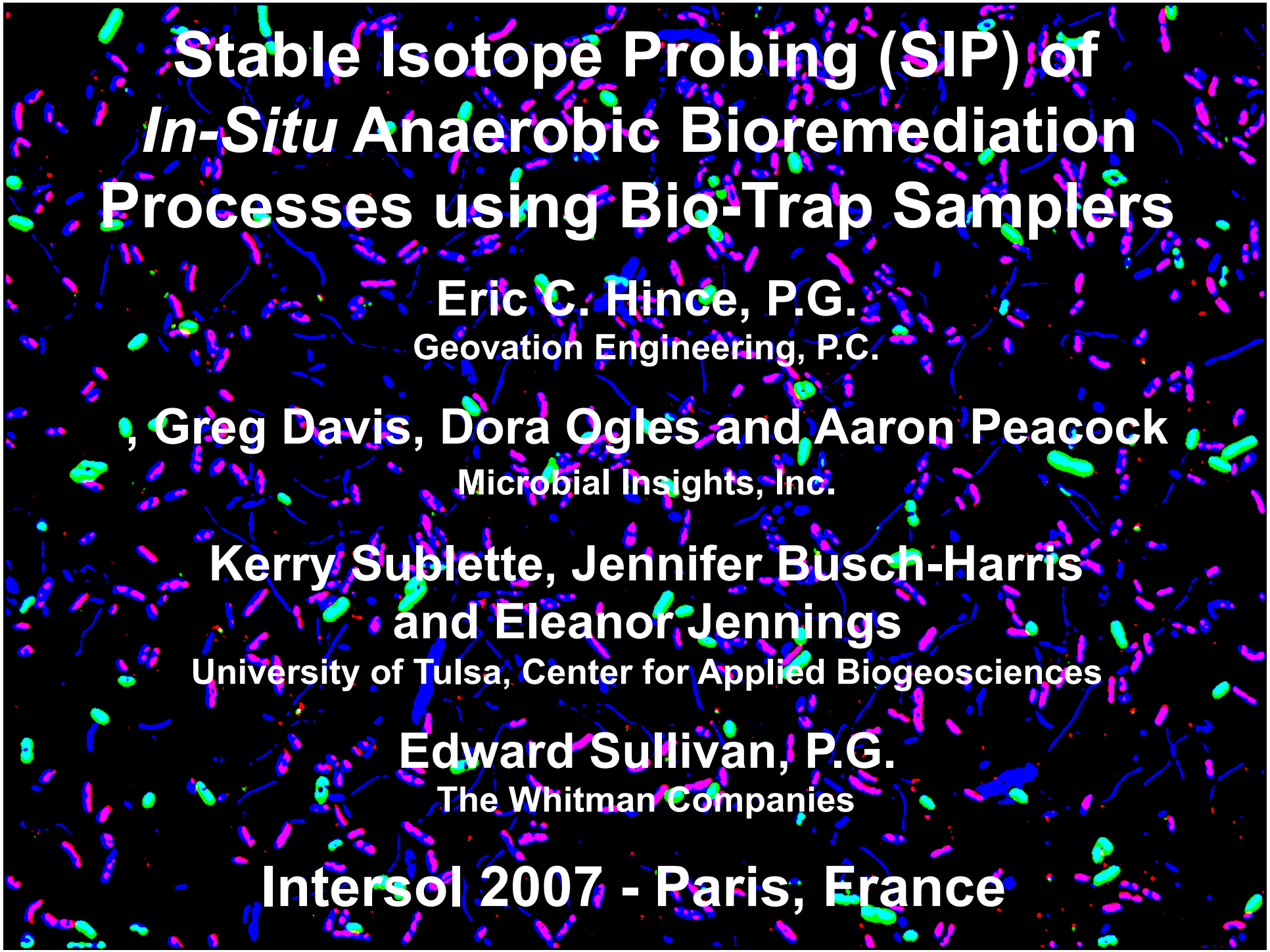

Stable Isotope Probing (SIP) of *In-Situ* Anaerobic Bioremediation Processes using Bio-Trap Samplers

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Geovation Engineering, P.C.

, Greg Davis, Dora Ogles and Aaron Peacock
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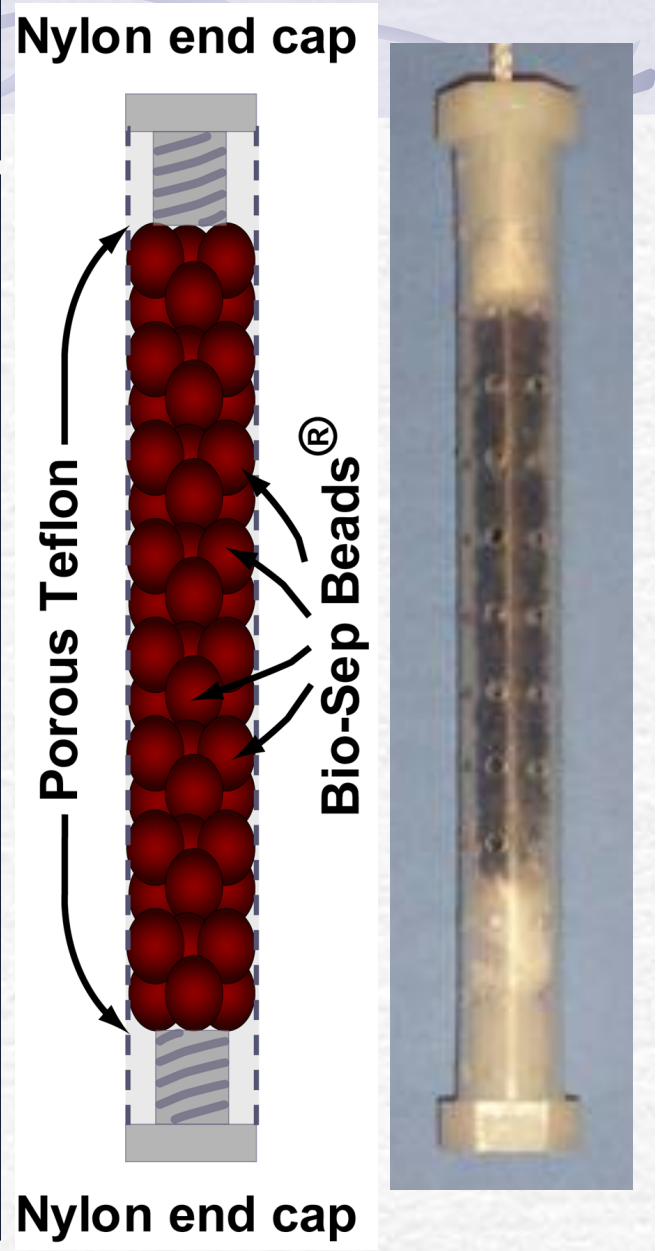
Bio-Trap Samplers

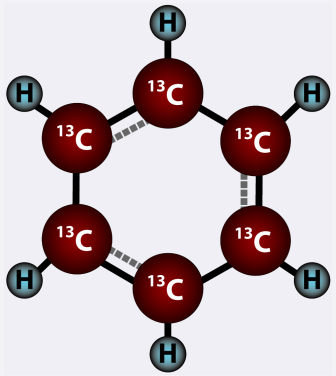
- Device for *in-situ* collection of microbial biomass
- Microbiological monitoring of:
  - Monitored Natural Attenuation
  - Enhanced Bioremediation
    - Biostimulation
      - DBB Program
      - SRC biobarrier
      - DBB/SRC Source Area Pilot
    - Bioaugmentation



Investigation of Contaminant Fate

- **Stable isotope probing (SIP)**: Bio-trap Samplers are pre-loaded with a known amount of a ^{13}C-labeled contaminant (or surrogate) of interest

^{13}C Benzene



- SIP surrogates are specially produced "heavy" compounds comprised of a 99% ^{13}C carbon content
- Molecular tracing of ^{13}C into biomarkers (PLFA, DNA, RNA) provides understanding of contaminant fate

The $^{13}C/^{12}C$ Isotope Ratio

- The amount of ^{13}C relative to ^{12}C in a sample is expressed by the $\delta^{13}C$ notation where

$$\delta^{13}C\,[‰] = \left(\frac{(^{13}C/^{12}C)_{\text{Sample}}}{(^{13}C/^{12}C)_{\text{Standard}}} - 1 \right) \cdot 1000$$

- The standard is a specific carbon-containing mineral from a specific location: Pee Dee Belimnite (PDB)
- $\delta^{13}C$ is delta C thirteen and units are °/oo or "per mill"

