Biocontrol in the Age of the Microbiome:
The Confluence of Predictive Understanding, Big Data, and Cultural Memes

Jay L. Garland
(321) 266-2646
jlgarland3@gmail.com

Goals of This Talk

- Review previous work on community-based biocontrol
  - Ecological/System Conceptual Models
  - Results of work
- The Emergence of “The Microbiome” in the last decade:
  - The technological basis (high throughout sequencing)
  - The Big Data nature of the work
  - As a Cultural Meme
- How to move forward with the biocontrol now

Problem Statement

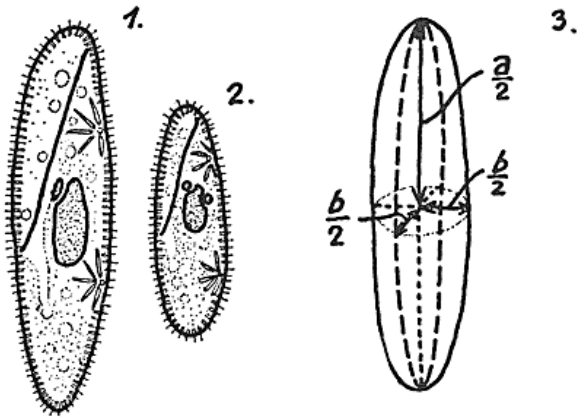
- Contamination of fresh sprouts by the human pathogenic bacteria *Salmonella enterica* and *Escherichia coli* O157:H7 has been linked to food poisoning outbreaks (Mohle-Betani et al., 2002; Brooks et al., 2001; Winthrop et al., 2001).

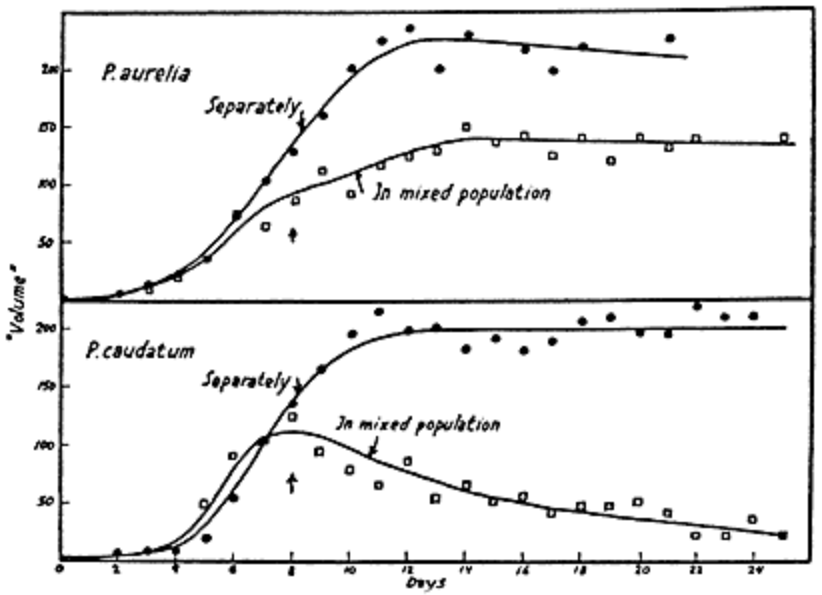
- Chlorine soak to sanitize sprout seeds has been recommended to reduce food safety risks, but this harsh treatment is not only counter to the goals of organic agriculture, it is of limited and variable efficacy (Montville and Schaffner, 2004)

- Rapid bacterial growth during the sprouting process (Howard and Hutcheson, 2003; Matos and Garland, 2005) allows for even a very few pathogens which survive the chlorine soak to reach disease-causing densities in harvested sprouts (Fett, 2002).

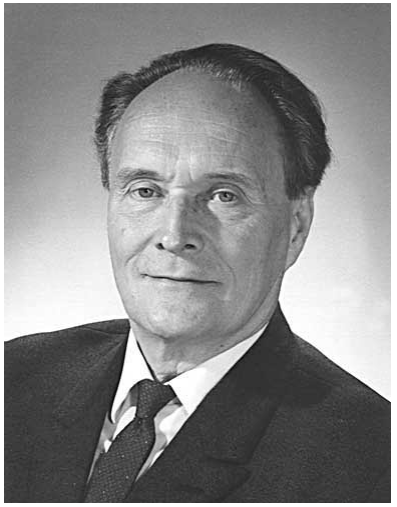
- Alternative approaches focused on reducing growth of the pathogens during sprout development are needed.

Competitive Exclusion Principle





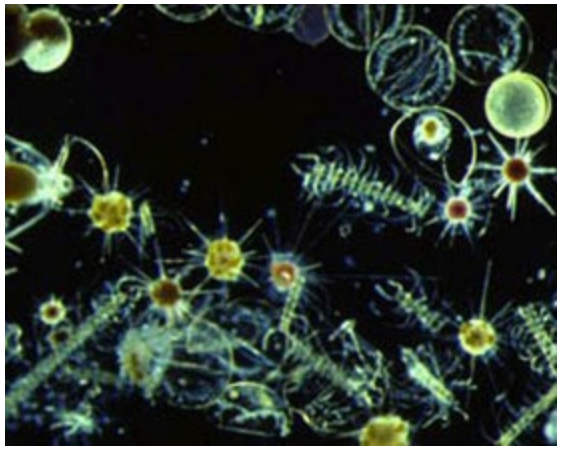
"Let us summarize the data of this chapter. We have studied the competition between two species for a source of energy kept continually at a certain level. …..*there is but a redistribution of the completely seized energy between the two species,* which is again controlled by the differential equations of competition. Owing to its advantages, mainly a greater value of the coefficient of multiplication, one of the species in a mixed population drives out the other entirely. "

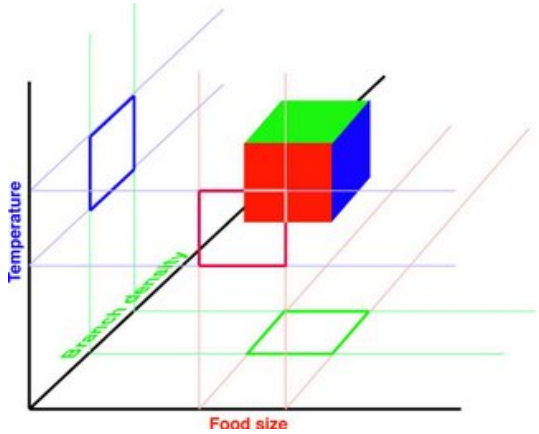


"The Struggle for Existence" G.F. Gause 1934

G.E. Hutchinson. Paradox of the Plankton (1961)

Why are there so many kinds of animals and how can they coexist in an apparently homogeneous habitat?





