

# Characterizing Root-Associated Bacteria for Improvement of Soil Health

**The Northeast Residuals and Biosolids Conference**  
**“Managing Residuals in a Complex World”**  
**Burlington, VT**



**Cairn Ely, Ph.D.**

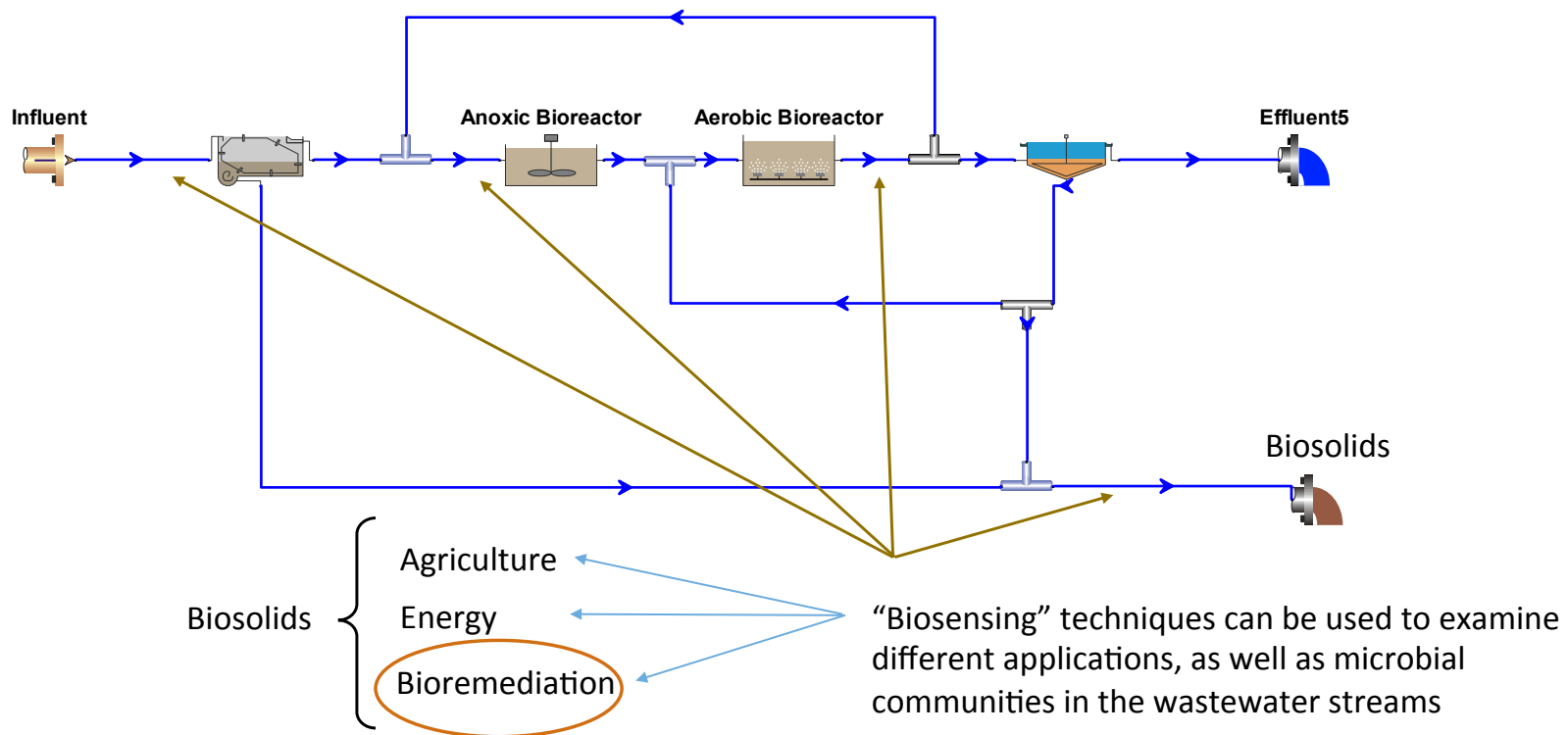
Assistant Professor in Civil Engineering  
Central Connecticut State University