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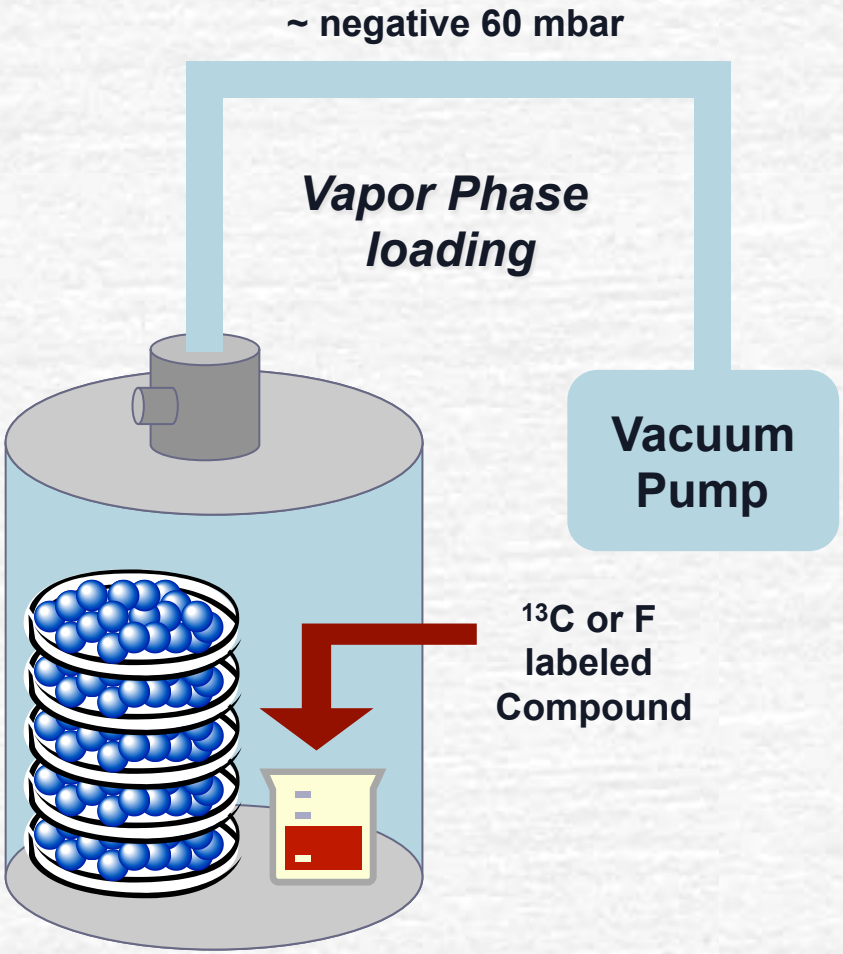
Loading Bio-Sep Beads with SIP Compounds, F-Analogs

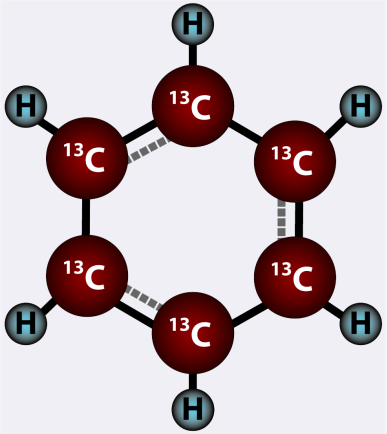


- ^{13}C-labeled compounds:
  - Benzene
  - Acetate
  - MtBE
  - cis-1,2-DCE
- Fluorinated analogs:
  - TCFE (F analog of TCE)
  - DCFE (F analog of cDCE)

SIP and FAP Compounds (This Study)

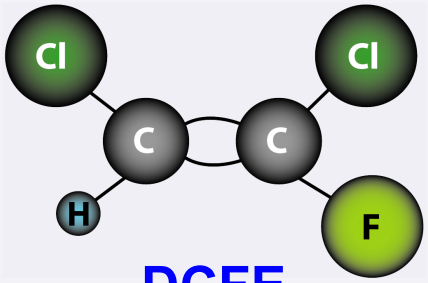
Site 1: DBB Treatment of Gasoline Plume

SIP using ^{13}C Benzene (right) to investigate the anaerobic oxidation potential of Benzene in a gasoline-contaminated aquifer undergoing denitrification-Based bioremediation ("DBB")



^{13}C Benzene

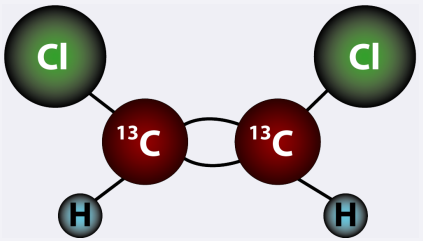
Sites 2 & 3: SRC/DBB Biostimulation of Chloroethene Plume



DCFE

cis-1,2-dichloro-fluroethene

SIP using ^{13}C-cDCE (right) and FAP using DCFE (left) to evaluate respective rates of anaerobic oxidation and reductive dechlorination in response to combined SRC / DBB treatments



^{13}C cDCE

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Loading of Bio-Sep Beads

- Surrogate compound can be loaded at different concentrations
- Minimal leaching of most compounds

Results of Leach Testing of Benzene-loaded Bio-Sep Beads

Sample Type	Initial	Day 15	Day 30
Bio-Sep Beads	1.05±0.04 mg/bead	0.99±0.02 mg/bead	0.97±0.03 mg/bead
Sterile Water	0.00099 mg/L	0.00098 mg/L	BDL

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Analysis of Bio-Trap Samplers

Molecular Biological Tools

- PLFA
- DGGE
- Real-time PCR (DNA or RNA)

Assessment of Contaminant Fate

- SIP – trace incorporation of ^{13}C into PLFA biomarkers (GC/IR-MS)
- FAP - Measure parent/daughters of fluorinated analogs of chlorinated compounds (GC/MS)

Case Study:
^{13}C-Labeled Benzene Bio-Trap Monitoring
DBB Treatment of BTEX Plume (DoD Site, ME)

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Denitrification-Based Bioremediation ("DBB™") Technology

- DBB is an alternative to aerobic bioremediation, chemical oxidation for treatment of hydrocarbon source-areas
- DBB uses nitrate reduction as the primary driver for microbial respiration and bioremediation processes
- DBB™ biogeochemistry, microbial ecology are advantageous for subsurface bioremediation
  - Practical, cost-effective means to deliver stoichiometric requirement of electron acceptors (nitrates)
  - N-blend (nitrate) diffusion rates much greater than for active oxygen, most oxidants
  - Can treat residual NAPLs, aquifer media with high sorbed-phase mass / concentrations

Comparison of Microbial Electron Acceptors

Electron Acceptor	Microbial Respiration Process	Gibbs Free Energy KJ/mole CH_2O	Eh/ORP (mV)	Solubility (mg/L)
O_2	Aerobic	-475	300	6
NO_3^-	Denitrification	-448	200	> 600,000
Mn(IV)	Mn(IV) Reduction	-349	100	Insoluble Mn(IV) minerals
Fe(III)	Fe(III) Reduction	-114	-70	Insoluble Fe(III) minerals
$SO_4^=$	Sulfate Reduction	-77	-210	> 1,300
CO_2	Methanogenesis	-58	-250	1,900

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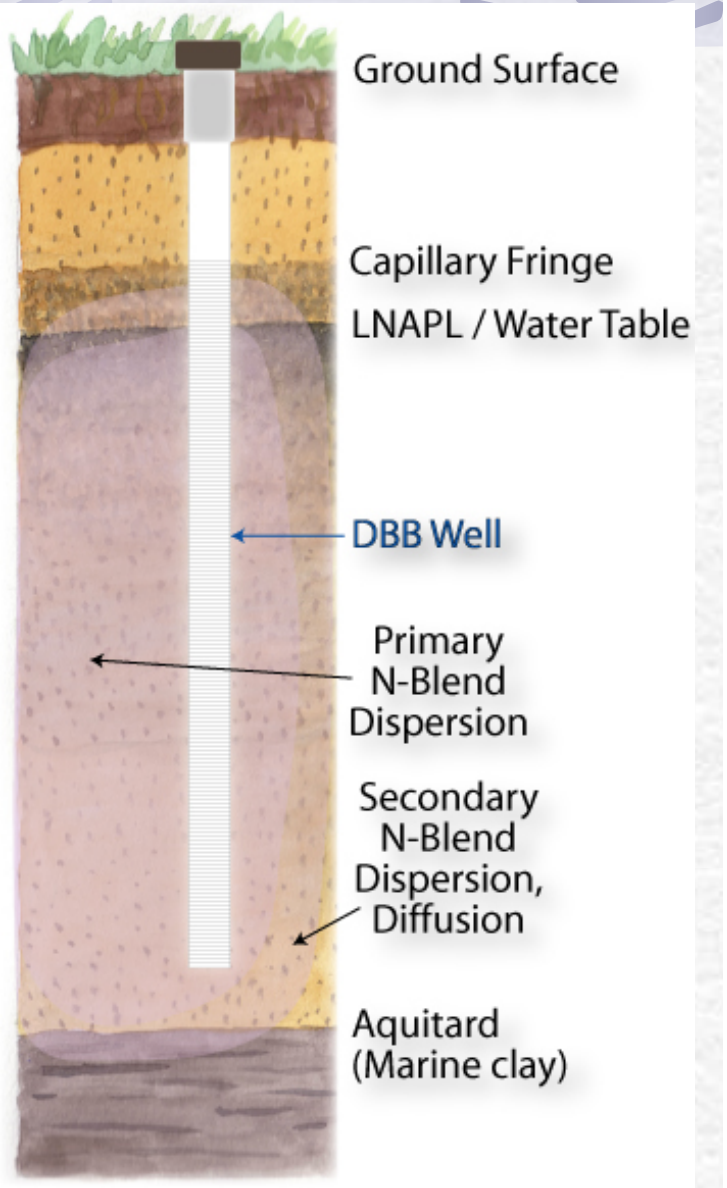
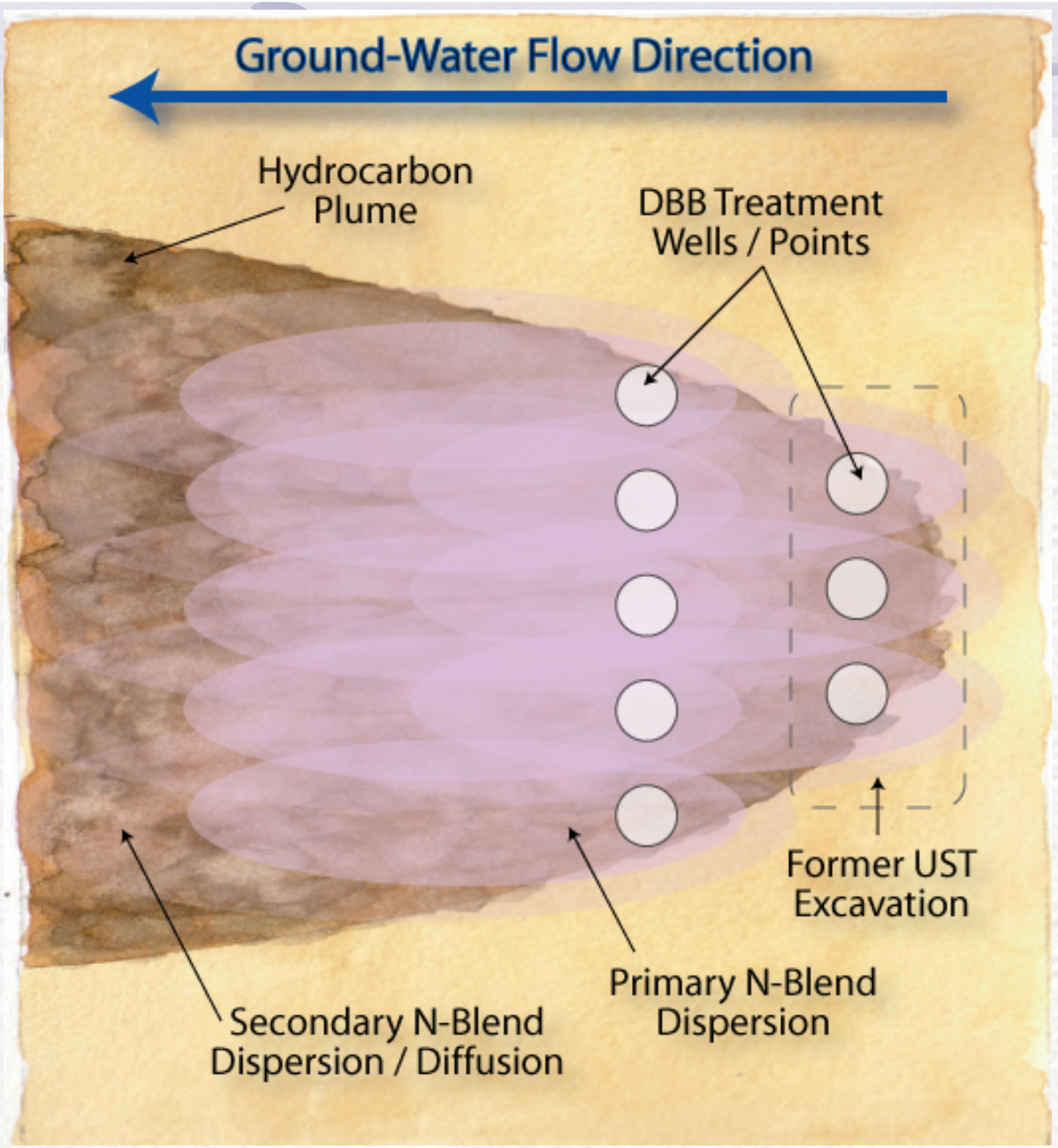
DBB™ Implementation

