>	2.332	0.135	11.46	0.002	0.956	0.334	4.643	0.037	0.069	0.794	15.071	<0.001	1.961	0.169
<i>Spartobacteria (gis)</i>	0.648	0.425	0.667	0.419	64.727	<0.001	4.032	0.051	2.008	0.164	1.872	0.179	3.035	0.089
<i>Povalibacter</i>	0.892	0.351	0.115	0.736	7.337	0.01	0.436	0.513	0.054	0.817	0.001	0.974	0.697	0.409
<i>Sphingomonas</i>	0.043	0.836	9.123	0.004	1.466	0.233	3.431	0.071	0.145	0.706	2.996	0.091	4.016	0.052

Interaction effect of NI treatment & Compartment affected relative abundance of fungal phylum Basidiomycota

Three-way repeated measures ANOVA of relative abundance of dominant phyla of fungal (ITS) communities

ITS Phyla	T		C		D		Tx C		Tx D		Cx D		Tx C x D	
	F	P	F	P	F	P	F	P	F	P	F	P	F	P
Mortierellomycota	0.037	0.849	1.505	0.227	12.566	0.001	1.561	0.219	2.924	0.095	7.27	0.01	2.407	0.129
Ascomycota	0.081	0.777	0.897	0.349	17.854	<0.001	2.676	0.11	4.004	0.052	7.177	0.011	2.161	0.149
Basidiomycota	0.044	0.836	2.415	0.128	25.571	<0.001	6.092	0.018	3.15	0.084	0.076	0.784	0.675	0.416
Blastocladiomycota	1.623	0.21	0.531	0.47	0.564	0.457	0.378	0.542	0.748	0.392	0.995	0.325	1.235	0.273

Relative abundance of Basidiomycota decreased in bulk & rhizosphere in NI treatments



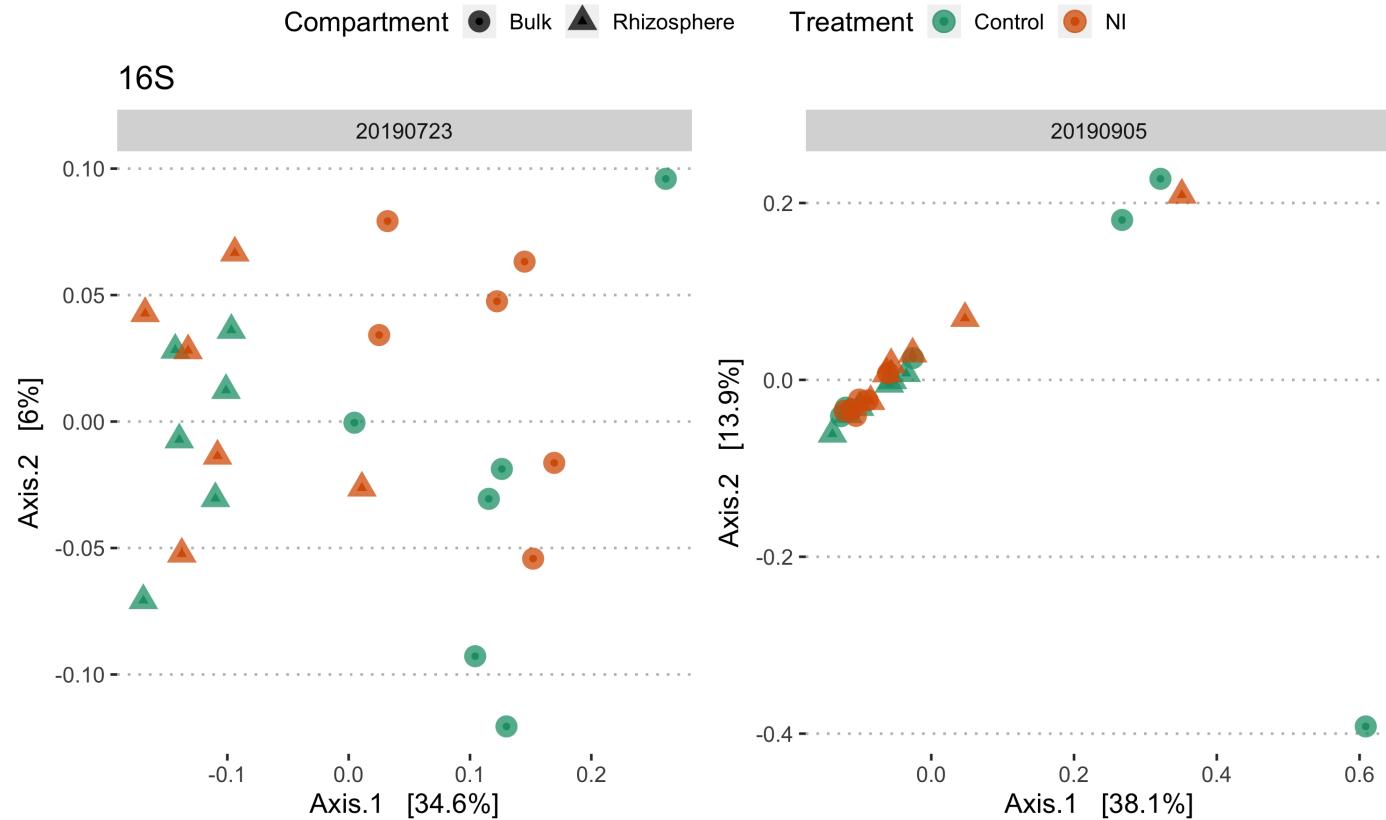
Not NI treatment, but Compartment & Date altered overall structure of bacterial and archaeal & fungal communities