October 26<sup>th</sup>, 2017

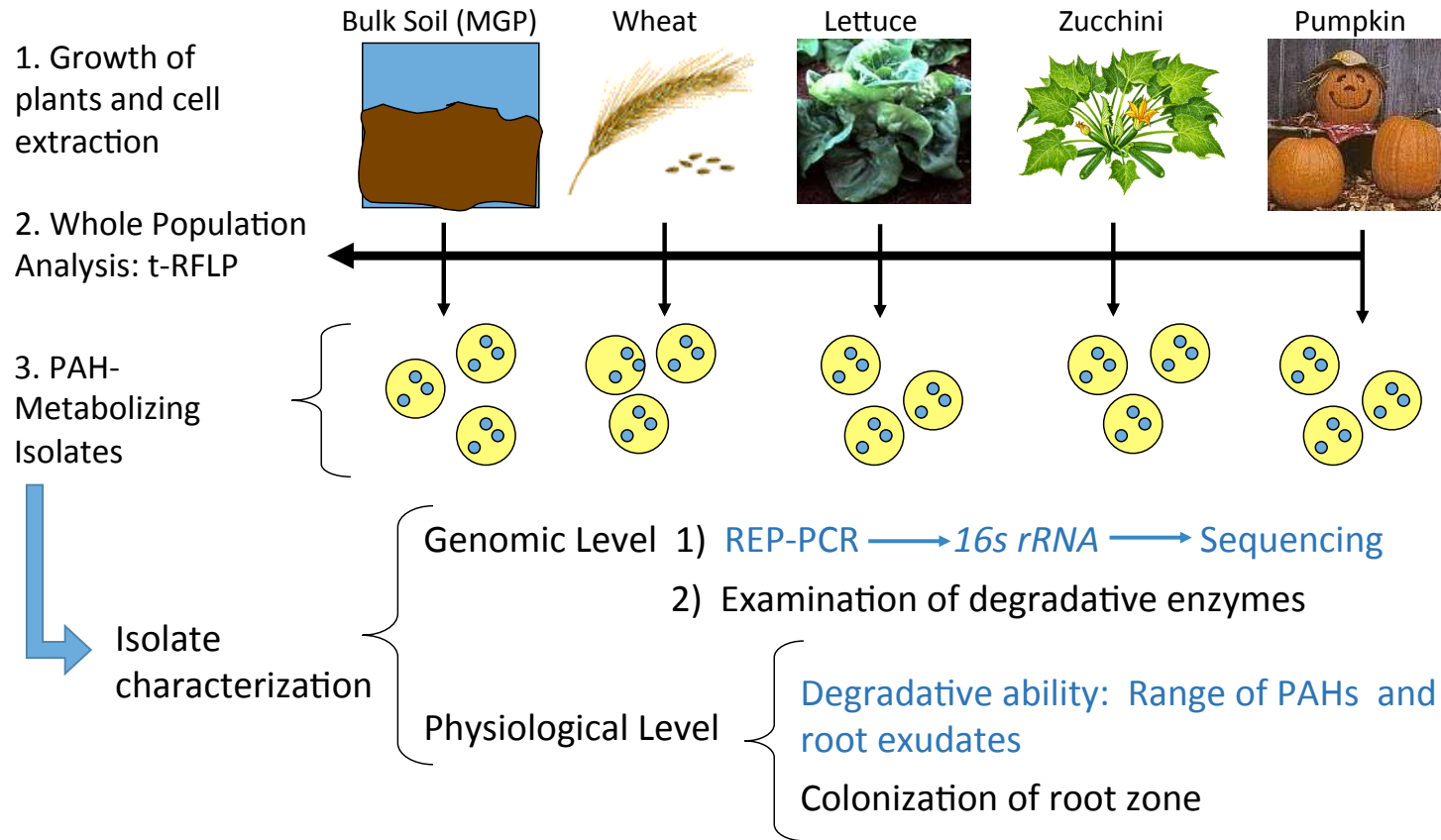
# Presentation Overview

- Research Topic in Environmental Biotechnology and Sustainable Design
- Performed under grant funding from EPA Star Program
- Based on publications in *Water, Soil and Pollution* and *International Journal of Phytoremediation*
  - Zelennikova, O., **C.S. Ely** and B.F. Smets. “Effects of PAH-contaminated Soil on Rhizosphere Microbial Communities” *Water, Air, and Soil Pollution* 2011.
  - **C.S. Ely** and B.F. Smets. “Bacteria from Wheat and Cucurbit Plants Metabolize PAHs and Aromatic Root Exudates: Implications for Rhizodegradation” *International Journal of Phytoremediation* 2017.
  - **C.S. Ely** and B.F. Smets. “Guild Composition of Rhizobacteria Shift in Response to Toxic Chemical Stress”
- Developed procedures to examine bacteria populations and metabolic activity
- Use procedures to understand how plants encourage changes in microbial communities and promote contaminant removal
  - Biotechnology procedures employed as means of “bio-sensing” changes occurring in soil
- Knowledge of stimulation mechanism can be applied to composting and other engineered bioreactors for different pollutants

# BioWin WWTP Design



# Experimental Approach

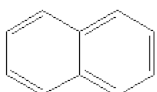


# Using PAHs as a Model Soil Pollutant

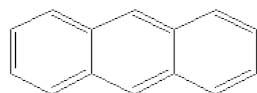
- Why focus on treating PAHs?

## Model Contaminant - Polycyclic aromatic hydrocarbons (PAHs):

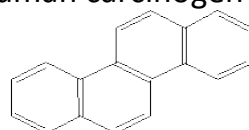
- Mutagenic and carcinogenic pollutants
- Ex. benzo[a]pyrene Category A human carcinogen – lung and kidney cancer



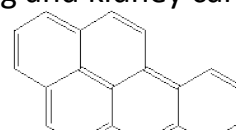
Naphthalene



Anthracene



Chrysene



Benzo[a]pyrene

## Manufactured Gas Plants

- Early 1800s to the mid-1900s manufactured gas plants (MGPs) produced gas from coal or petroleum for lighting, heating, and cooking
- Approx. 50,000 MGPs in operation
- 7-17.5 million m<sup>3</sup> total residual waste
- Cost of remediating all sites: \$26-\$128 billion
- Tar (“pitch”) waste dumped in pits, ponds, or rivers
- Polycyclic aromatic hydrocarbons primary constituents of pitch

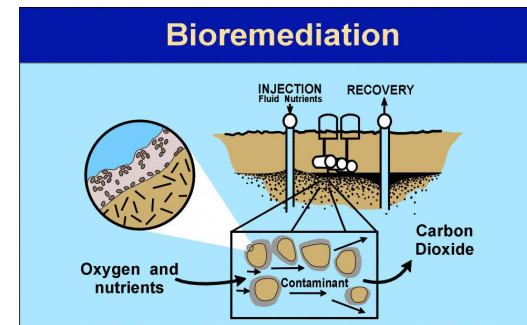
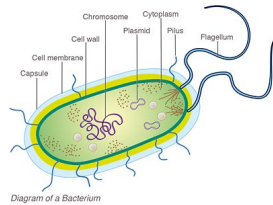
Sample of MGP locations