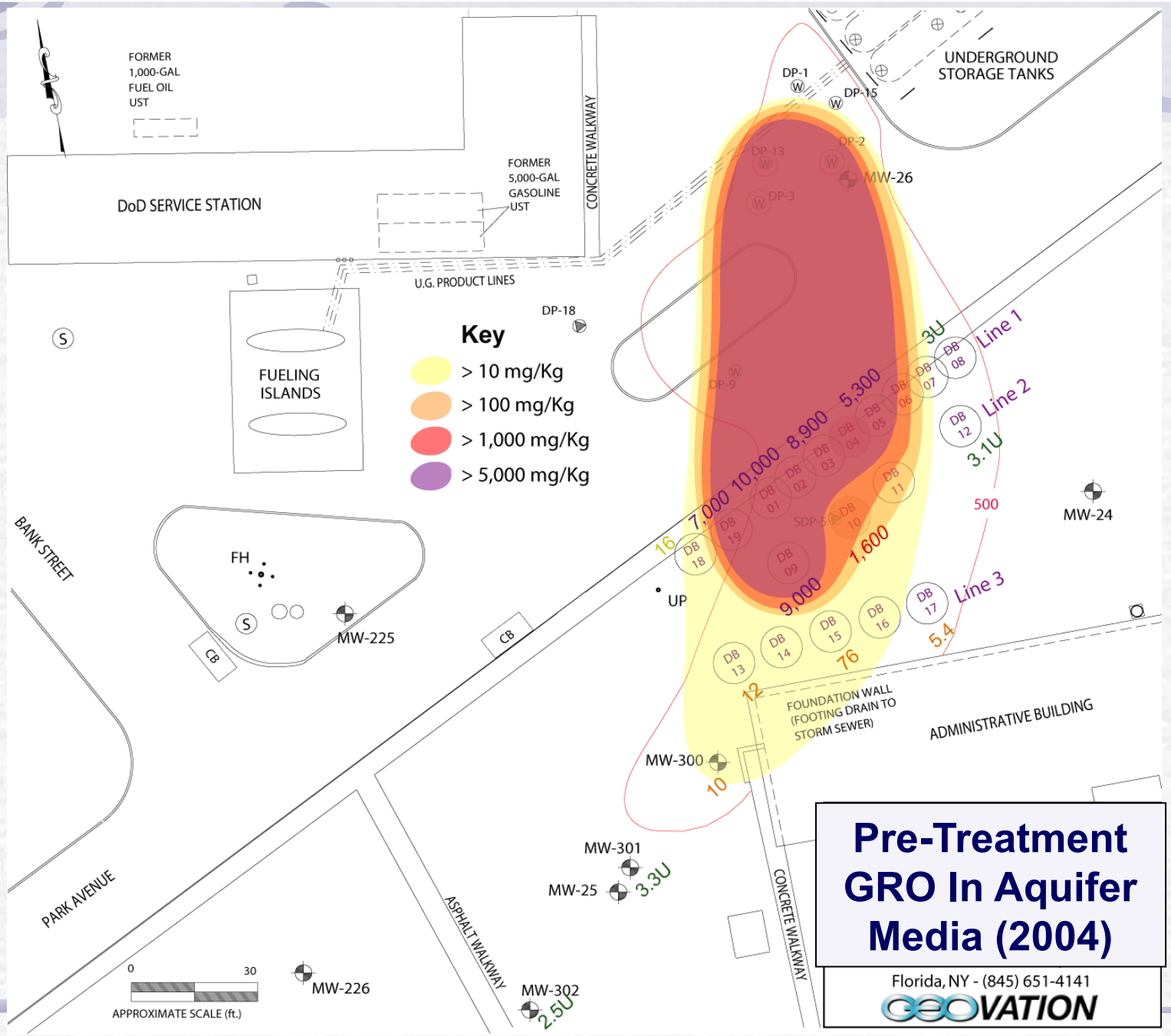
- Goal: optimize N-blend (nitrate + nutrient) addition to meet biological demand created by sorbed-phase hydrocarbon mass
- Target treatment of residual product and sorbed-phase hydrocarbon mass in the smear zone and saturated zone
- Treatment before/during high water table will help to treat capillary ("smear zone")





Overview of DBB™ Pilot Program: Gasoline Plume, DoD Service Station, ME

- **DBB/N-Blend** treatments:
  - 11 major N-Blend treatment events conducted from 11/04 - 4/06
  - Most treatment/monitoring in zone of high residual sorbed-phase GRO mass within and downgradient of source area
  - Up to ±320 Gal. N-Blend injected in plume per event
  - N-Blend concentrations / volumes increased over time
- Chemical (gasoline), biogeochemical and microbiological monitoring conducted before and during DBB program
- ^{13}C-labeled benzene **Bio-traps** installed in DP-13, DB-04 from 11/05 – 12/05 (approx. 11 weeks after last N-blend treatment)



Pre-Treatment GRO In Aquifer Media (2004)