PERMANOVA of bacterial, archaeal (16S) and fungal (ITS) community structure

	16S		ITS	
	R^2	P	R^2	P
Treatment	0.019	0.244	0.016	0.422
Compartment	0.05	0.001	0.035	0.020
Date	0.124	0.001	0.187	0.001
Treatment × Compartment	0.041	0.004	0.031	0.048
Treatment × Date	0.015	0.464	0.016	0.408
Date × Compartment	0.057	0.002	0.033	0.038
Treatment × Date × Compartment	0.036	0.019	0.029	0.076

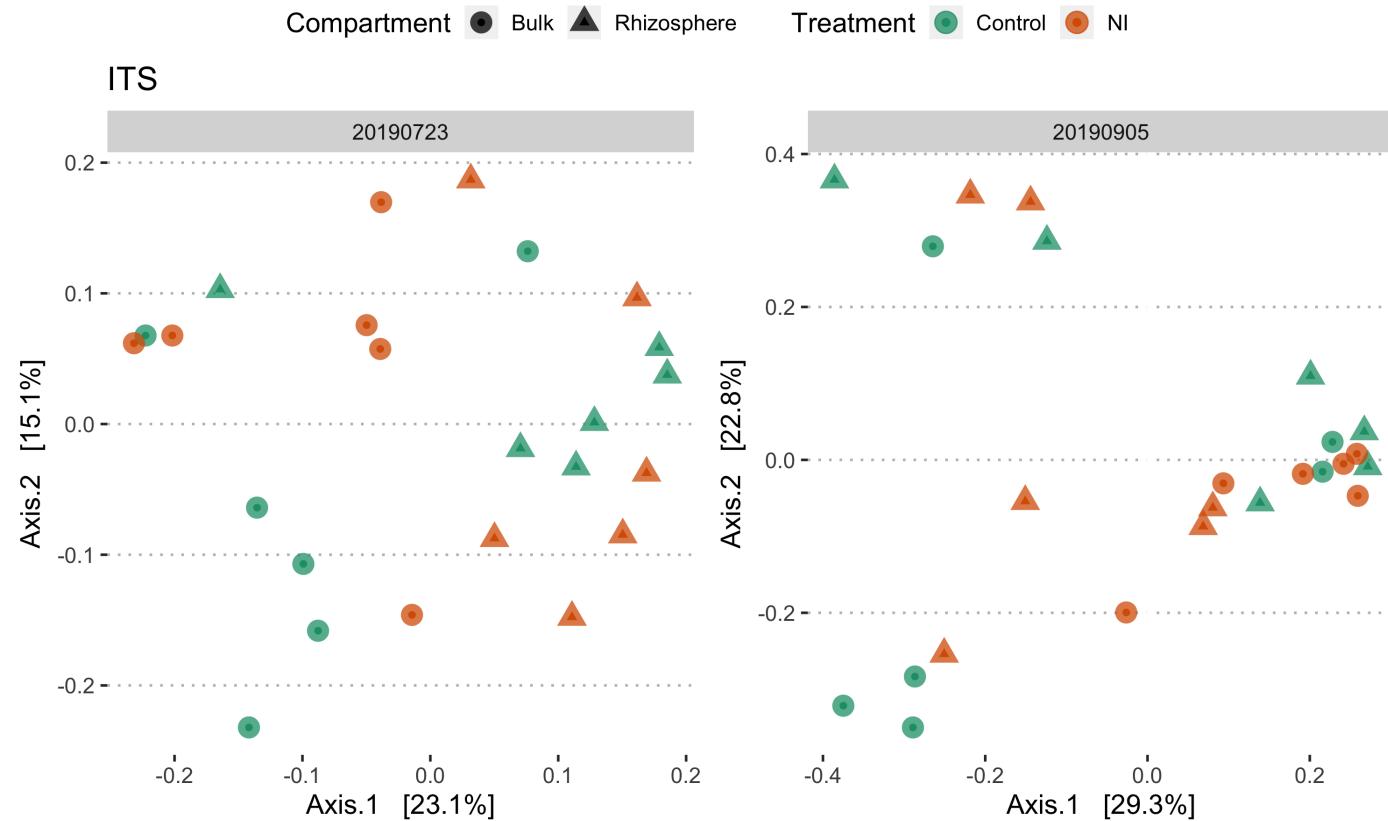
Date separated between rhizosphere and bulk soil (16S)