Niche: n-dimensional space (axes x1, x2. ,,, x_n) represent physical and biological variables) corresponding to state of the environmental which would permit a species to exist

Organisms with complete niche overlap could not coexist, but this is rare:

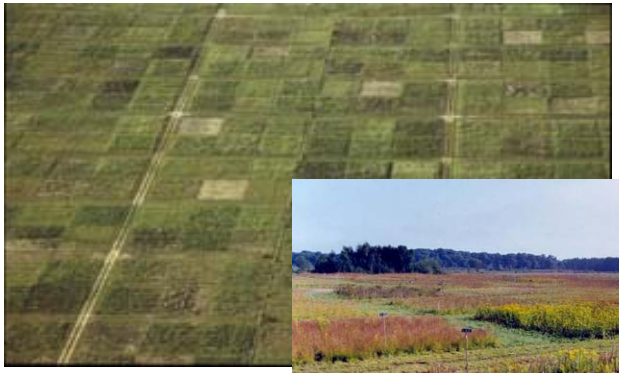
1) Complexity of the niche
2) Niche differentiation
3) Non-equilibrium (e.g., temporal variation)

Old dogma in ecology that diversity confers stability

Little theoretical or empirical support

More recent manipulative studies

Diversity stabilizes function by allowing for variation (destablization) of structure



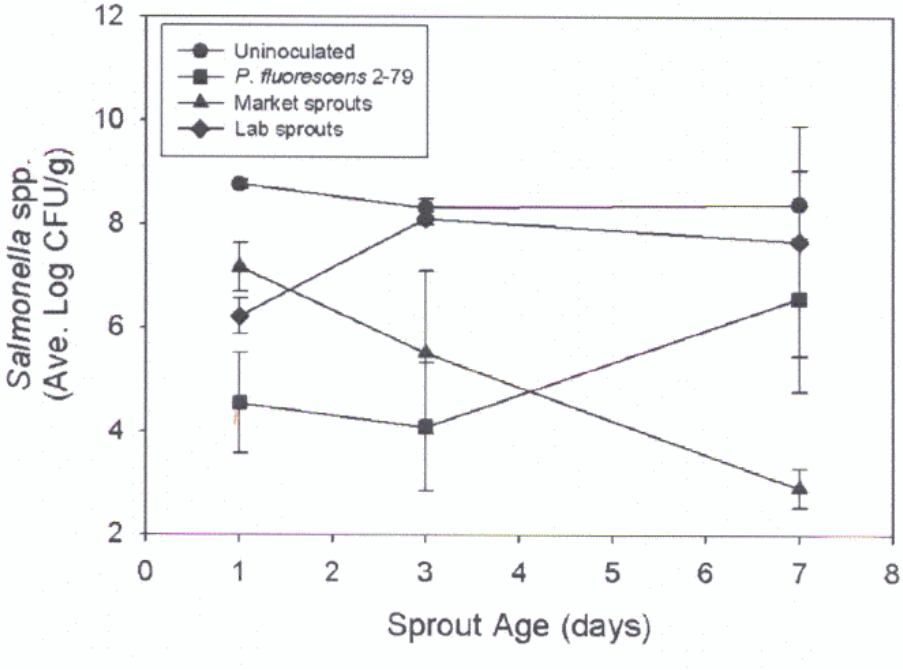
Consequences of Diversity

- Is it beneficial?
  - Growing body of literature suggests that it may increase productivity, increase efficiency, and stability
- If so, why?
  - Complementarity – interactive effects
  - Sampling effect – the more organisms the more likely you will have a “star”
- Can mixtures of organisms provide better bicontrol than single strains?

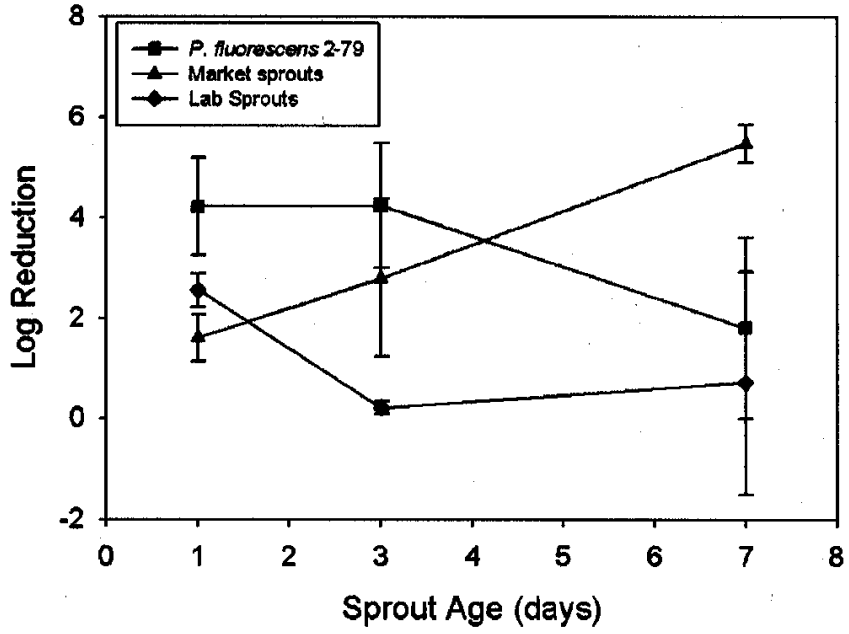
Methods

Community vs Single Strain Biocontrol

- Alfalfa seeds inoculated with a mixture of 4 strains of antibiotic resistant *Salmonella*
- Seeds soaked in suspensions of either:
  1) Pure culture of *Pseudomonas fluorescens* 2-79
  2) Microorganisms rinsed from sprouts grown from seeds for 7 days (Lab Sprouts Inoculum)
  3) Microorganisms rinsed from alfalfa sprouts purchased at a local store (Market Sprout Inoculum)
  4) Nothing (negative control)
- Seeds grown in capped glass vials containing moistened filter paper
  - Sampled after 1, 3 and 7 days growth



The Market Sprout Community caused greater reduction in Salmonella numbers. particularly over time



Conclusions

- The complex mixed microbial community provided greater biocontrol of *Salmonella* (> 5 log reduction)

- The mixed microbial community showed greater stability and more efficient resource utilization
  - Effects consistent with diversity theory (complementarity or sampling effect?)

Issues for Further Research

- Unknown inocula (i.e., market sprout community) not a viable approach for food safety
  - Can you guarantee the lack of deleterious organisms?

- Can you rationally assemble communities (using known isolates) which provide effective biocontrol?

What's Happened in the Past Decade?

- The cost of sequencing dramatically decreased
  - Human Genome Project (2003) cost about $54 million to sequence and analyze one human genome
  - Now $4,000 for a eukaryotic genome, and about one tenth of that for a bacterial genome
  - Metagenomics is the simultaneous sequencing and analysis of multiple genomes, such as those found in a microbiome, can now cost less than $1,000 for a high level analysis of a metagenome
- The Microbiome as a meme
  - An idea, belief or belief system, or pattern of behavior that spreads throughout a culture
  - Microbes are good

Microbiome Meme (NPR Stories)

Do We Really Need Probiotics in Our Coffee, Granola, and Nut Butter. Apr. 19, 2016

Your Invisible Neighbors: Each City has Unique Microbes. April 19, 2016

The Human Body's Complicated Relationship with Fungi. April 16, 2016

Researchers Test Microbe Wipe to Promote Babies' Health After C-Sections Feb. 1, 2016

Stomach of Ancient Iceman Held Microbes Like Ours. Jan. 7, 2016

Is This A Snowy Wonderland or the World Inside a Petri Plate Dec. 25, 2015

Tiny Witnesses: Microbes Can Tell When a Murder Victim Died. Dec. 10, 2015

Missing Microbes Provide Clues About Asthma Risk. Sept. 30, 2015

Wherever You Go, Your Personal Cloud of Microbes Follow. Sept. 22, 2015

Does This Phylum Make Me Look Fat? Aug. 20, 2015

Microbe Mix May Play Role in Preterm Birth Risk. Aug. 17, 2015

Spore Microbe Helps Fen Off Life-Threatening Bacterial Infections. May 5, 2015

How Modern Life Depletes our Gut Microbes Apr. 21, 2015

Origins of the Term

Whipps, JM., K. Lewis, RC Cooke. 1988. Mycoparasitism and plant disease . In NM Burge, Ed. Fungi in Biological Control Systems Manchester University Press.