Florida, NY - (845) 651-4141

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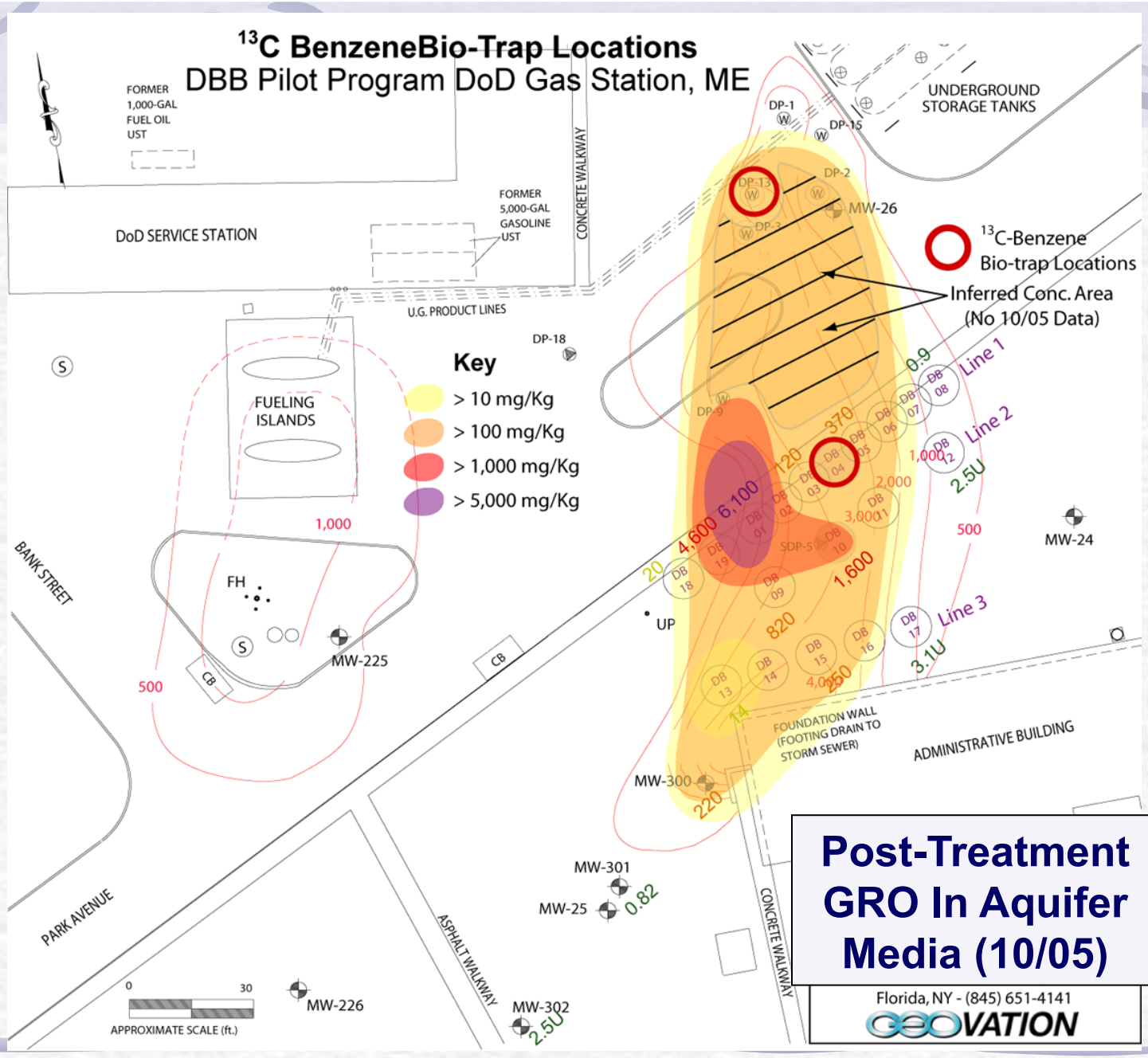

Objectives of ^{13}C-Benzene Bio-Trap Investigation of DBB™ Process

- Prior "conventional wisdom" suggested benzene was recalcitrant under denitrifying conditions
- However, past DBB experience has not shown benzene to be recalcitrant -- could we prove it?
- Coates et al. (2005) reported finding of anaerobic benzene oxidation coupled to denitrification by *Dechloromonas* spp.
- Stable isotope probing (SIP) with Bio-traps could:
  - test hypothesis that DBB stimulates anaerobic benzene oxidation
  - prove DBB degrades benzene by tracing incorporation of ^{13}C from ^{13}C-benzene into PLFA biomarkers

^{13}C-Benzene Bio-Trap Findings

- Approximately 78% (DP-13) and 43% (DB-04) of the ^{13}C-labeled benzene was degraded in 30 days
- Biomarkers associated with the *Proteobacteria* dominated the PLFA profiles from both wells
- Predominant DGGE sequences: *Gammaproteobacteria* (*Pseudomonadaceae*) and *Betaproteobacteria* (*Comamonadaceae*)
- *Gammaproteobacteria* and *Betaproteobacteria* numerically dominant (mFISH)
- *Betaproteobacteria* generally more numerous than *Gammaproteobacteria* (mFISH)

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^{13}C-Benzene Bio-Trap Conclusions

Benzene degradation, δ-^{13}C enrichment

- Clear evidence of benzene degradation coupled to DBB (Among the most conclusive SIP Bio-trap results to date)
- Benzene degradation, ^{13}C enrichment of biomass proportionately higher in DP-13 than DB-04

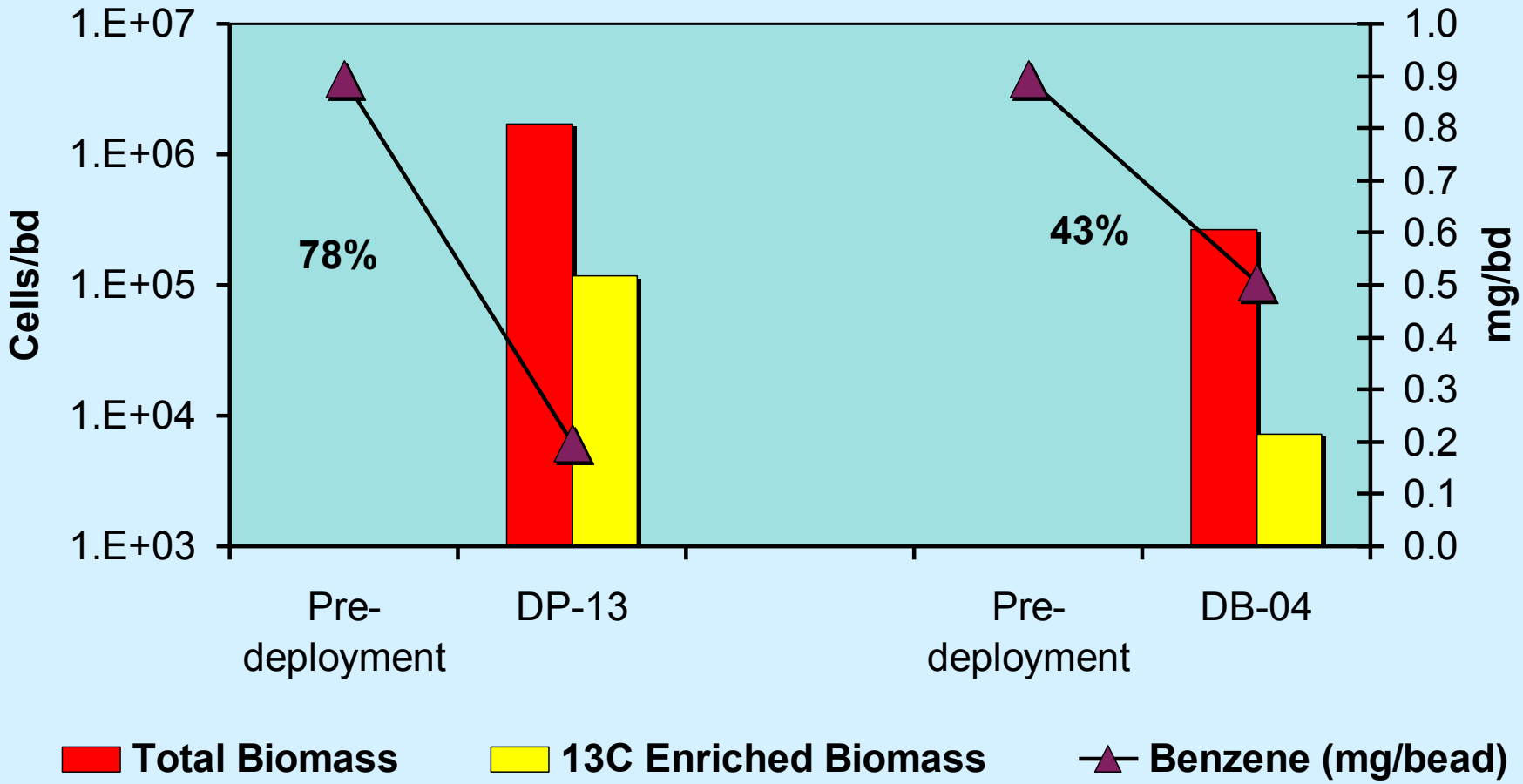
Positive Correlations:

- Contaminant mass / concentration in aquifer
- Biodegradation / loss of ^{13}C-labeled benzene
- PLFA biomass
- δ-^{13}C enrichment of lipid biomarkers
- total microbial cell counts in groundwater (DAPI staining)

^{13}C-Benzene Bio-Trap PLFA Data Summary

Sample Name	Pre-Deployment	DP-13	DB-04
Sample Date		12/28/2005	12/28/2005
No. Beads		245	257
Total Picomoles of PLFA		20,799	3,414
Biomass Concentrations			
Total Biomass		1.70E+06	2.65E+05
13C Enriched Biomass		1.18E+05	7.14E+03
% 13C Incorporation		7%	3%
Contaminant Concentration			
Benzene (mg/bd)	0.893	0.198	0.506
	0.04	0.02	0.05
% loss		78%	43%
Community Structure: (% of Total PLFA)			
Firmicutes (TerBrSats)		1.1	4.7
Proteobacteria (Monos)		64.8	64.6
Anaerobic metal reducers (BrMonos)		0.6	2.3
Actinomycetes (MidBrSats)		2.5	4.5
General (Nsats)		14.0	17.8
Eukaryotes (polyenoics)		17.0	6.2
Metabolic Status: (Ratio)			
group A (cy17:0/16:1w7c)	Growth Rate	0.37	0.63
group B (cy19:0/18:1w7c)	Membrane Permeability	0.05	0.07