Principal Coordinates Analysis (PCoA) of the bacterial & archaeal (16S) community composition based on Bray-Curtis dissimilarity



Date separated between rhizosphere and bulk soil (ITS)

Principal Coordinates Analysis (PCoA) of the fungal (ITS) community composition based on Bray-Curtis dissimilarity



Summary (16S&ITS results)

- Nitrapyrin treatment, among strong effect of compartment and sampling date, affected relative abundance of many bacterial, archaeal and fungal taxa (e.g. Proteobacteria, Nitrospirae, Basidiomycota)
 - Proteobacteria contain AOB
 - Nitrospirae (nitrite-oxidizing bacteria often in close association with AOB or AOA)
 - Basidiomycota contain denitrifiers that play key roles in N cycle
- Nitrapyrin had significant effects on expected targets, ammonia-oxidizers
 - AOA expected to increase and AOB decrease due to stronger inhibition of AOB
 - AOA *Nitrososphaera* and Thaumarcheota increased
 - BUT: no observed effect for AOB (below detection limit)

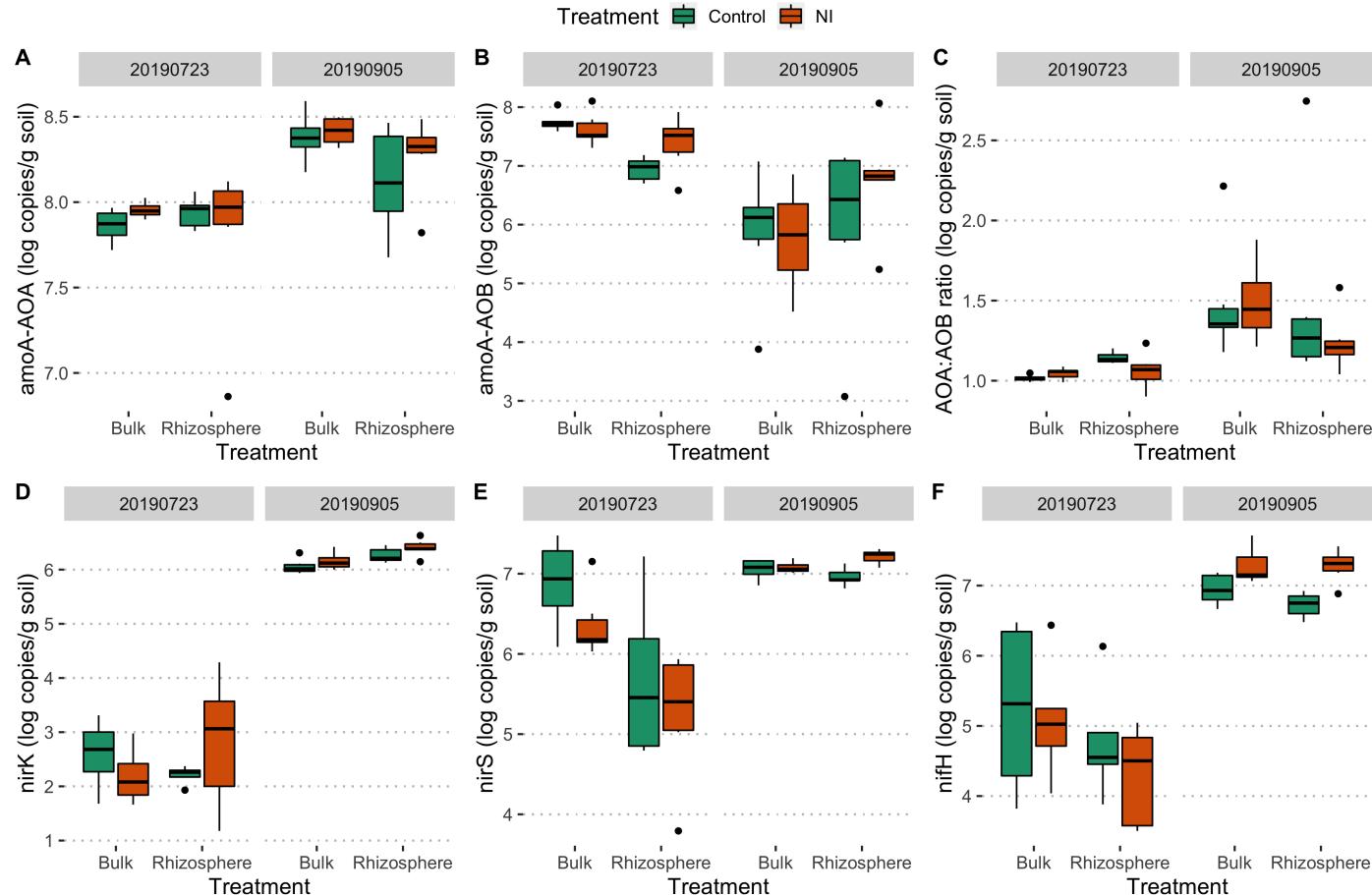
Abundance of functional genes of the N cycle

Abundances of the N-cycling genes not affected by NI treatment, but strongly affected by Date

Kruskal-Wallis test of abundance of functional genes involved in N cycle