"A convenient ecological framework in which to examine biocontrol systems is that of the microbiome. This may be defined as a characteristic microbial community occupying a reasonably well defined habitat. The term thus refers not only to the microorganisms involved but also encompasses their theatre of activity."

The "biome" of microorganisms

Ledeberg, Joshua. 2004. Of men and microbes. New Perspectives Quarterly

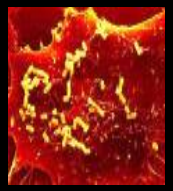
"The great scientific news that greeted thus century was the campaign to decode the human genome, We must now remind ourselves that much of the biological composition of our bodies consists of the genomes other than the human. Multitudes of bacteria and viruses occupy our skin, our mucous membranes, and our intestinal tract….Understanding this cohabitation of genomes within the human body – what I call the microbiome is central understanding the dynamics of health and disease".

Emphasis on the new technology of gen-"omics". And human centric

The 3-Domain System Of Biological Classification (Carl Woese 1977)

100-150 MYA

Salmonella



Escherichia

100 MYA

Homo sapien

Heterocephalus glaber

gram positive bacteria

green non-sulfur bacteria

purple bacteria

Deinococci

Cyanobacteria

Flavobacteria

Thermotoga

BACTERIA

animals

fungi

plants

flagellates

Microsporidia

EUCARYA

Korarchaeota

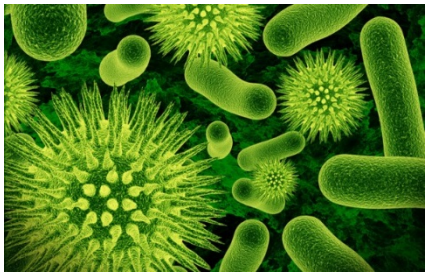
extreme halophiles

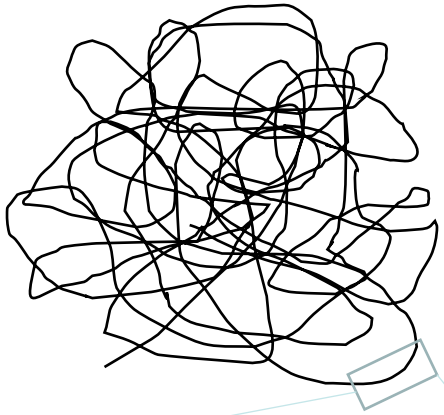
methanogens

extreme thermophiles

ARCHAEA

Microbiome Approach



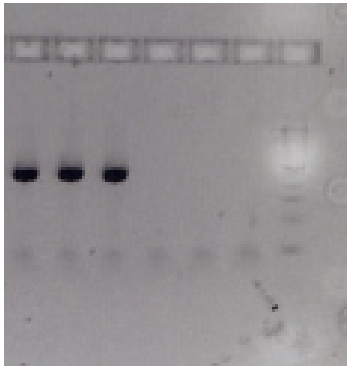


16S rRNA gene,
1509 bp

V1 V2 V3 V4 V5 V6 V7 V8 V9

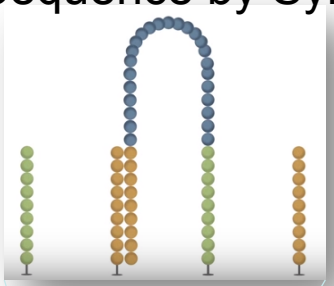
375 bp

PCR



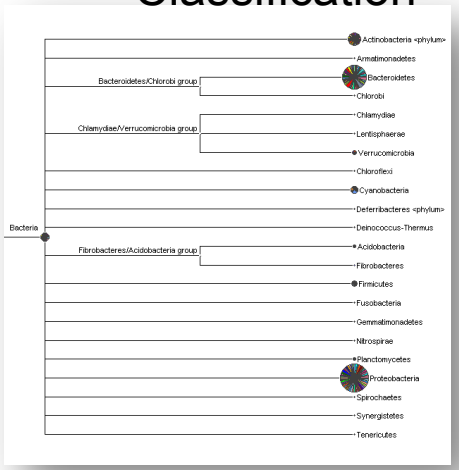
16S Sequencing and Analysis

Sequence by Synthesis (SBS)