^{13}C-Benzene Degradation and ^{13}C Enrichment of Biomass



^{13}C-Benzene Bio-Trap Results

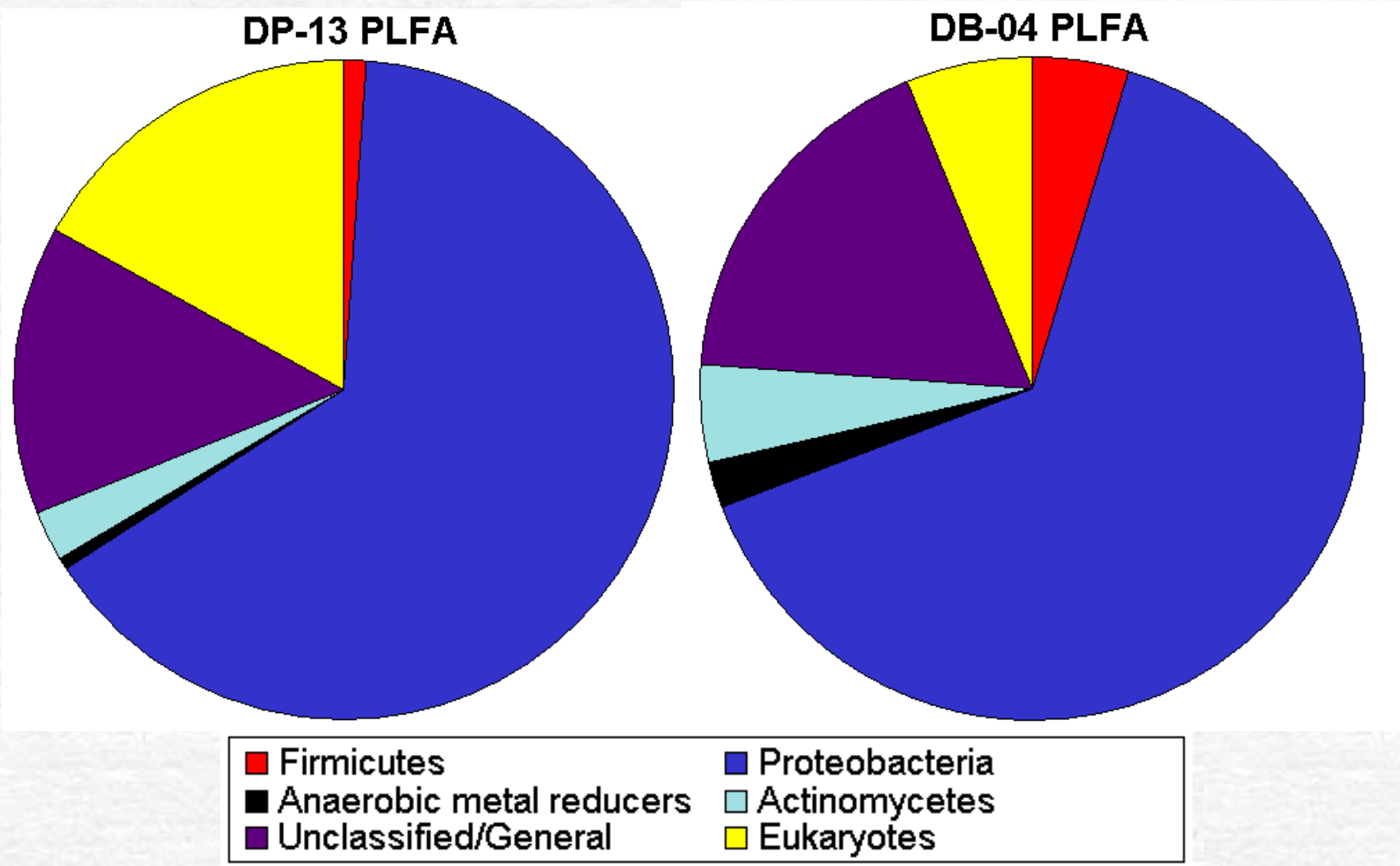
Benzene loss

- DP-13: 78% benzene loss ; 1° rate = 0.050 day^{-1}
- DB-04: 43% benzene loss ; 1° rate = 0.019 day^{-1}

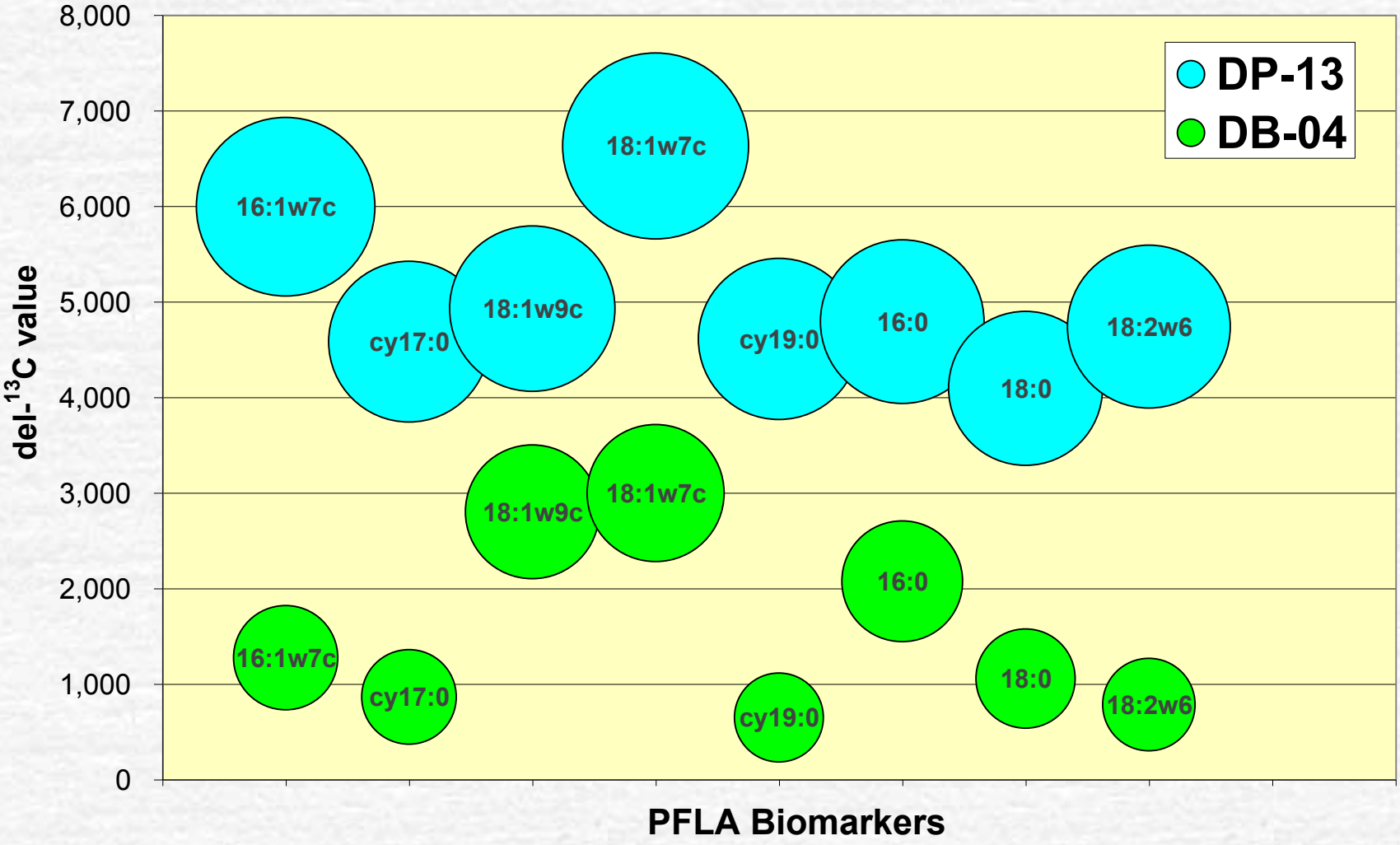
δ-^{13}C enrichment of PLFA biomarkers

- DP-13: Several PLFA markers exhibited values approaching theoretical maximums.
  - δ-^{13}C of *Proteobacteria* biomarkers: **16:1ω7c** (+ 5,992), **18:1ω7c** (+ 6,627)
  - δ-^{13}C of Fungal biomarkers: **18:2ω6** (+4,738), **20:4ω6** (+5,106)
- DB-04: δ-13C enrichment proportionately lower than in DP-13, but still high:
  - δ-^{13}C of *Proteobacteria* biomarkers **16:1ω7c** (+1,274) and **18:1ω7c** (+ 2,996)
  - δ-^{13}C of Fungal biomarkers: **18:2ω6** (+ 4,738) and **20:4ω6** (+5,106)

Taxa based on ^{13}C Incorporation into PLFA



^{13}C Enrichment of PLFA Biomarkers



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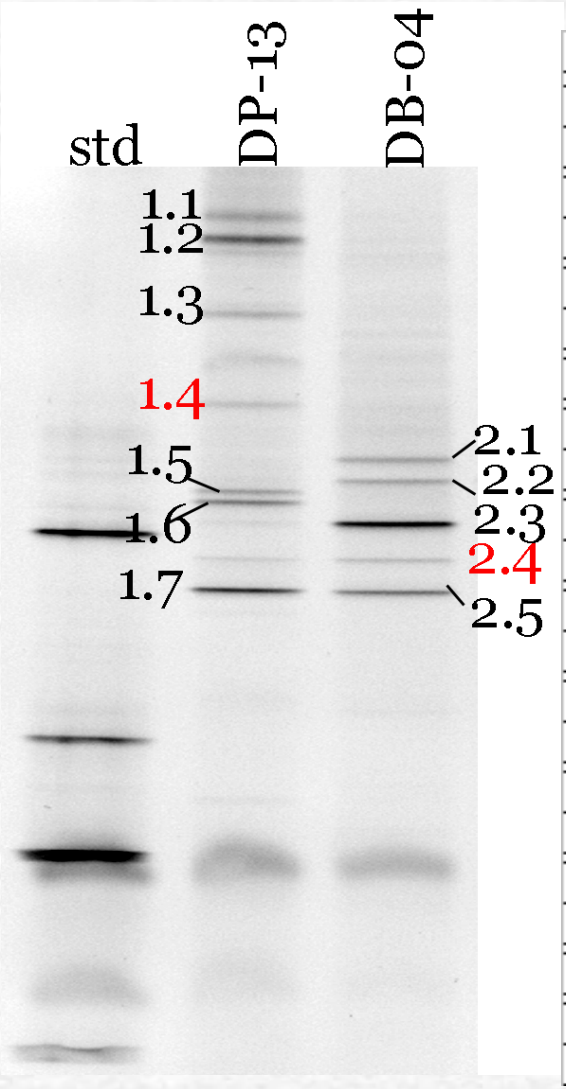
^{13}C-Benzene Bio-Trap DGGE Results