	T		C		D		TxC		TxD		CxD		Tx CxD	
	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P
amoA-AOA	1.021	0.312	1.150	0.284	21.716	<0.001	2.205	0.531	22.741	<0.001	25.724	<0.001	27.049	<0.001
amoA-AOB	0.435	0.509	0.17	0.68	22.491	<0.001	2.24	0.524	22.954	<0.001	28.582	<0.001	30.816	<0.001
AOA:AOB ratio	0.188	0.665	0.043	0.837	27.215	<0.001	1.71	0.635	27.445	<0.001	32.125	<0.001	28.16	<0.001
nirK	0.002	0.962	1.728	0.189	32	<0.001	1.774	0.621	32.683	<0.001	34.569	<0.001	35.624	<0.001
nirS	0.051	0.821	2.521	0.112	14.551	<0.001	4.314	0.23	19.38	<0.001	21.324	<0.001	28.16	<0.001
nifH	1.734	0.188	0.322	0.57	32.87	<0.001	2.121	0.548	36.287	<0.001	33.454	<0.001	37.265	<0.001

Abundance of *amoA*-AOA, *nirK*, *nifH* & *nirS* increased, while *amoA*-AOB decreased over time



Summary (qPCR results)

- Shift (increase) in relative abundance of AOA phyla and genera confirmed by qPCR of archaeal *amoA* gene (dependent on sampling date)
- Similar trends (decrease) observed for AOB *amoA* gene abundance in rhizosphere, suggesting weak efficiency of nitrappyrin on target group
- AOA:AOB ratios >1 (soils low in AOB)
- Sampling date was overarching factor affecting abundance of all N-cycle genes, with stronger effects on first sampling date (limited residence time in soil)