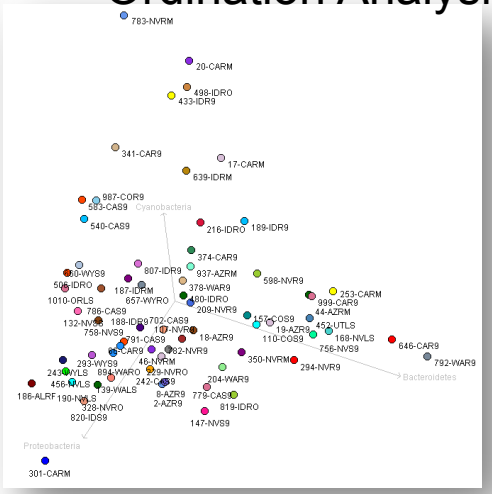


MiSeq

Classification



Ordination Analysis

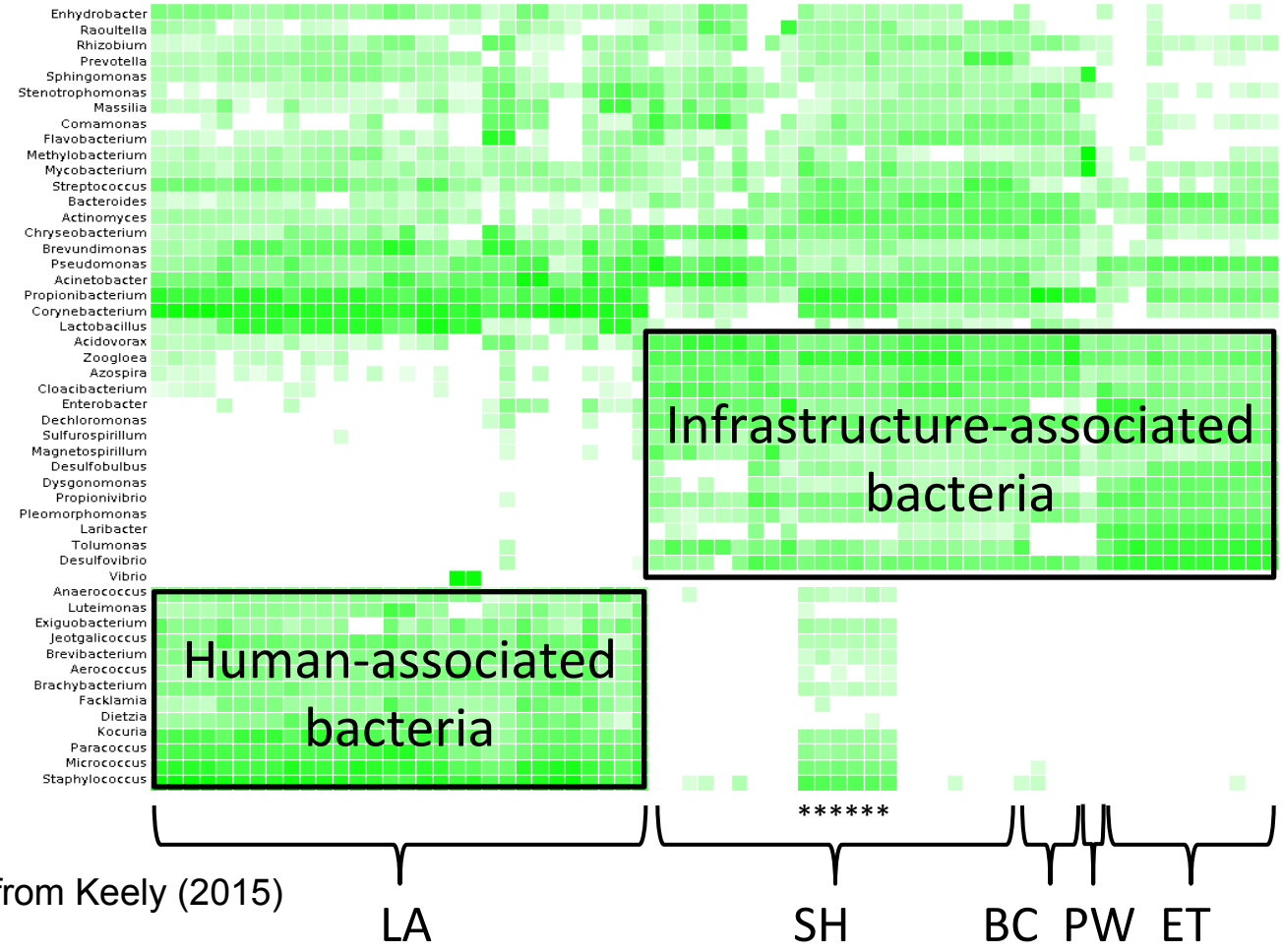


An Example of the Big Data Nature of High Throughput Sequencing

- From a collection of ~50 water samples
- Over 1.8 million raw reads generated
  - Average over 35,000 raw reads per sample

Sample Type	Number of Samples	Average Number of Genera Detected	Total Number of Genera Detected
SH	18	86	191
ET	6	53	90
BC	3	82	107
PW	1	37	37
LA	24	105	295

Log_{10}-scale Heat Map of Genera Detected



Adapted from Keely (2015)

Fast Track Committee of Mapping the Microbiome (FTAC-MM)

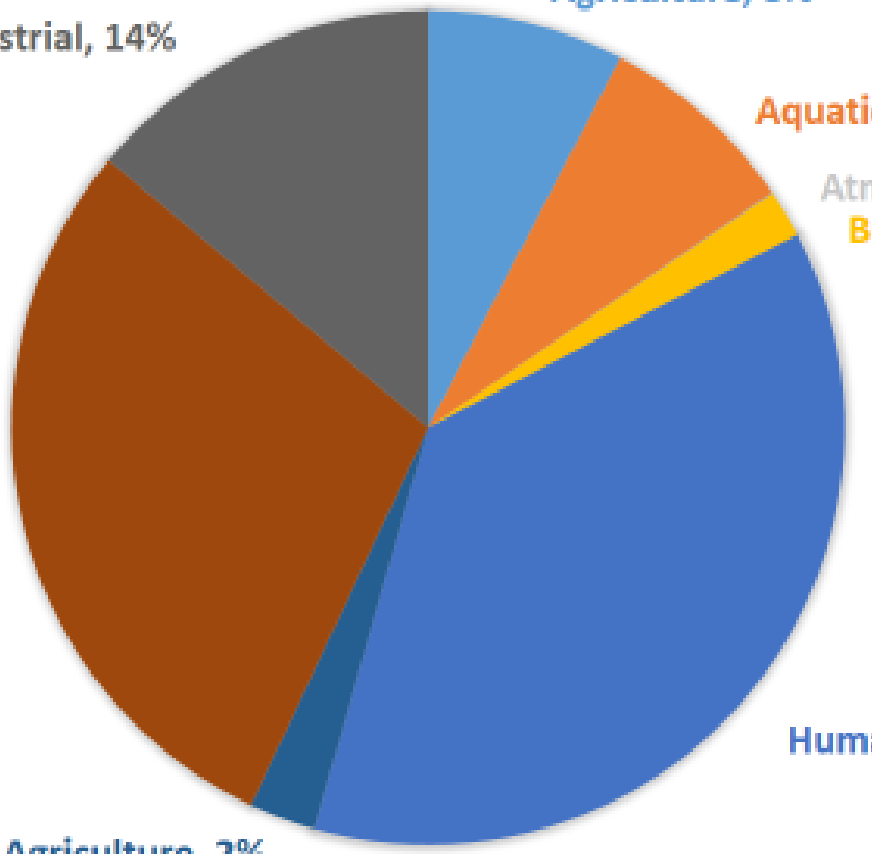
- Describe existing federally supported research and development activities in microbiome research, including a clear description of current investments and individual agency priorities;

- Identify and prioritize technology needs and cross-cutting challenges common to all microbiome research, with a **specific focus on enabling predictive understanding and modeling of microbiomes**; and

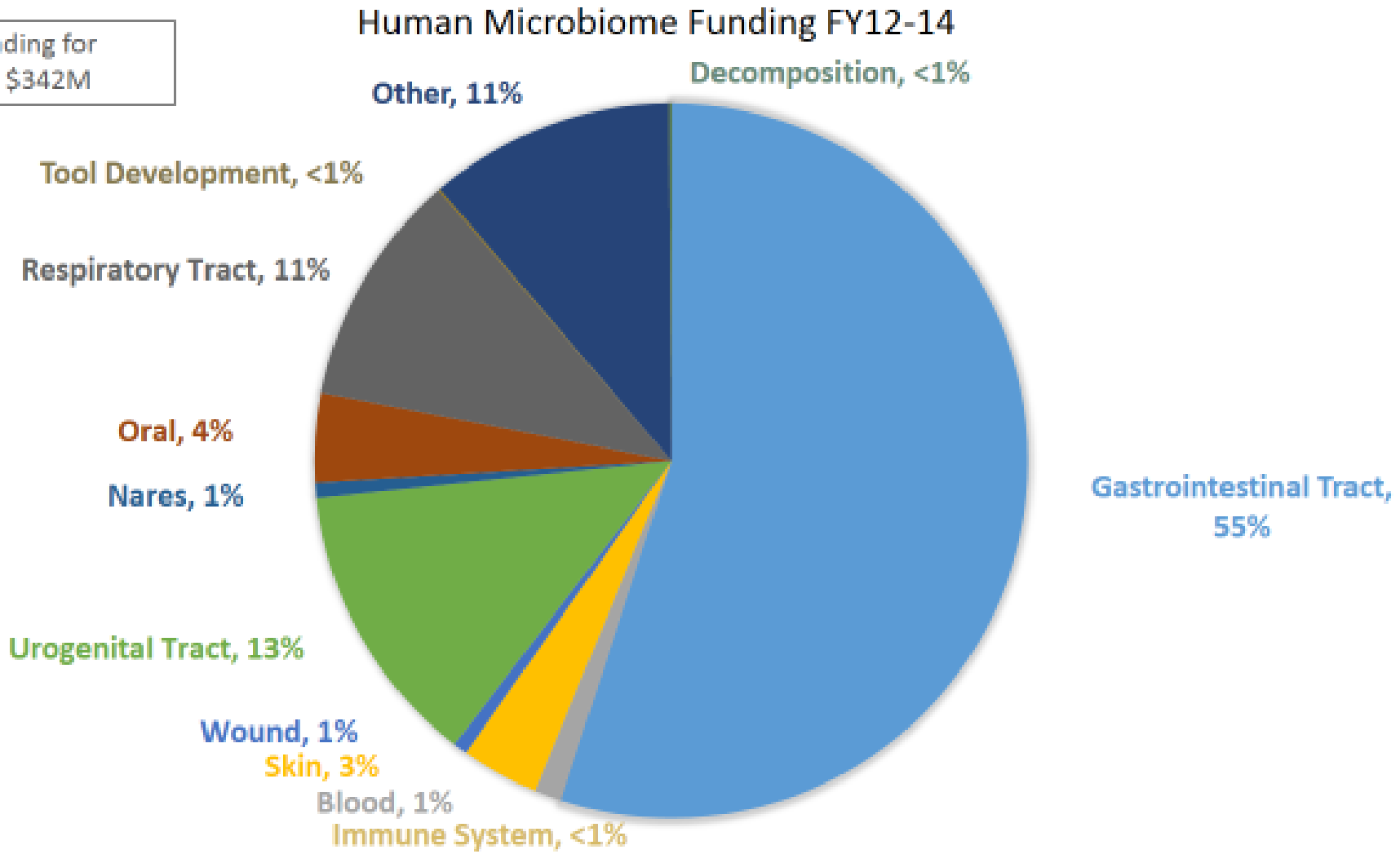
- Outline a coordinated plan for Federal investment to address research and development gaps for microbiome research required to achieve a **predictive understanding of microbiomes and their functions.**