Band	Well	Database	% Match	Closest Genus / Species
1.1	DP-13	NCBI	96%	**Diaphorobacter nitroreducens**
	DP-13	RDP	0.821	**Diaphorobacter nitroreducens**
1.2	DP-13	NCBI	99%	**Pseudomonas** sp. MFY116
	DP-13	RDP	0.903	**Pseudomonas brenneri**
1.3	DP-13	NCBI	98%	**Pseudomonas** sp. XJ-2
	DP-13	RDP	0.897	**Pseudomonas extremorientalis**
1.4	**DP-13**	**Sequencing Failed**		
1.5	DP-13	NCBI	97%	**Pseudomonas** sp. isolate Ki-1w
	DP-13	RDP	0.871	**Pseudomonas sp. Ki-1w**
1.6	DP-13	NCBI	100%	**Pseudomonas** sp. WT OTU2
	DP-13	RDP	0.987	**Pseudomonas tolaasii**
1.7	DP-13	NCBI	98%	**Rhodoferax antarcticus** strain Fryx1
	DP-13	RDP	0.916	Rhodoferax antarcticus
2.1	DB-04	NCBI	99%	**Pseudomonas** sp. PNP4
	DB-04	RDP	0.942	Pseudomonas fluorescens
2.2	DB-04	NCBI	100%	**Pseudomonas** sp. PNP4
	DB-04	RDP	0.987	Pseudomonas fluorescens
2.3	DB-04	NCBI	100%	**Comamonadaceae** bacterium PIV-20-1
	DB-04	RDP	0.981	Comamonadaceae bacterium PIV-16-1
2.4	**DB-04**	**Sequencing Failed**		
2.5	DB-04	NCBI	99%	**Pseudomonas** sp. PNP4
	DB-04	RDP	0.942	Pseudomonas reactans

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Case Study:
^{13}C-Labeled cis-1,2-DCE and DCFE Bio-Trap Monitoring
Combined Anaerobic Oxidation and Reductive Dechlorination of Chloroethenes (New York)

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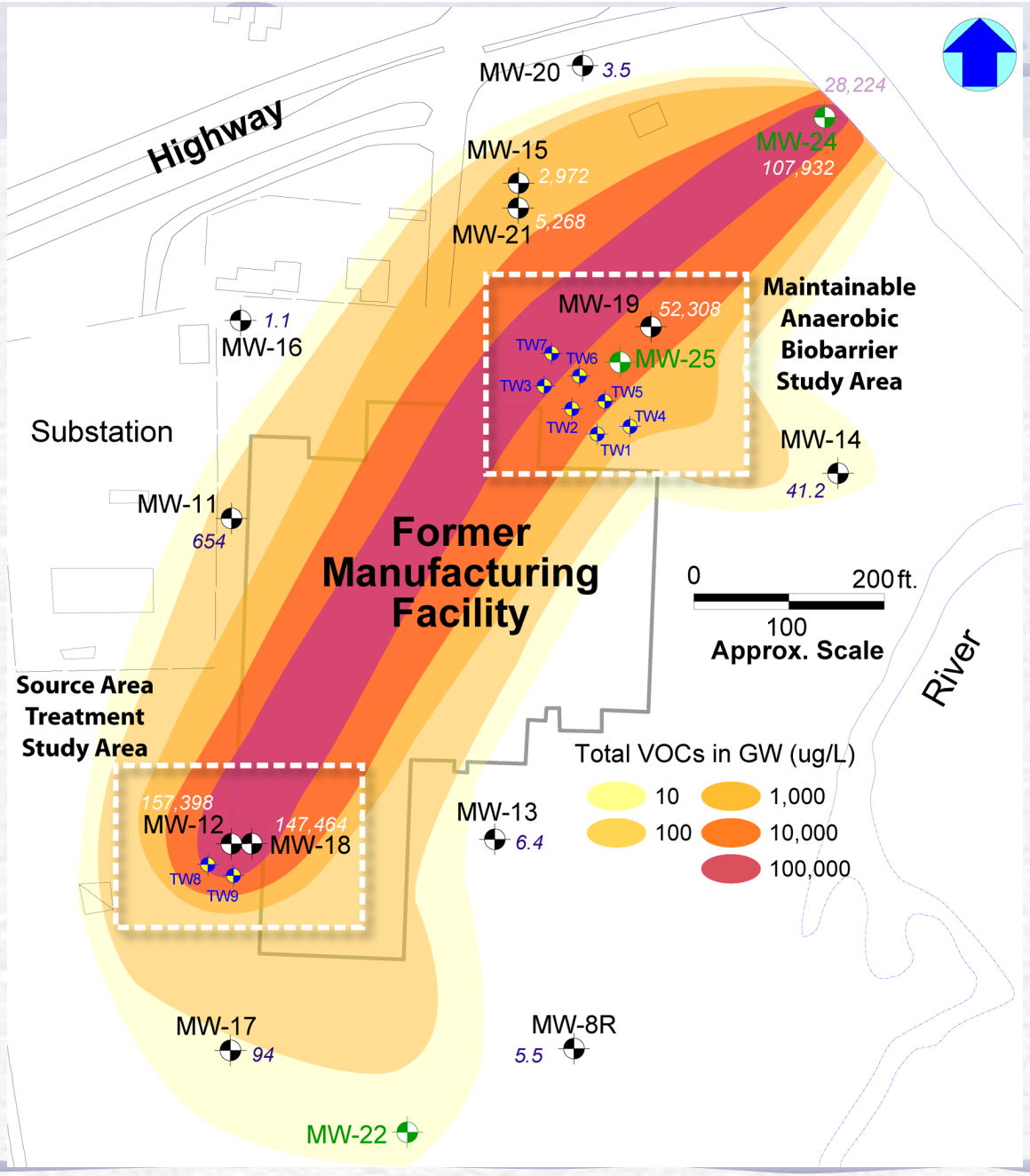
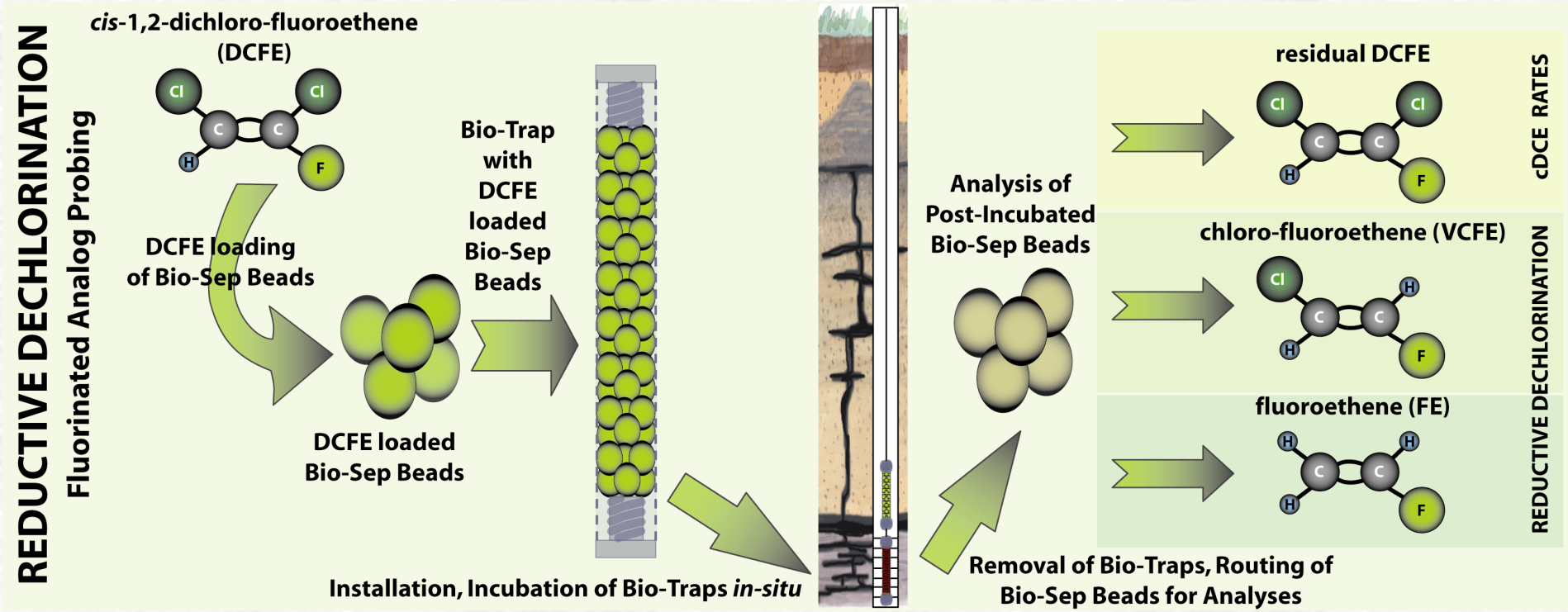
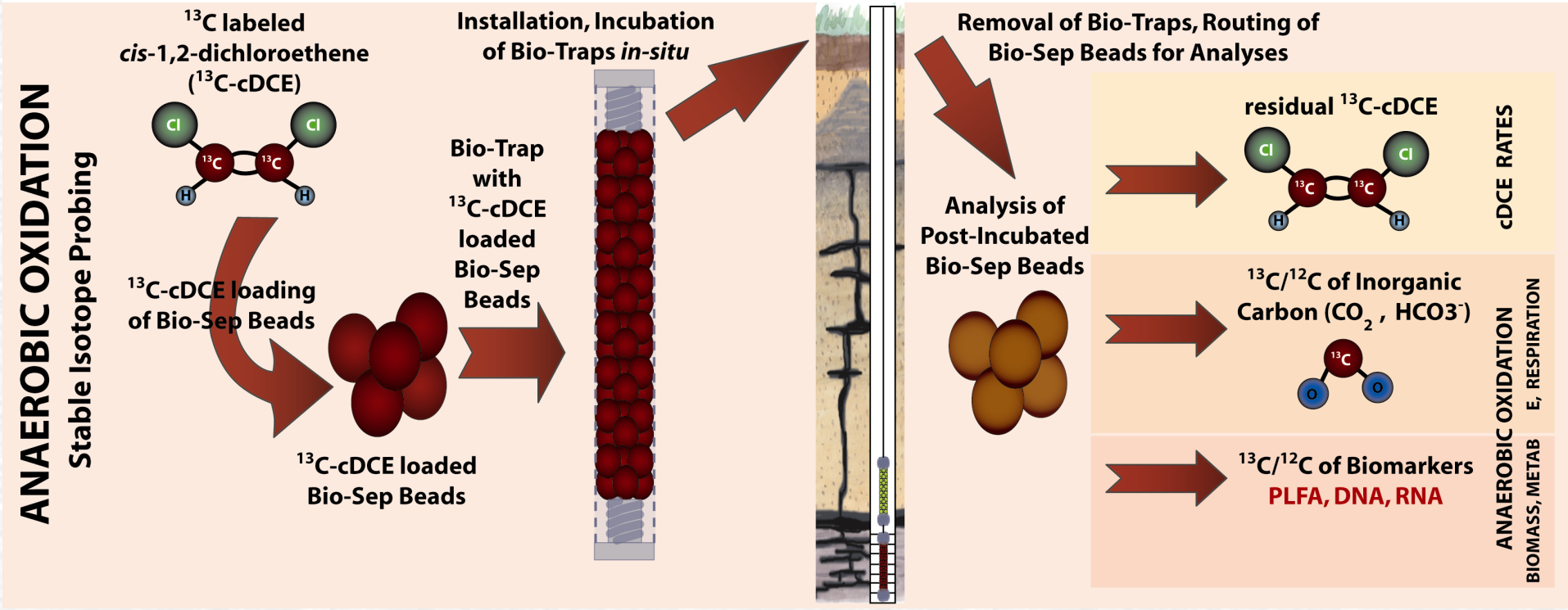