Volatile organic compound (VOC) profiles

Before we dive into the results, let's have a glimpse at the metabolomics data

Metabolomics data structure after normalization

```
# glimpse() is like transposed version of print()
# data.table() is an enhanced version of read.table (detects sep, colClasses and nrows automatically), e.g. fread()
library(data.table)
mset = data.frame(fread("/Users/ruthschmidt/Dropbox/Work/INRS/Data/NI_experiment/Files/VOC/data_normalized.csv", header=T))
glimpse(mset)

## #> #> Rows: 397
## #> Columns: 25
## #> $ ID <chr> "2:39.0239@4.34 (2-Picoline, 6-nitro-)", "5:41.0393@2.65 (Acetic acid, trifluoro-, 1,1-dimethylethy...
## #> $ C1_1 <dbl> 0.99612847, 1.17741294, 1.30116310, 0.11315459, 0.58418700, 1.14554990, 0.56823013, -0.39126432, 0...
## #> $ C1_2 <dbl> -0.98692061, -0.82511936, 0.89388240, 0.67710410, 0.91347540, -1.21960350, -4.49007811, -0.36844694...
## #> $ C2_1 <dbl> 0.77056605, 1.26379973, -1.01594020, 0.12086544, 0.49139500, 1.21053260, 0.48056031, -0.07304908, 0...
## #> $ C2_2 <dbl> -1.03844604, -0.75240572, 0.59400100, 0.46031433, 0.79126180, 0.10756470, -0.04640221, -1.19476863,...
## #> $ C3_1 <dbl> 1.0072837, 0.5954468, 1.7478495, 0.6275328, -1.1834992, 0.8704402, 0.5413666, -0.1621676, -2.076392...
## #> $ C3_2 <dbl> 0.70390366, -0.11760454, -1.00852080, 0.44338935, 0.78026210, 0.56424430, 0.28653018, 0.82146821, 0...
## #> $ C4_1 <dbl> -0.305153220, 0.171339670, -1.040703100, 0.523495940, -1.297018700, -1.175799400, 0.107818050, -0.8...
## #> $ C4_2 <dbl> 0.20962962, -0.96142020, 0.60655030, 0.54596879, 0.53573230, -1.14054200, 0.17270478, 0.81501908, ...
## #> $ C5_1 <dbl> 0.297530520, 0.573360510, -1.042560900, -1.927893540, -1.299380000, 0.644202200, 0.302834960, -0.00...
## #> $ C5_2 <dbl> -0.168318480, -0.687110460, 1.372546400, 0.800043910, 0.916068900, 0.286085800, 0.160191980, 1.5886...
## #> $ C6_1 <dbl> 0.861060470, 1.323099010, -1.007170600, -1.877575530, -1.254399400, 1.165107700, 0.369109020, -0.79...
## #> $ C6_2 <dbl> -1.14258605, -0.61457896, 0.61751670, 0.63317136, 0.81936700, -1.15752780, -0.06613528, 0.48511005, ...
## #> $ N1_1 <dbl> 0.8627581, 0.9203053, -0.9945753, -1.8596675, -1.2383910, 0.9880515, 0.5126458, -0.4886315, 0.22438...
## #> $ N1_2 <dbl> 0.65735836, 1.26171425, -1.15217180, 0.61546751, -1.43869380, -1.27358740, 0.21015793, -1.05698860, ...
## #> $ N2_1 <dbl> 1.25086663, 1.1869419, -0.9030305, 0.5024116, 0.7001255, 0.7433047, 0.6940271, 1.6688031, 0.1411357, ...
## #> $ N2_2 <dbl> -1.88570010, -1.59109300, 0.55924830, 0.19878731, 0.74244550, -1.29980740, -0.43214363, -1.33193544...
## #> $ N3_1 <dbl> 1.24246639, 1.19845886, -0.92040620, -1.75421373, -1.14412310, 1.20021080, 0.55006091, 2.05411820, ...
## #> $ N3_2 <dbl> -1.18474878, -1.04092505, 0.36092020, 0.58820241, 0.93040390, 0.12812140, -0.12119062, 0.16235694, ...
## #> $ N4_1 <dbl> -0.01077812, -0.08877474, 0.81224630, 0.29850596, -1.09446820, 0.51601060, 0.01361097, 1.31079876, ...
## #> $ N4_2 <dbl> -0.04228815, -0.96642186, -1.03139870, 0.73180790, 0.95307030, -1.16763700, 0.17058058, 0.67657073, ...
## #> $ N5_1 <dbl> 0.41268699, 0.46180647, 0.90301740, 0.07013754, 0.38094690, 0.60726600, 0.11060688, -1.62168055, 0...
## #> $ N5_2 <dbl> -0.50972304, -0.83613079, 0.65094450, 0.77991340, 0.91012510, -1.21398210, 0.16167093, -0.04941278, ...
## #> $ N6_1 <dbl> 0.3938863, 0.2694553, 0.7975968, -1.9218008, -1.2939336, 0.7004945, 0.1997543, -0.8423605, 0.447939...
## #> $ N6_2 <dbl> -2.39146230, -1.92155613, -1.10100500, 0.61087692, 0.79504020, -1.22870040, -0.45651153, -0.3566936...
```