Total Microbiome Research Funding FY12-14 by Subject or Ecosystem

Total Funding for FY12-14: $922M

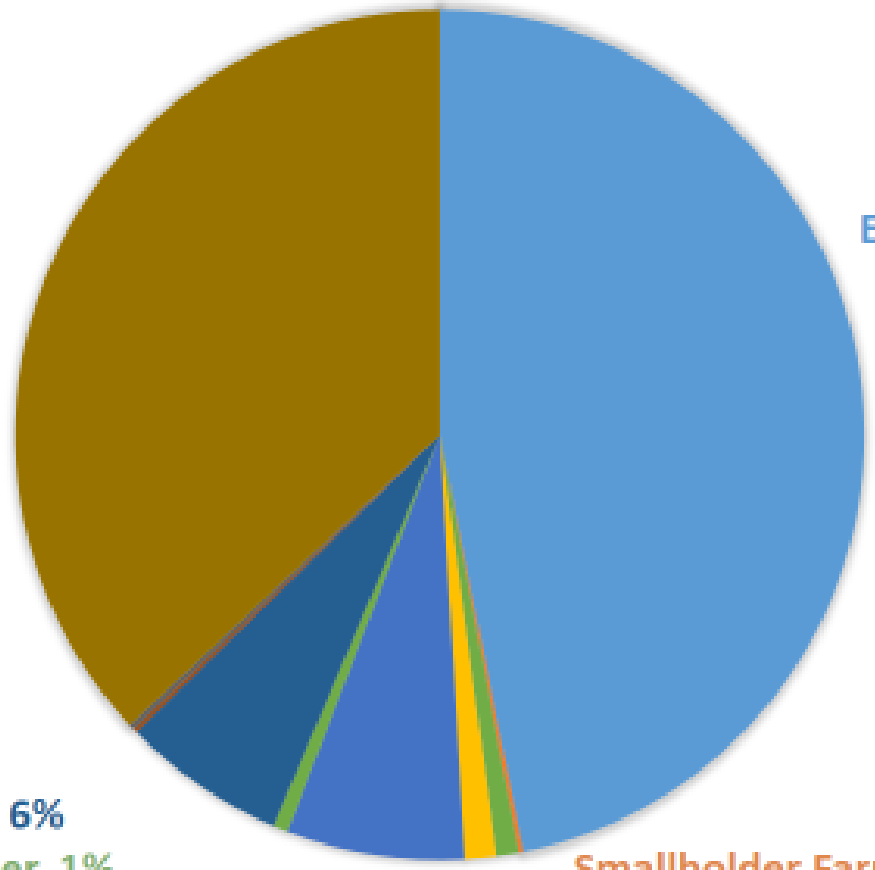


Total Funding for FY12-14: $342M



Agriculture Microbiome Funding FY12-14

Total Funding for FY12-14:
$70.5M



From FTAC-MM Report

- Probiotics are currently a $35 million industry, projected to reach $650 million by 2023
  - A microbial therapeutic for treatment of Clostridium difficile is in clinical trials
- Predictability in treatment outcomes and increased understanding of the microbiome could lead to impacts on costs of:
  - Obesity ($147 billion)
  - Foodborne illness ($14.6 billion)
  - Antimicrobial resistance ($55-70 billion)
  - Cancer ($90 billion)

FTAC-MM Report (Cont'd)

- The potential for applied microbiome research to impact the health care industry is substantial, but **understanding the basic mechanisms of microbiome assembly and communication within the context of any system**, human or otherwise, will provide benefits to many additional areas, such as energy, agriculture, manufacturing, and environmental health.

- Answering the fundamental questions that lie at the heart of microbiome dynamics, such as "***What is a healthy microbiome?"*** **and** ***"What makes a microbiome resilient?"*** require a coordinated, interdisciplinary effort that should include geochemists, statisticians, environmental engineers, mathematical modelers, medical professionals, and others.

- If you want to assemble an ecological community, you need to know:
  - Are there important assembly rules concerning timing, density, or sequence of addition I should be aware of?
    - How many discrete elements (i.e., species) should I add?
      - » Biodiversity issues
  - What changes can I expect to occur over time once I have added my components together?
    - Successional dynamics
    - Evolutionary changes
  - The good news:
    - The situation is easy(or easier) with sprouts
    - Short term race (not long term maintenance of an ecosystem)
  - The bad news
    - Working with microbes is difficult

Proposed Work

- Maximize isolation of organisms from sprouts
  - Sonicate samples, uses more dilute media, replace agar with gellum gum, long incubations, look for small colonies
  - Select organisms from this library
    - without any biosafety concerns
    - with benefical health effects (?)
  - Compare to high-throughput sequencing of sprouts

- Conduct testing with different mixtures of organisms
  - Different levels of richness (i.e., 1, 3, 5, 10, etc.)
  - Replicate with randomly selected mixtures (in order to eliminate “sampling effect”)
  - Or “intelligently” select different types based on genomic analysis of community
  - Test for ability to resist invasion by targeted pathogens

- Commercial product - seed preparations with optimal microbial “cocktails”
  - Cultures of microorganisms (think large fermenters)
  - Carrier (e.g. calcium alginate)
  - Adhesive (e.g., carboxymethyl cellulose)
  - Existing commercial examples (e.g., Rizobacter, Inc.)

Back to the Title

- Increased acceptance of the approach
  - Leveraging the meme that microbes are good

- Using big data to better inform:
  - the selection of specific organisms (or ingredients) for biocontrol “cocktail”
  - Or, alternatively, to QA the composition of undefined mixtures used for biocontrol

How do you perform manipulative experiments with microbial diversity when you can only culture a small percentage of microorganisms?