Fluorinated Analog Probing of cDCE Reductive Declorination using DCFE Loaded Bio-Traps



Stable Isotope Probing of cDCE Anaerobic Oxidation using ^{13}C-cDCE Loaded Bio-Traps



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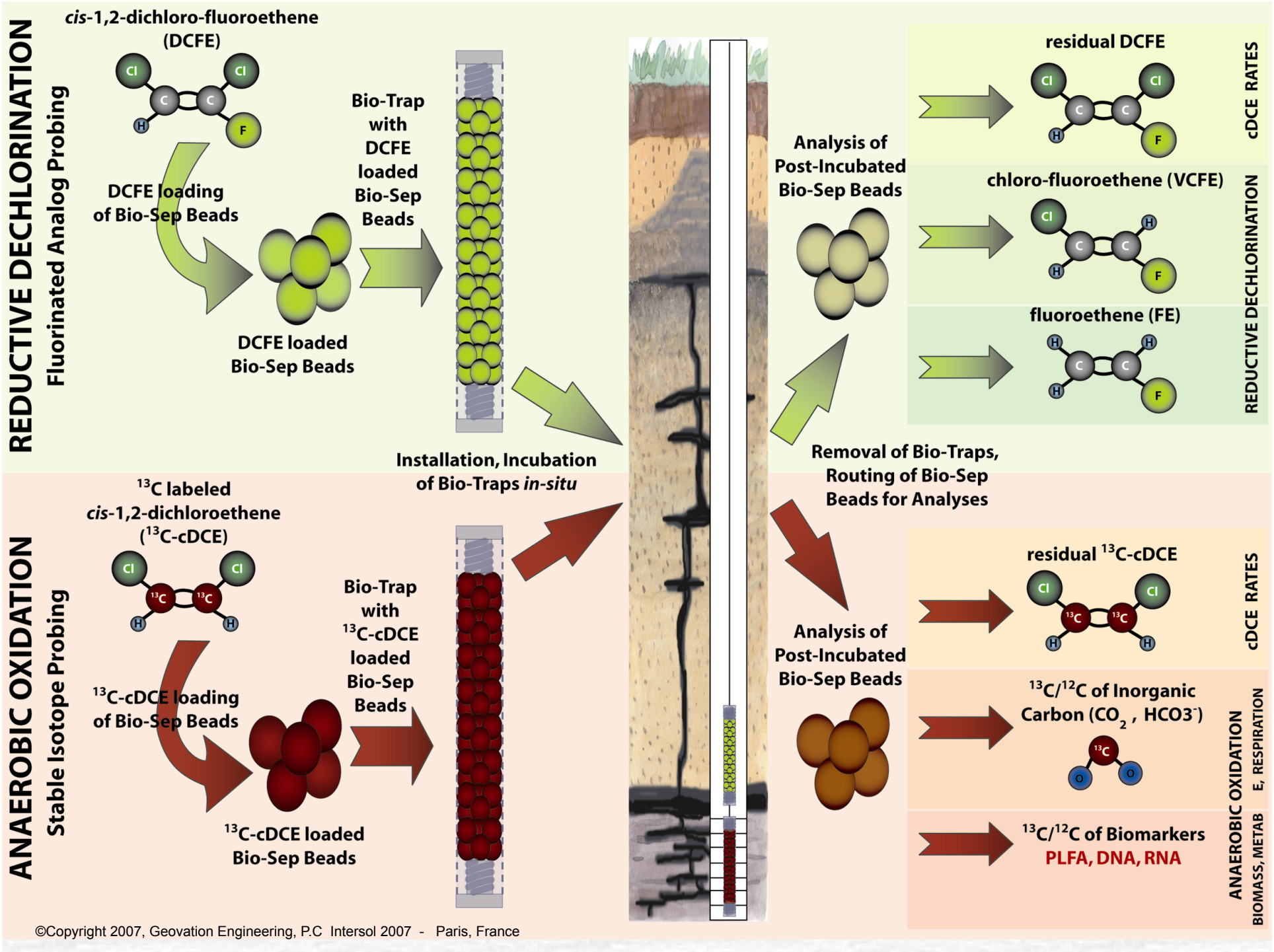

^{12}C-cDCE "Calibration" Bio-Trap Results

cDCE loss from Bio-Sep Beads:

- MW-12: 83% loss from 30-120 Days; 1° rate = 0.0202 day^{-1}
- MW-19: 70% loss from 30-120 Days; 1° rate = 0.0194 day^{-1}

PLFA Biomass and Biomarkers:

- Biomass increased significantly by 120 days vs. 30, 60 days
  - MW-12: Big increase from 60 to 120 days
  - MW-19: Steady increase from 30 to 120 days
- *Proteobacteria* biomarkers dominate PLFA
  - **16:1ω7c** (11-35%), **18:1ω7c** (7-35%)
  - MW-12: 59-80% Proteobacteria PLFAs
  - MW-19: 71-90% Proteobacteria PLFAs

^{12}C-cDCE "Calibration" Bio-Trap PLFA Data Summary

Sample Name	MW-12	MW-12	MW-12	MW-19	MW-19	MW-19	MW-24
Sample Date	7/31/2006	8/30/2006	12/20/2006	7/31/2006	8/30/2006	12/20/2006	7/31/2006
Sampling Event	Day 30	Day 60	Day 120	Day 30	Day 60	Day 120	Day 30
Total Picomoles of PLFA[1]	1,169	1,614	10,600	1,068	4,994	12,007	1,497
Biomass:							
pmols PLFA/bd	3	3	18	3	9	21	4
Cells/bd	5.70E+04	5.54E+04	3.70E+05	6.27E+04	1.85E+05	4.24E+05	8.22E+04
Contaminant Concentration							
DCE (ug/bd)	796	366	129	681	688	119	846
1st Order Degradation Rate (Day^{-1})	0.000	0.0259	0.0202	0.000	0.000	0.0194	0.000
Community Structure: (% of Total PLFA)							
Firmicutes (TerBrSats)	0.0	17.8	12.9	0.0	10.0	5.3	0.0
Proteobacteria (Monos)	80.8	69.0	59.4	90.1	75.8	70.9	90.1
Anaerobic metal reducers (BrMonos)	0.0	0.0	0.4	0.0	0.0	0.3	0.0
Actinomycetes (MidBrSats)	0.0	0.0	4.1	0.0	0.0	7.5	0.0
General (Nsats)	19.2	13.2	22.6	9.9	12.7	12.7	9.9
Eukaryotes (polyenoics)	0.0	0.0	0.7	0.0	1.6	3.2	0.0
Metabolic Status: (Ratio)							
Starvation							
group A (cy17:0/16:1w7c)	0.26	0.23	0.00	0.23	0.42	0.03	0.17
group B (cy19:0/18:1w7c)	0.39	0.38	0.29	0.38	0.13	1.12	0.29
Total	0.65	0.61	0.29	0.61	0.55	1.15	0.46
Membrane Stress[2]							
group A (16:1w7t/16:1w7c)	NC	0.10	0.08	NC	0.00	0.06	NC
group B (18:1w7t/18:1w7c)	1.82	0.53	0.09	0.53	0.04	0.06	0.09
Total	1.82	0.63	0.17	0.53	0.04	0.12	0.09