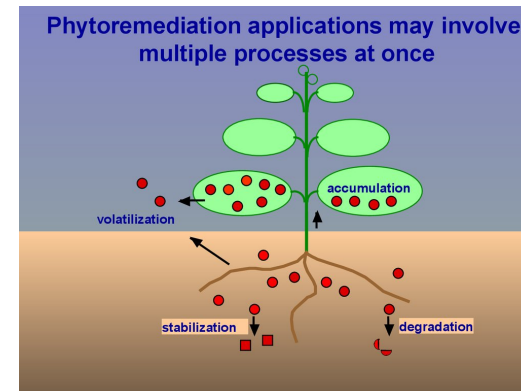
[http://www.hatheway.net/state\\_site\\_pages/pa\\_main.htm](http://www.hatheway.net/state_site_pages/pa_main.htm)

# Bioremediation and Phytoremediation

- Bioremediation
  - Use of microbes (bacteria) to degrade pollutants in soil or water
  - Bacteria can be naturally occurring or augmented
- Limits to Bioremediation
  - Food supply
  - Nutrition
  - Oxygen
  - **Stimulants**
- Phytoremediation
  - Use of plants to remove pollutants from soil or water through 3 primary methods
    - Extraction/accumulation/volatilization
    - Degradation
    - Containment/stabilization
- Defining Rhizodegradation
  - Roots grow into contaminated areas to provide access
  - Bacteria in plant root zone (rhizosphere) degrade pollutants
  - Degradation rates are increased by plant stimulation



<http://4.bp.blogspot.com/-ZutDbPCW9Q/TqfCuE9zJSI/AAAAAAAAALg/9Q59qZ8vk/s1600/bioremediation.jpg>

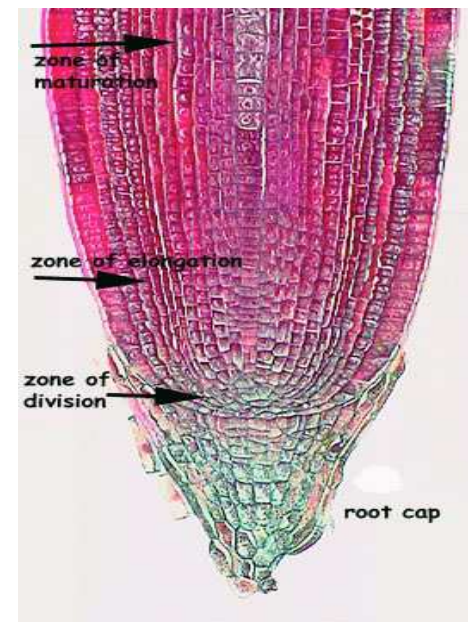


<http://cf.ppt-online.org/files/slide/z/ZmDdEi12cf5BQzHTn0FM7UsrqRNpY34hOWGSvV/slide-45.jpg>

# Why are Plant Environments More Active?

- Provides consortia of substrates/nutrients through roots and gas exchange
  - Amino acids, sugars, organic acids
  - Specialized exudates
    - Allelopathic
    - Antimicrobial
    - Stimulating
- Greater microbial population and activity than bulk soil
- Vast area for attachment/interaction

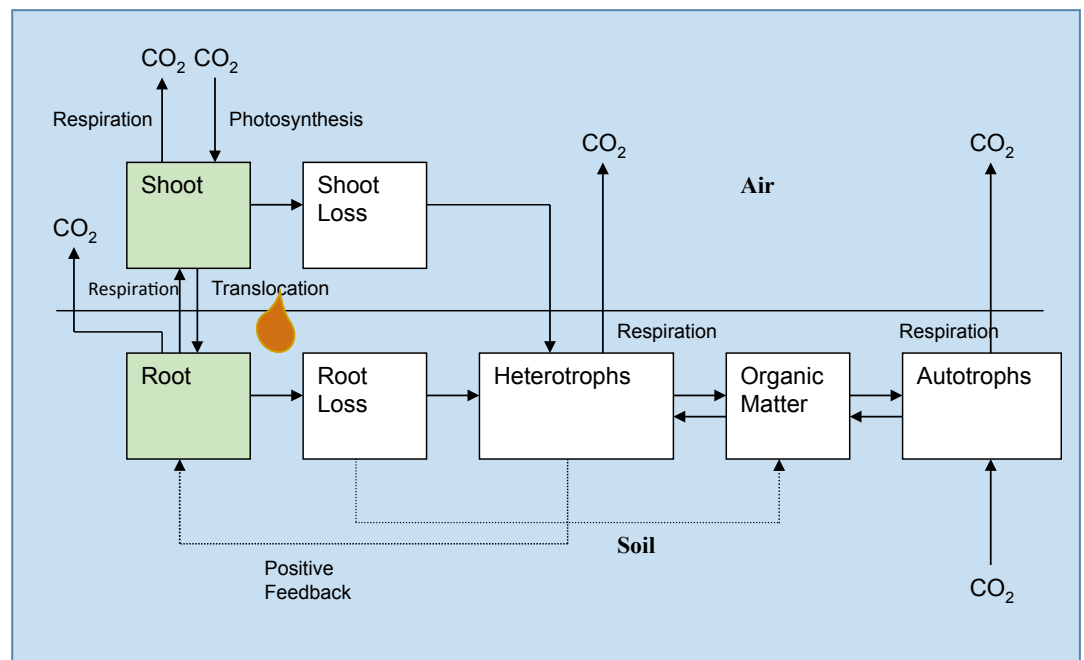
Wheat root at 40x magnification showing numerous root hairs protruding from main root



Plant root protruding into soil sloughs off cells from root cap, providing constant source of substrates/nutrients

# The “Tritrophic Trinity”

- Under normal conditions, plants establish feedback loops with bacteria in the root zone to maintain homeostasis
- The symbiosis between the host plant and rhizobacteria, in context with the surrounding soil has been termed the “tritrophic trinity” (Singer et al., Current Opinion in Microbiology)
- When pollutants are introduced, the plants experience phytotoxic effects and must find a mechanism to reduce the pollutant concentration
- Research in this field has shown that plants exude particular phytochemicals (“exudates”) which aid in the rhizodegradation process
- The exudates which stimulate bacteria to metabolize the pollutant are similar in structure to catabolic intermediates of PAH degradation