The culturable minority

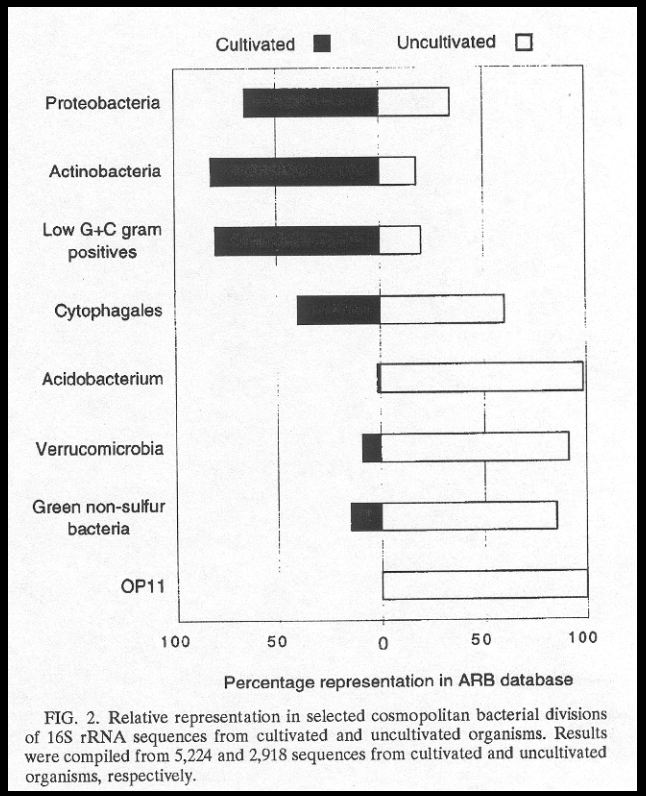


FIG. 2. Relative representation in selected cosmopolitan bacterial divisions of 16S rRNA sequences from cultivated and uncultivated organisms. Results were compiled from 5,224 and 2,918 sequences from cultivated and uncultivated organisms, respectively.

Rhizosphere Characterization

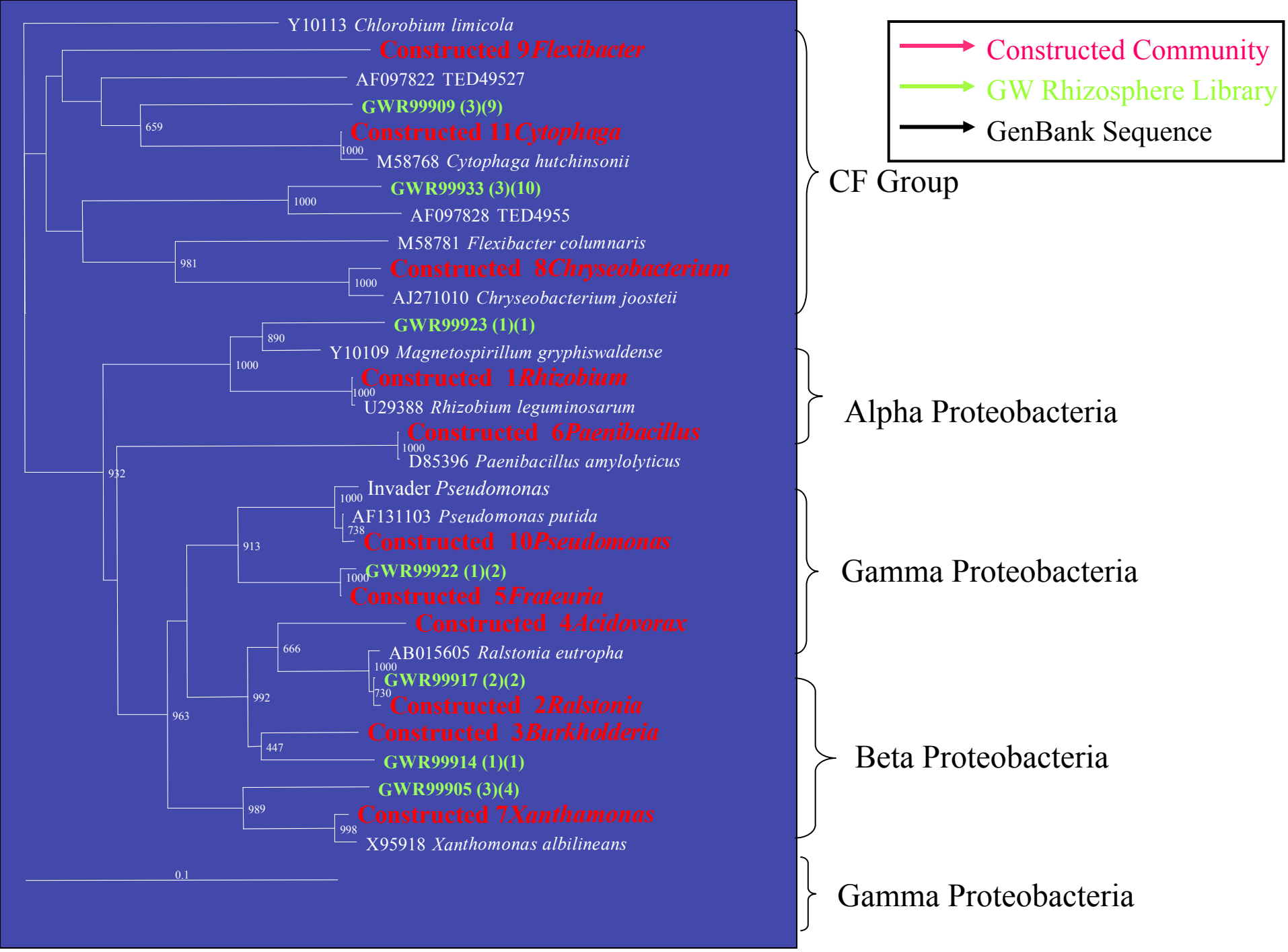
Isolate Microbial Population from Rhizosphere of Plants Used for Waste Processing

Isolates & Enrichment Cultures

Extract DNA & Prepare 16S rDNA Clonal Libary

16S rDNA Sequence Analysis

Selection of Isolates to Include in Constructed Community


Y10113 Chlorobium limicola
Constructed 9 Flexibacter
AF097822 TED49527
GWR99909 (3)(9)
659
Constructed 11 Cytophaga
1000
M58768 Cytophaga hutchinsonii
GWR99933 (3)(10)
1000
AF097828 TED4955
M58781 Flexibacter columnaris
981
Constructed 8 Chryseobacterium
1000
AJ271010 Chryseobacterium joosteii
GWR99923 (1)(1)
890
Y10109 Magnetospirillum gryphiswaldense
1000
Constructed 1 Rhizobium
1000
U29388 Rhizobium leguminosarum
Constructed 6 Paenibacillus
1000
D85396 Paenibacillus amylolyticus
932
Invader Pseudomonas
1000
AF131103 Pseudomonas putida
738
913
Constructed 10 Pseudomonas
1000
GWR99922 (1)(2)
Constructed 5 Frateuria
Constructed 4 Acidovorax
666
AB015605 Ralstonia eutropha
1000
GWR99917 (2)(2)
730
992
Constructed 2 Ralstonia
963
Constructed 3 Burkholderia
447
GWR99914 (1)(1)
GWR99905 (3)(4)
989
Constructed 7 Xanthamonas
998
X95918 Xanthomonas albilineans
0.1
Constructed Community
GW Rhizosphere Library
GenBank Sequence
CF Group
Alpha Proteobacteria
Gamma Proteobacteria
Beta Proteobacteria
Gamma Proteobacteria