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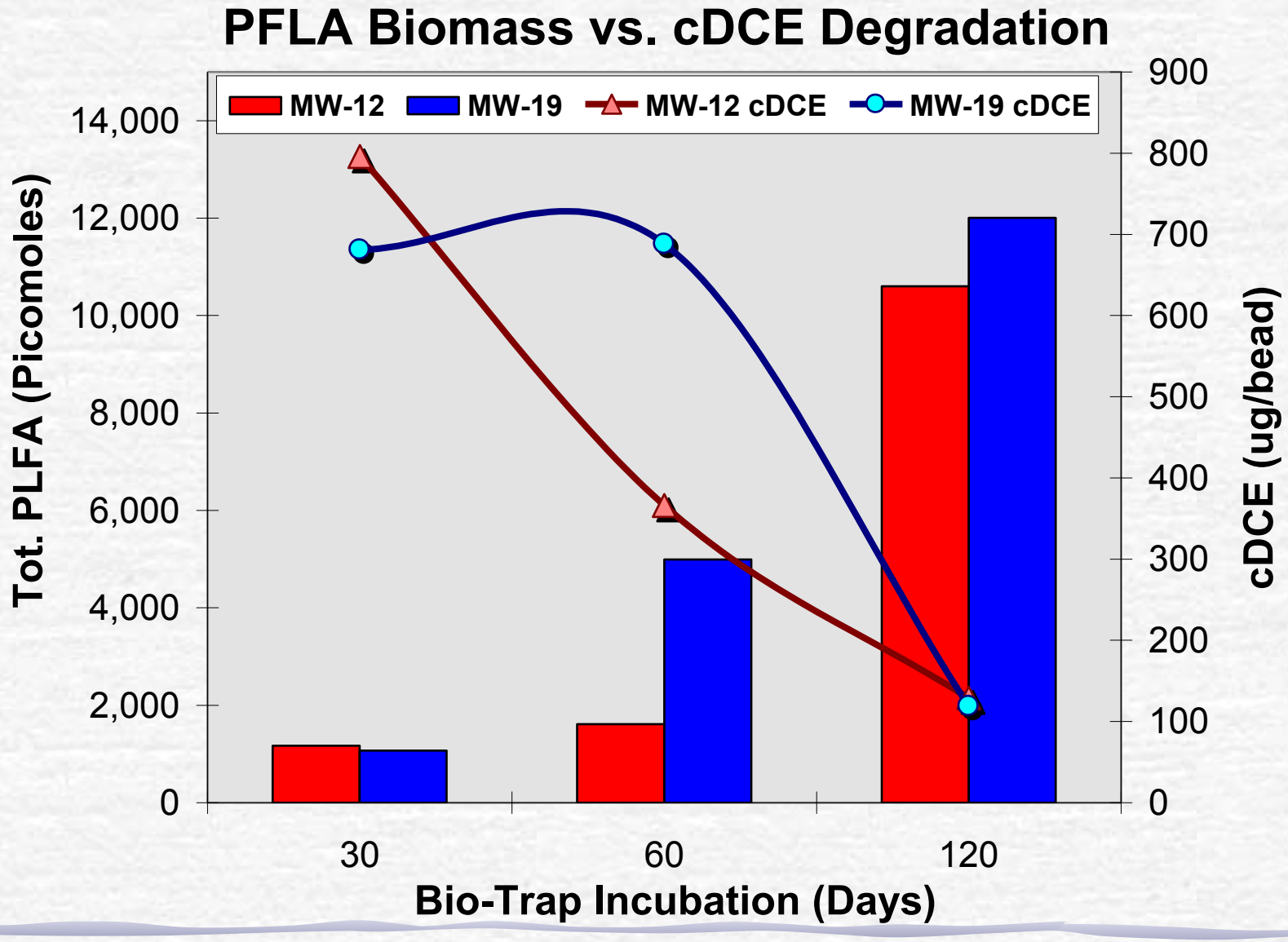
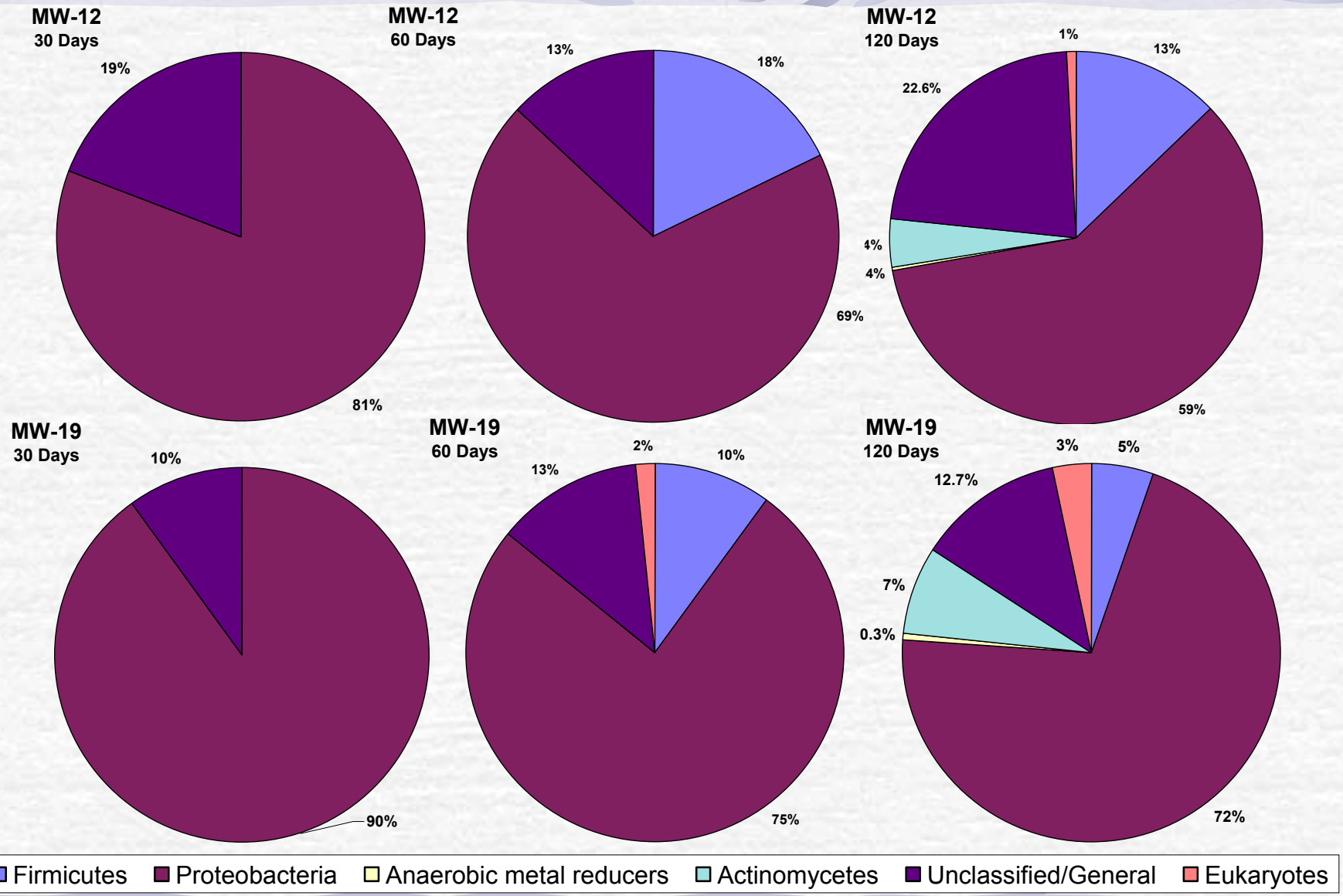
^{12}C cDCE Calibration Bio-Traps - Results



^{12}C-cDCE Grown Taxa from PLFA Markers



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Ongoing / Future SIP-FAP Bio-Trap Work

- Combined SRC-DBB Reductive Dechlorination-Anaerobic Oxidation Demonstration Program Initiated in Dec. ’06
- First ^{13}C-cDCE (SIP), DCFE (FAP) Bio-Traps Installed
  - Bio-Trap pairs deployed in MW-12, MW-25 (near MW-19) at New York site in January 2007
  - 3 rounds of SIP-FAP Bio-Traps planned for 2007-08
  - SIP-FAP Bio-Traps to be retrieved for analyses after 90-120 days incubation
- Analyses: ^{13}C/^{12}C PLFA profiles by GC-IR/MS; PCR-DGGE; qPCR (*Dehalococcoides*, *Rdase* genes); FISH
- Start up of SIP-FAP Bio-Trap work at second site in New Jersey pending