Carbon flow in the plant and soil. (Adapted from Whipps, The Rhizosphere)

\*Heterotrophs obtain carbon and energy from organic compounds

\*Autotrophs obtain carbon from CO<sub>2</sub> and energy from inorganic compounds



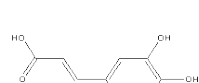
# Experimental Setup

- Plants grown in MGP soil
  - From former MGP site in Winsted, CT
  - Contaminated with 760 ppm PAHs
- Plants harvested after 2 months
  - Roots separated and sonicated in buffered solution to remove bacterial cells
- Bacteria inoculated on solid plates amended with a coating of PAHs
  - Bacteria selected based on metabolism of PAHs, indicated by clearing zone around colony

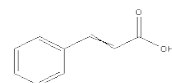


# Catabolic Pathways for PAH Degradation

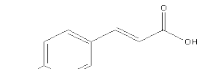
- Catabolic pathways follow complex enzymatic process which begins with ring-hydroxylating dioxygenases and proceeds to pyruvate and simple phenolics
- Root exudates which stimulate this degradative process are aromatic (flavonoids and simple phenolics) and induce translation of genes for enzymes in the pathway



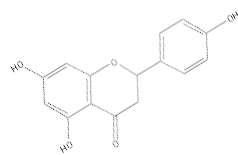
### Caffeic Acid



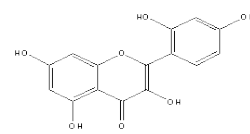
### Cinnamic Acid



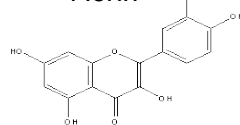
Coumaric Acid



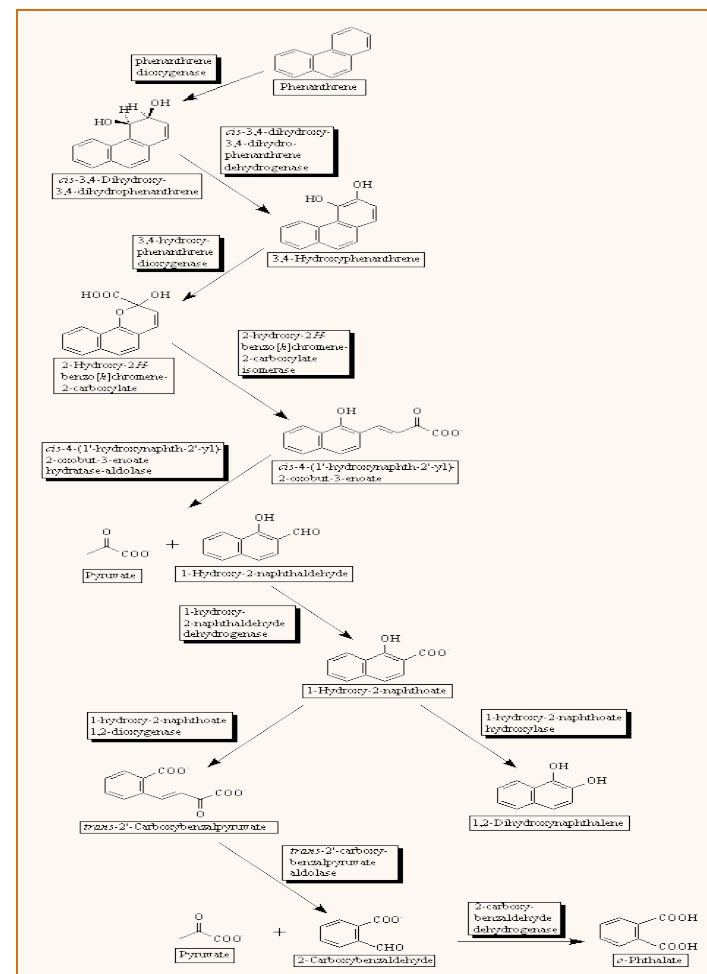
## Naringenin



Morin



## Quercetin



Catabolic pathway for phenanthrene degradation (Biocatalysis and Biodegradation database, <http://umbbd.ahc.umn.edu>).

# Metabolic Assay Results

Compound	Wheat	Cucurbits	Weighted Avg.
Anthracene	40.8	44.0	41.9
Chrysene	30.6	48.0	36.5
Fluoranthene	69.4	44.1	60.9
Phenanthrene	46.9	48.0	47.3
Pyrene	46.9	48.0	47.3
Protocatechuate	98.0	100.0	98.7
Gentisate	26.5	48.0	33.8
Salicylate	4.1	0.0	2.7

Pink = PAHs, Purple = PAH catabolic intermediates

The simple phenolic caffeic acid shows almost 100% metabolism by bacteria tested. Similar result to protocatechuate, which is a catabolic intermediate of anthracene and phenanthrene.