Constructed Community

Designation	Identification[a]	Phylogenetic Placement	Surfactant Degradation
1	*Rhizobium leguminosarum* (U29388)(99%)	Proteobacteria, Alpha subdivision	-
2	*Ralstonia eutropha* (AB015605)(99%)	Proteobacteria, Beta subdivision	+
3	*Burkholderia cepacia* (U96927)(98%)	Proteobacteria, Beta subdivision	+
4	*Aquaspirillum metamorphum* (Y18618)(98%)	Proteobacteria, Beta subdivision	-
5	*Frateuria aerantia* (AJ010481)(95%)	Proteobacteria, Gamma subdivision	+
7	*Xanthomonas axonopodis* (AF123090)(99%)	Proteobacteria, Gamma subdivision	+
10	*Pseudomonas putida* (AE016774)(99%)	Proteobacteria, Gamma subdivision	+
8	*Chryseobacterium joosteii* (AJ271010)(97%)	Flavobacteriaceae, Chryseobacterium	-
9	*Flexibacter sp* (AF361187)(94%)	Flexibacteraceae, Flexibacter	+
11	*Cytophaga hutchinsonii* (M58768)(98%)	Flexibacteraceae, Cytophaga	ND
6	*Paenibacillus amylolyticus* (D85396)(100%)	Firmicutes, Bacillales	ND

[a]Closest match from GenBank (Accession number)(%16S rDNA sequence similarity)
ND, not determined

Selected based on cultured specimens who matched the major clusters from a library of clones from 16sRNA sequence analysis

Testing Community Assembly Ideas

- Effects of Diversity on Stability
  - specifically, resistance to invasion
- Compare gnotobiotic vs. selectively enriched communities
  - Including a diversity gradient treatment
- Gnotobiotic approach
  - Characterize community using non-cultural methods, select representative members
- Diversity gradient approach
  - Dilution extinction of resident community

Complexity Gradient Formation

Surfactant Addition

Sludge Community

Dilution of Sludge & Inoculation into Rhizosphere

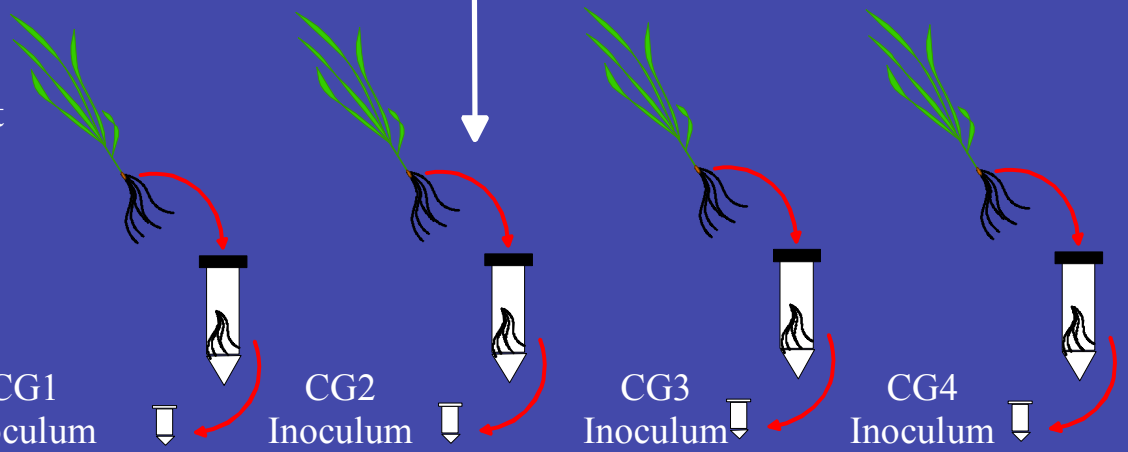
10^{-1} 10^{-3} 10^{-5} 10^{-7}

Gradient of Cell Types and of Cell Density

14 Days after Inoculation

Gradient of Cell Types but Cell Density is the Same Across all Treatments

Root Mass is Harvested, Cells are Removed from Root and Aliquots are Frozen in Glycerol for Use as Inocula



CG1 Inoculum

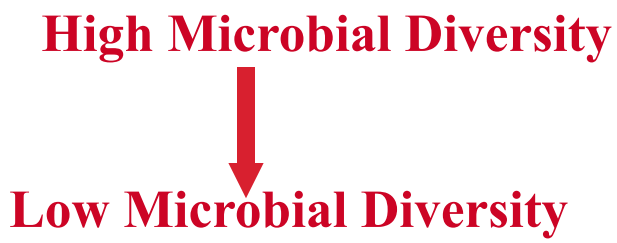
CG2 Inoculum

CG3 Inoculum

CG4 Inoculum

Experimental Design

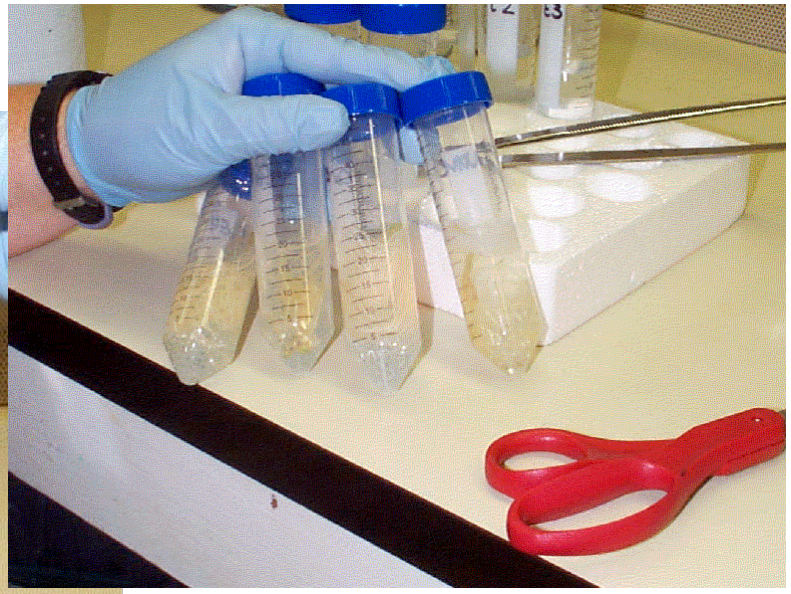
- Six treatments:
  - Constructed Community
  - Propitious Community
  - CG1
  - CG2
  - CG3
  - CG4