VOC profiles not affected by NI treatment, but differed among Date

Principal Component Analysis (PCA)
of rhizosphere VOC composition

PERMANOVA of rhizosphere VOC
composition

		R^2	P
●	Control	Treatment	0.013 0.384
●	NI	Date	0.665 0.001
		Treatment × Date	0.016 0.304

Monoterpene α -Pinene most significant compound

```
# DT(): R data objects (matrices or data frames) can be displayed as tables on HTML pages, and DataTables provides filtering,
# pagination, sorting, and many other features in the tables
library(DT)
voc_table <- fread(file="/Users/ruthschmidt/Desktop/images/voc_table.txt")
DT::datatable(
  voc_table,
  rownames = FALSE,
  extensions = 'FixedColumns',
  fillContainer = FALSE, options = list(pageLength = 8))
```

Show 8 entries

Search:

Compound ID	Class	RT	RI	P-value
1377_alpha-Pinene	Terpene	6.93	938.8	5.28e-9
386_Hexanoic acid, methyl ester	Ester	6.75	927.8	0.00000157
325_Uncertain		2.04	613.2	0.00000418
279_2,2-Diethoxyethanol	Alcohol	2.52	639.4	0.00000564
602_Uncertain		7.07	947.3	0.000016
1099_Toluene	Alcohol	4.34	769.4	0.0000266
364_Isopropyl acetate	Ester	3.13	680.7	0.0000459
638_Uncertain		14.57	1459.9	0.000141