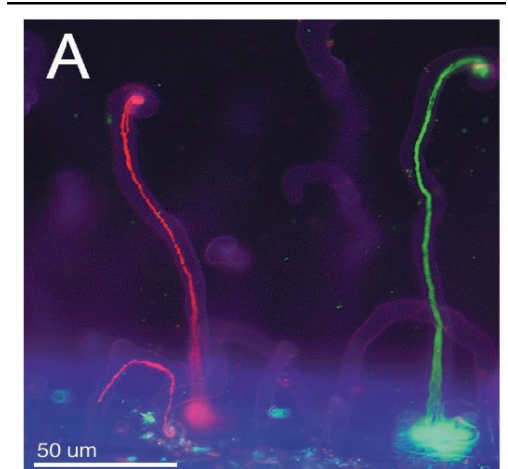
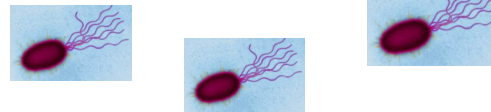
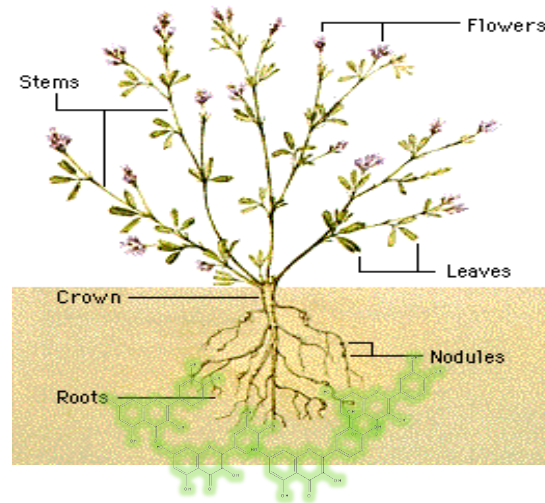
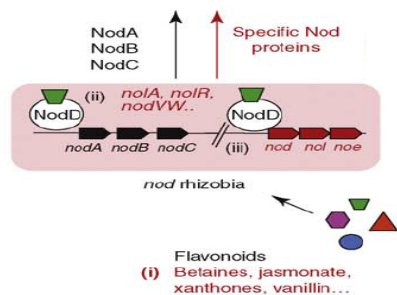
Compound	Wheat	Cucurbits	Weighted Avg.
Caffeic Acid	98.0	100.0	98.6
Cinnamic Acid	71.4	88.0	77.0
Coumaric Acid	79.6	80.0	79.7
Naringenin	53.1	80.0	62.2
Quercetin	89.8	92.0	90.5
Morin	93.9	100.0	96.0
Gibberellic Acid	32.7	56.0	40.5
Indole-acetic Acid	22.5	8.0	17.6

Blue = simple phenolics, Green = flavonoids, Orange = Plant hormones

The flavonols quercetin and morin show high rates of metabolism by bacteria tested (90.5% and 96%). These high rates suggest that there is a link between PAH degradation and the presence of aromatic exudates.

# Stimulation Example: Nodulation

- Plants (legumes) that require nitrogen will stimulate bacteria to infect the root and form nodules
  - Inside the nodules the bacteria will fix nitrogen, thereby supplying it to the plant
  - Plants exude isoflavonoids from the root which then induce nodulation genes
  - Genes are translated and transformed in to proteins for necessary components of infection thread and nodule formation

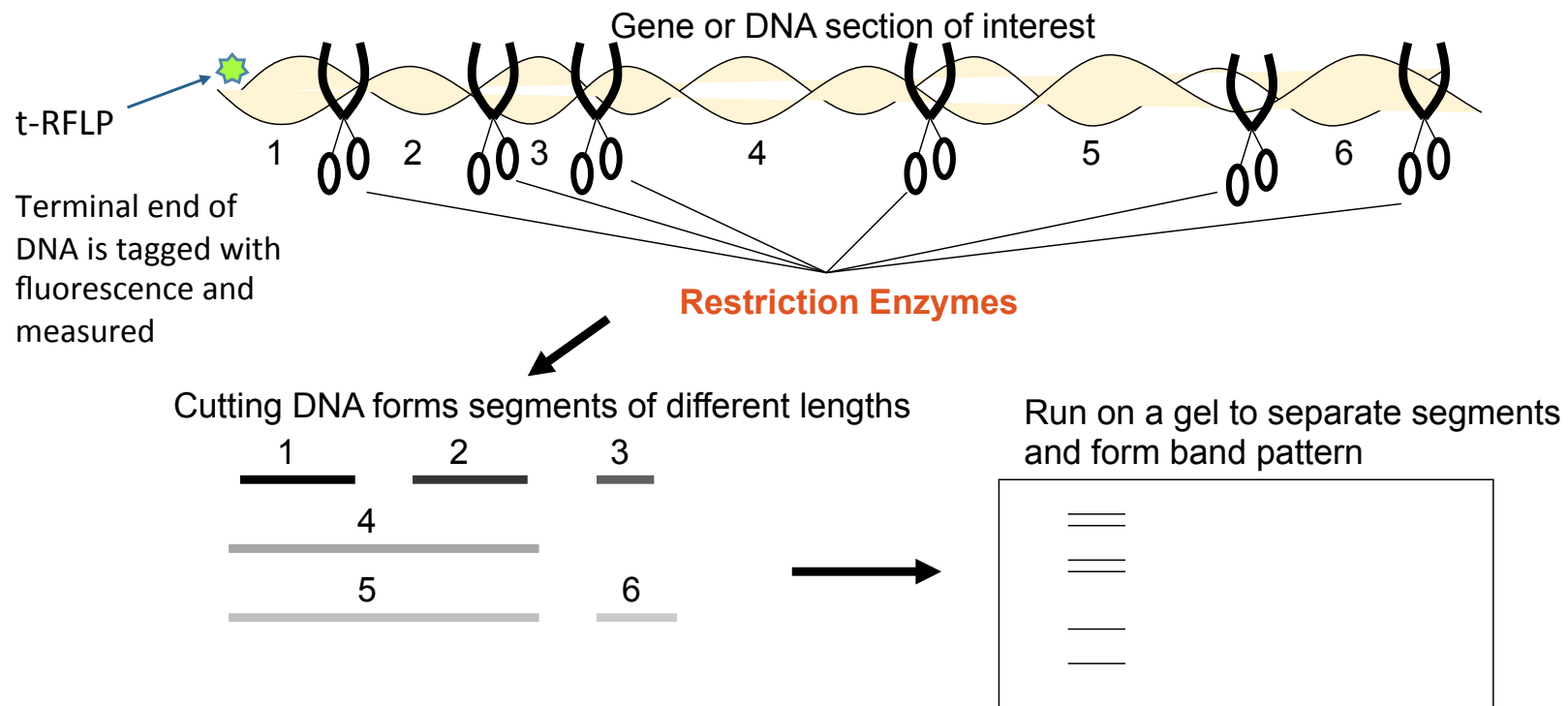


Microscopic photo showing the infection threads for two types of rhizobia – one tagged with red fluorescent protein and one with green fluorescent protein. (Dr. Dan Gage, Uconn, Molecular and Cellular Biology)