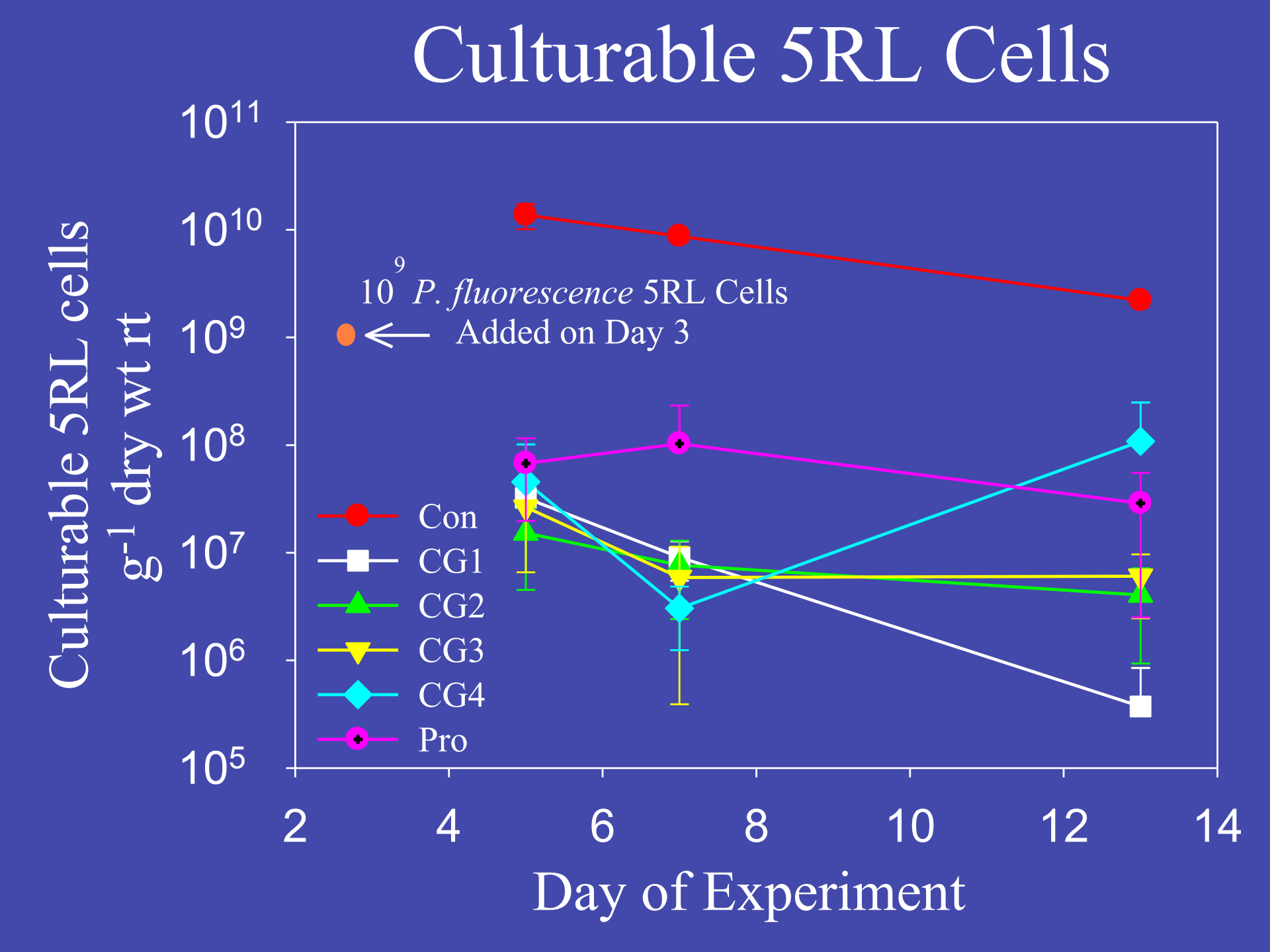


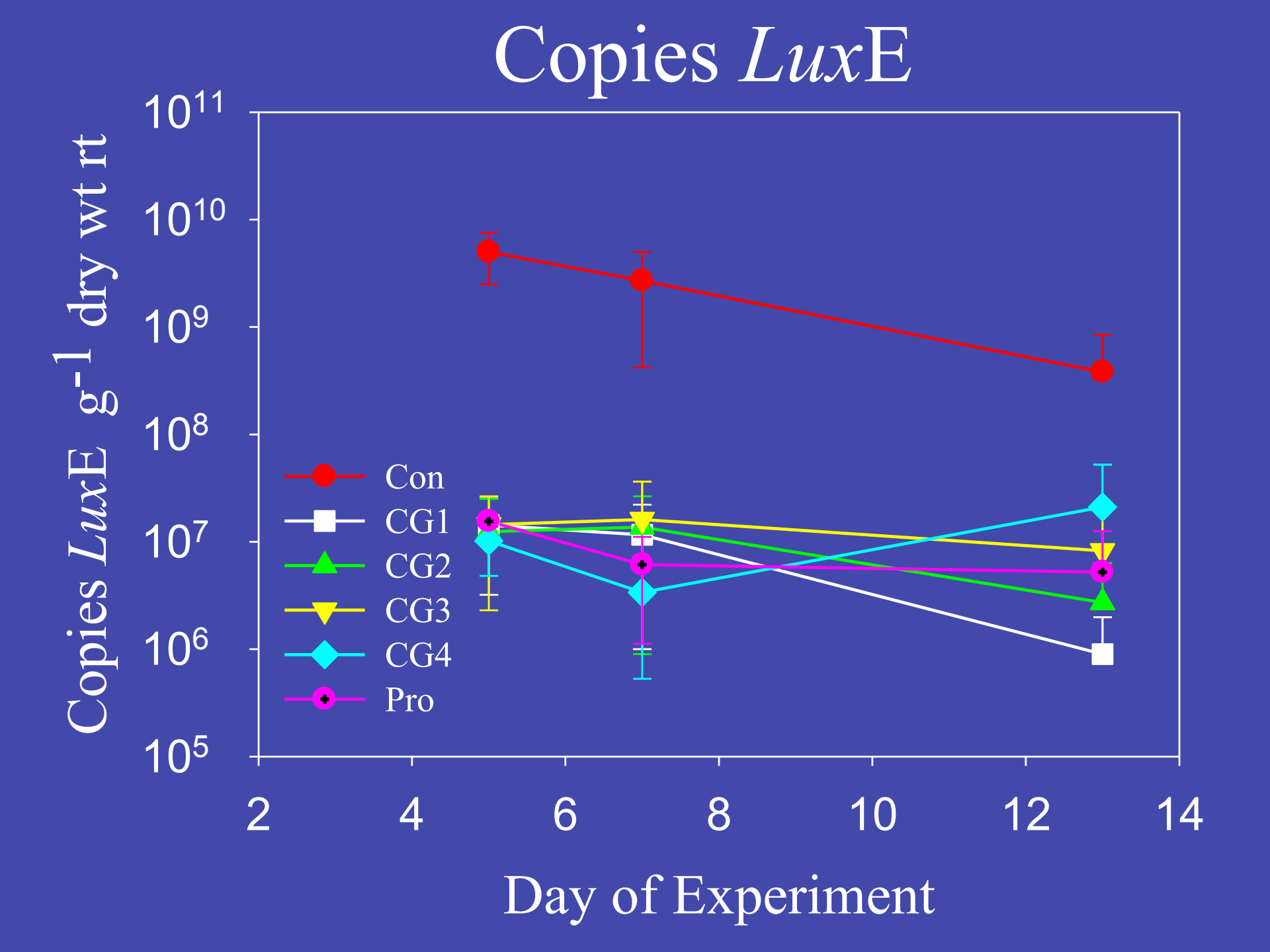
- Invader (*Pseudomonas fluorescens* 5RL)
  - Bioluminescent Bioreporter
  - Rhizosphere-associated organism

- Experimental crop
  - Wheat (*Triticum aestivum* L. Cv. Apogee) obtained from Utah State

Samples taken on days
3, 5, 7 and 13




Culturable 5RL Cells
Culturable 5RL cells
g-1 dry wt rt
10^11
10^10
10^9
10^8
10^7
10^6
10^5
10^9 P. fluorescence 5RL Cells
Added on Day 3
Con
CG1
CG2
CG3
CG4
Pro
2
4
6
8
10
12
14
Day of Experiment


Copies LuxE
Copies LuxE g-1 dry wt rt
10^11
10^10
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10^8
10^7
10^6
10^5
Con
CG1
CG2
CG3
CG4
Pro
2
4
6
8
10
12
14
Day of Experiment

Conclusions

- The constructed community and the complexity gradient communities:
  - Established in the rhizosphere and persisted over time
  - Exhibited functional diversity

- The constructed community was much more susceptible to invasion compared to the more “naturally” inoculated communities