Showing 1 to 8 of 13 entries

Previous

1

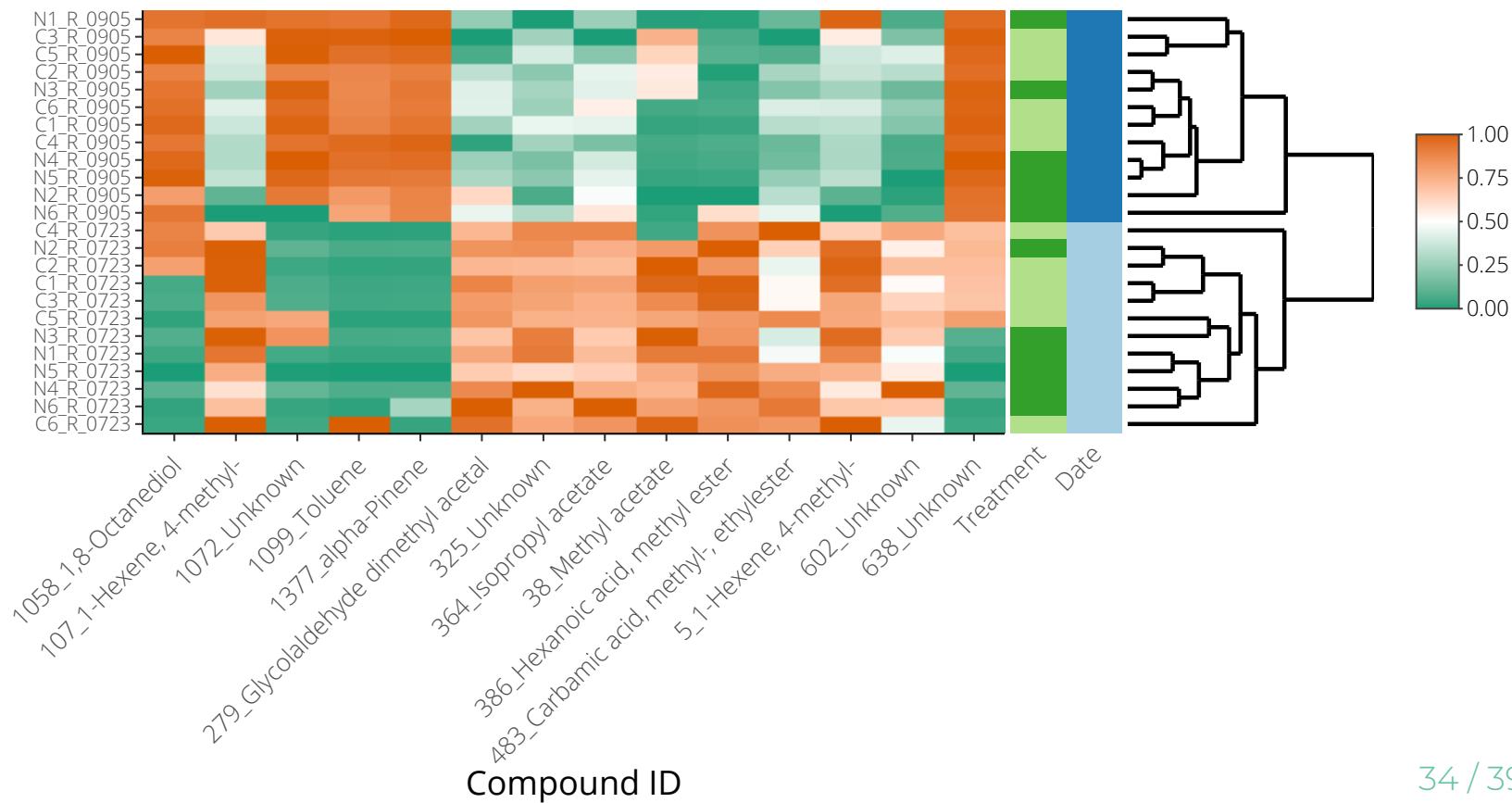
2

Next

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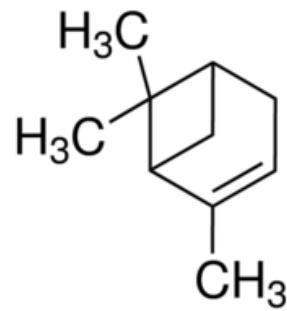
Monoterpene α -Pinene most significant compound in response to Date, with increase over time

Heatmap of significant VOCs ($P < 0.01$) based on Euclidian distance-clustering



Summary (VOC results)

- Contrary to expectations, we did not find significant direct effect of nitrappyrin treatment on VOC profiles (sampling time)
- But VOC profiles clearly differed according to sampling date
- α -Pinene (monoterpene) previously shown to inhibit nitrification by targeting the ammonia monooxygenase enzyme - linked to large decrease in AOB *amoA* abundance at the second sampling date?
- AOB might be more susceptible to inhibitory effects of alpha-pinene than AOA



Conclusions

- Results confirm hypothesis that nitrpyrin alters the relative abundance of non-target bacteria, archaea, fungi and associated nitrogen-cycle functions
- Effects of nitrification inhibitors might have far reaching but difficult to determine if shifts caused directly (e.g. off-target toxic effects) or indirectly (through reduced activities of ammonia-oxidizers)
- Shifts not reflected in VOC profiles of rhizosphere, suggesting that nitrpyrin did not affect soil functionality
- Nitrpyrin affected abundance of AOA, but positively
- Nitrpyrin effects also constrained by sampling date and by the plant compartment, suggesting interaction with environmental conditions

A HUGE THANKS TO ALL OF YOU!



Some useful resources

Courses I took:

- Cleaning data in R (Datacamp)
- Udemy data science courses (R, Python) (regular offers)
- Coursera data science courses (audit for free)

Papers I read:

- Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible
- Rarefaction, Alpha Diversity, and Statistics
- Normalization and microbial differential abundance strategies depend upon data characteristics

Documentations:

- Markdown (Presentation R package [xaringan](#), [Tutorial](#), [TutorialCheat Sheet](#))
- Interactive web-based data visualization with R, [plotly](#), and [shiny](#)
- Plotly (R)

R packages:

- [phyloseq](#), microbiome, microbiomeutilities, [heatmaply](#), tidyverse, [tidyverse](#), [data.table](#)

Ask me!

But most importantly, don't give up!

... even if the road is difficult