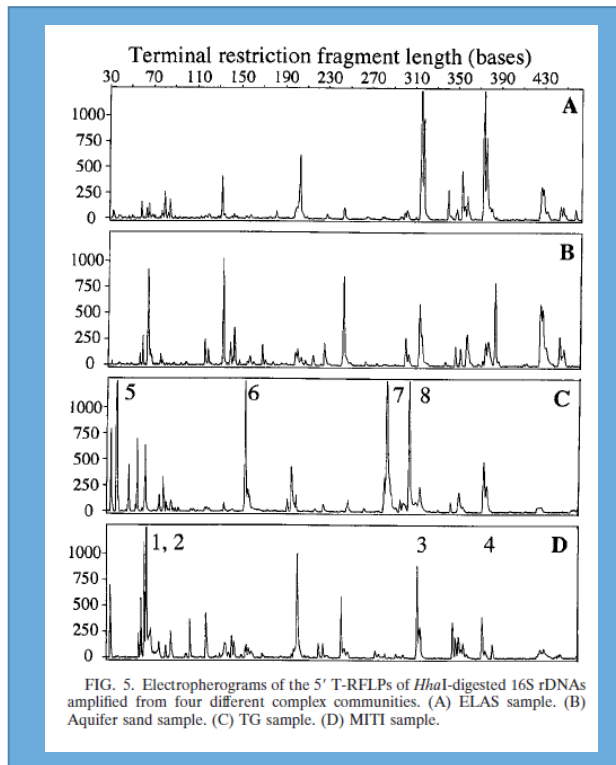
# Biosensing Techniques

- Terminal Restriction Fragment Length Polymorphism (t-RFLP)
  - Whole bacteria population (uncultured)
- REP-PCR genome fingerprints
  - PAH-metabolizing isolates from host plants (cultured)
- 16s rRNA gene PCR sequencing and species identification
  - PAH-metabolizing isolates from host plants (cultured)
  - Performed on each unique genome fingerprint

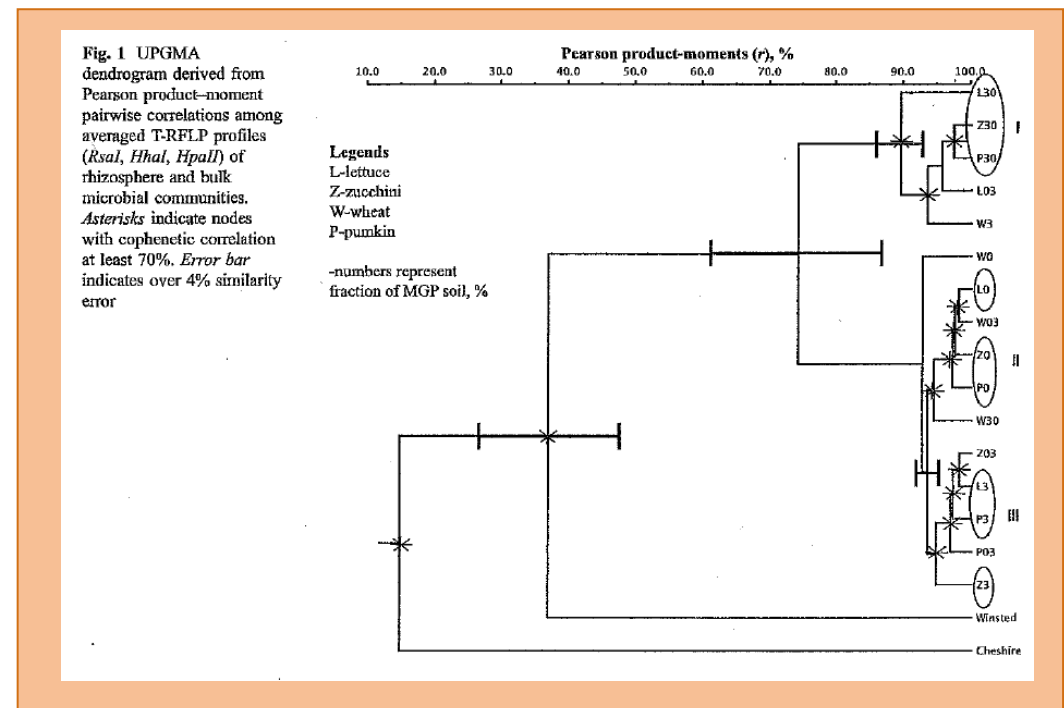
# Restriction Fragment Length Polymorphism



# t-RFLP Results

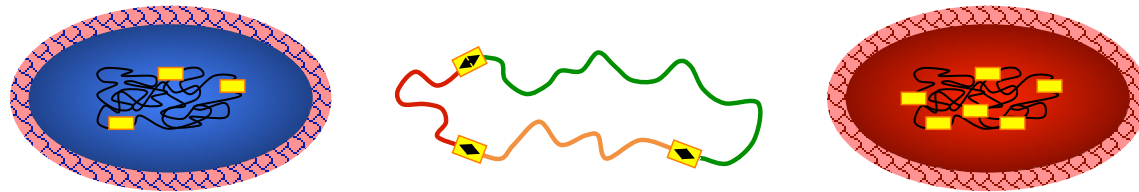


Liu et al. Applied and Environmental Microbiology, Vol. 63, 1997.

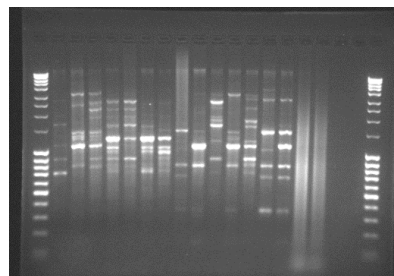


Zelennikova, Ely, and Smets. Water, Air, and Soil Pollution, 2011.

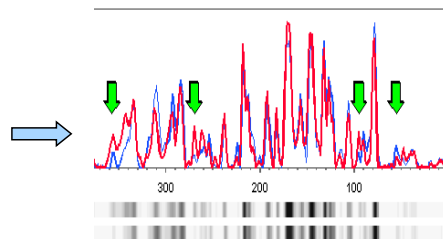
# Typing Strain Diversity: Genome Fingerprints



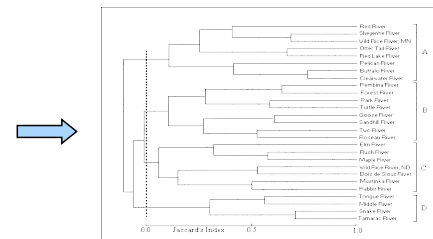
- REP-PCR fingerprints: genome wide fingerprint based on PCR targeting repetitive sequences on the chromosome
- Pearson's product-moment correlation coefficient: Similarity Coefficients
- UPGMA (unweighted pair group method with arithmetic mean) algorithm: Cluster analyses



Gel Images



Pattern analysis



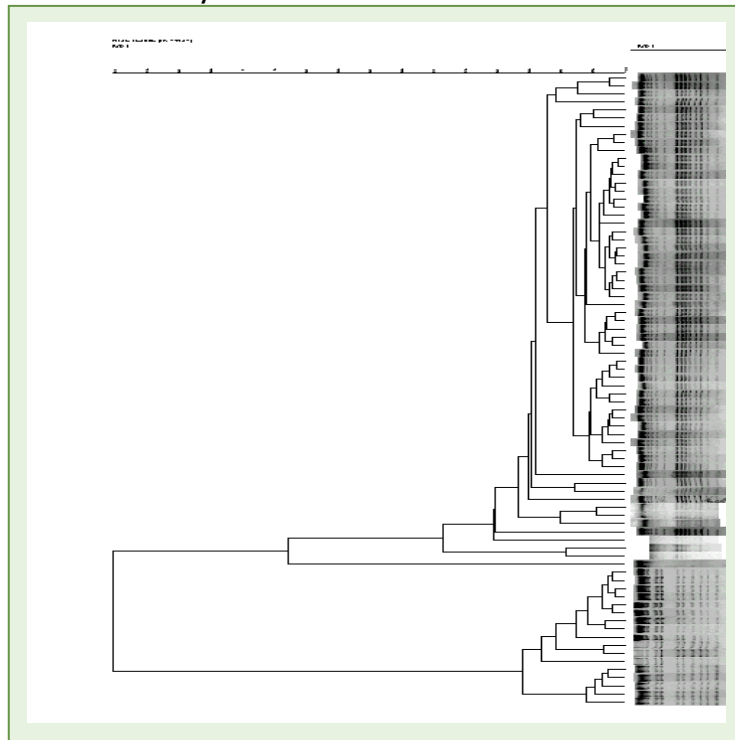
Cluster analysis

Oda, Y., W. Wanders, et al. (2002). Applied and Environmental Microbiology **68**(7): 3467-3477.



## REP-PCR Results

By Treatment: 0% MGP



### By Host Plant Type: Lettuce and Pumpkin, 3% MGP



# Identifying Isolated Bacteria using 16s rRNA gene

Each unique cluster  
from REP-PCR



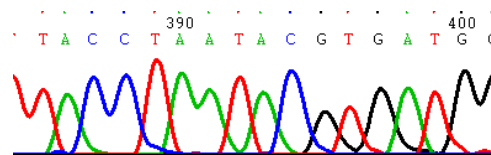
16S rRNA  
gene PCR



Sequencing  
(Macrogen)



Sequence editing (Chromas)

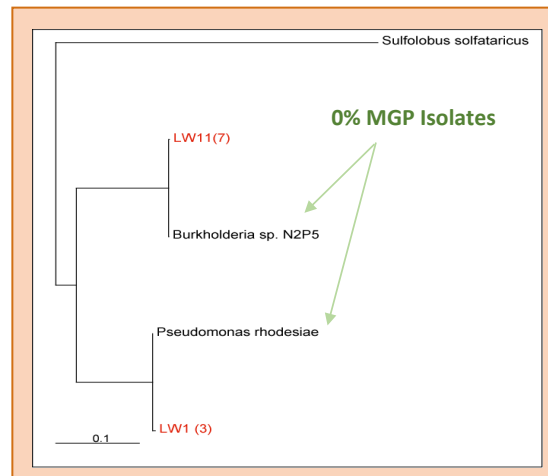


Sequence alignment in  
Ribosomal Database Project

My seq. --	AGGGNTACCGCCGAT	} RDP Sequences
1 <sup>st</sup> neighbor --	AGGGTTACCGCCGAT	
2 <sup>nd</sup> neighbor --	AGGGTTACCGCCGAT	
3 <sup>rd</sup> neighbor --	AGGGTTACCGCCGAT	



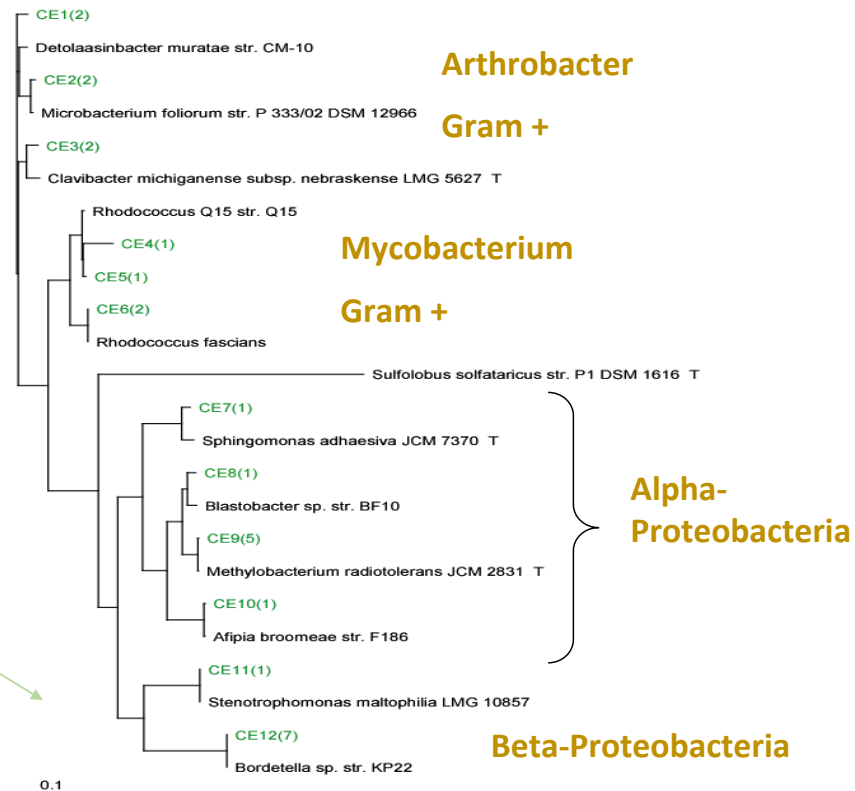
Phylogenetic Tree  
showing similarity to  
known sequences  
(Identification)



# 16s rRNA gene sequencing and identification

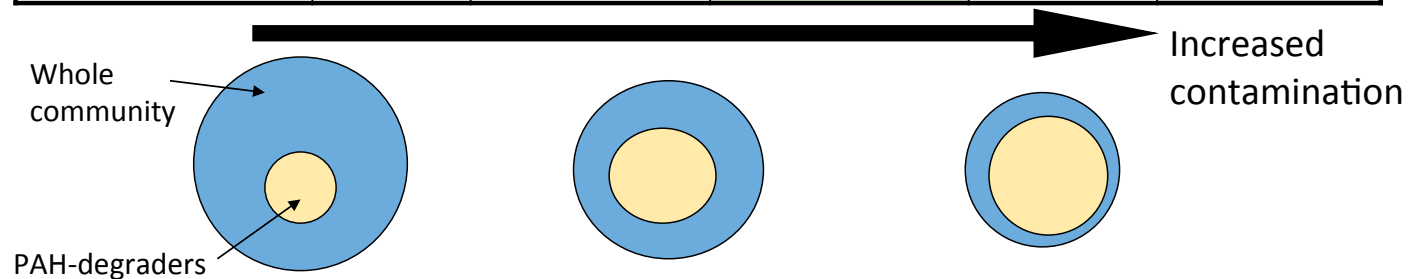
- As PAH contamination increases, more species in Gram + and Alpha-proteobacteria are found

30% MGP Isolates



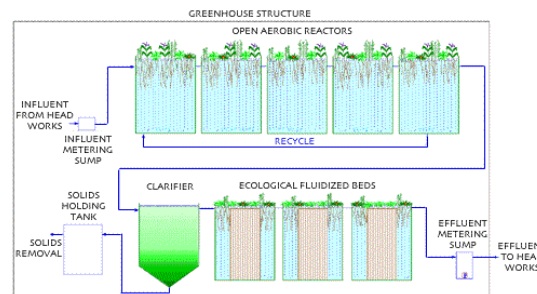
## Diversity of PAH-degraders changes with higher % contamination

% Rhizosphere Contamination	Total Isolates	REP-PCR Fingerprints	Isolates per Fingerprint	16s rDNA Species	Isolates per Species
0	139	12	11.6	2	69.5
3	386	86	4.5	19	20.3
30	90	39	2.3	12	7.5



# Conclusions and Applications

- Numerous bacteria found in plant rhizospheres which can degrade toxic PAH soil pollution
- These bacteria not only degrade PAHs but also metabolize simple phenolics and flavonoids high rates (ex. caffeic acid, quercetin, and morin)
  - This finding implies that these aromatic root exudates stimulate the PAH catabolic pathway in a similar manner to the stimulation process in nodulation
- Bacterial communities change in response to contamination level and differ from those in bulk soil
- Bio-Engineered Composting System
  - Treatment of toxic wastes in composting process through augmentation
  - Addition of bacterial species that can quickly degrade pollutant
  - Addition of stimulant phytochemicals to enhance the rate and degree of degradation
- Biological Water Treatment
  - Similar to Wastewater Treatment
- Techniques can “biosense” changing bacterial communities in different settings



# Acknowledgements

- Dr. Barth Smets, PI – Technical University of Denmark
- Co-advisors: Dr. Thomas Wood and Dr. Dan Gage at UConn
- Grants: GAANN Environmental Biotech. and EPA Star Program
- Collaborators: Dr. Jason White and colleagues at the Connecticut Agricultural Experiment Station
- Undergraduate researchers: Laura Waters, Jocelyn Michellini, Christopher Shores, and Bill Fusco

