Assessment of *In Situ* Biodegradation Potential of Benzene Using Stable Isotope Probing (SIP)

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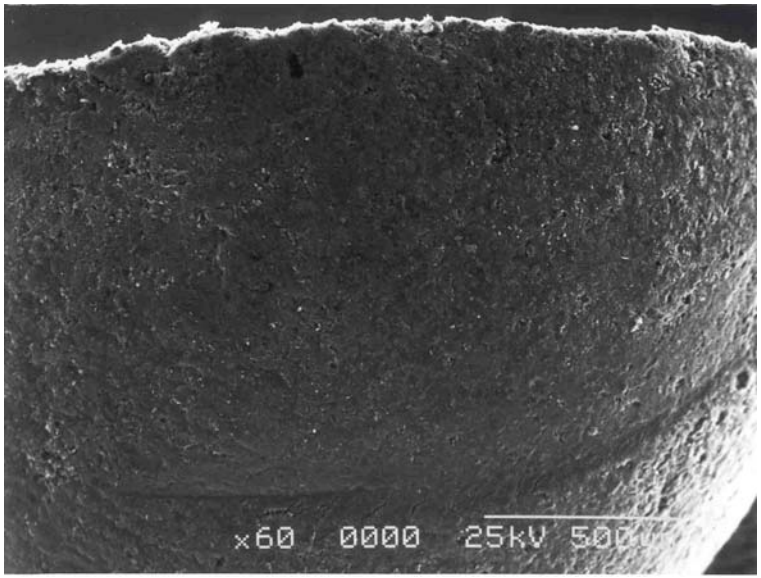
A New Tool: Bio-Sep Bio-traps

- Demonstrate biodegradation of benzene and other hydrocarbons by indigenous microbes in
  - Aquifers
  - Surface waters
  - Sediments
  - Soil
- Characterize the microbial ecology of benzene biodegradation

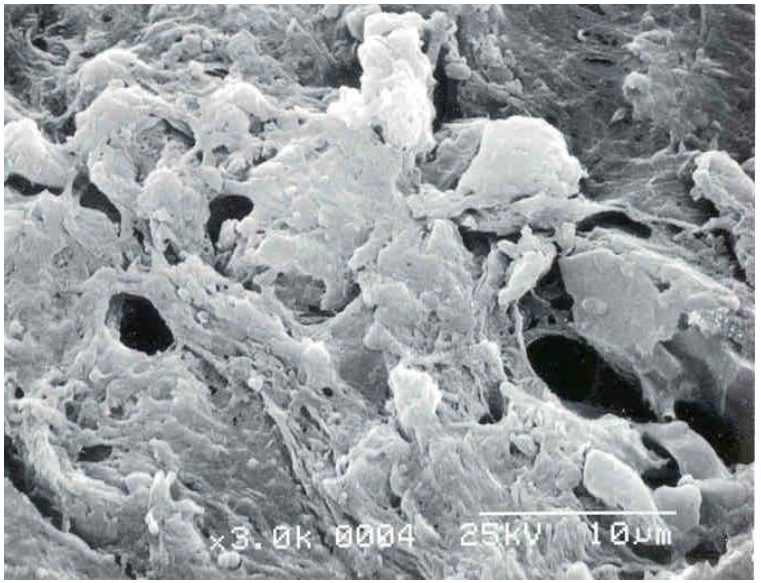
Bio-Sep Beads

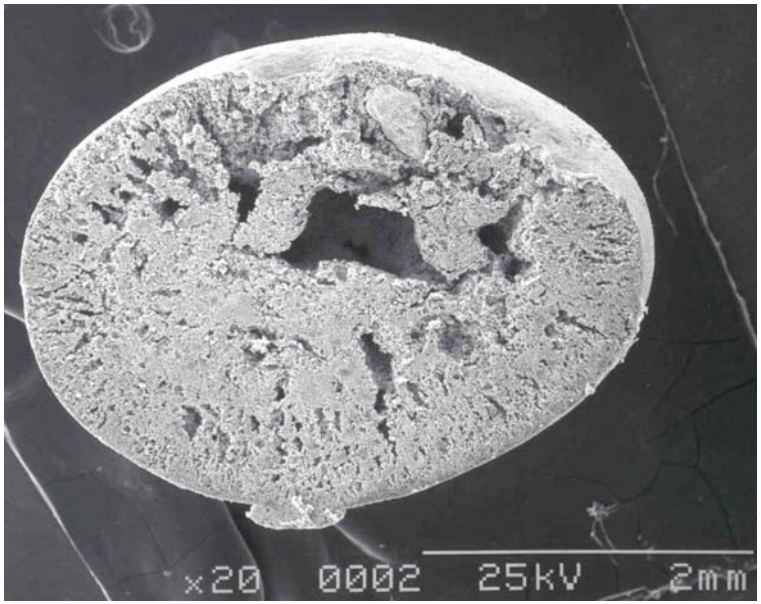
- 3-4 mm in diameter
- 25 % Nomex, 75% PAC
- 74% porosity
- 600 m^2 of surface area/g
- Surrounded by ultrafiltration-like membrane with 1-10 micron holes
- Autoclavable
- Cleaned of fossil biomarkers by heating to 300 ^oC

SEM of Bio-Sep Beads



Surface

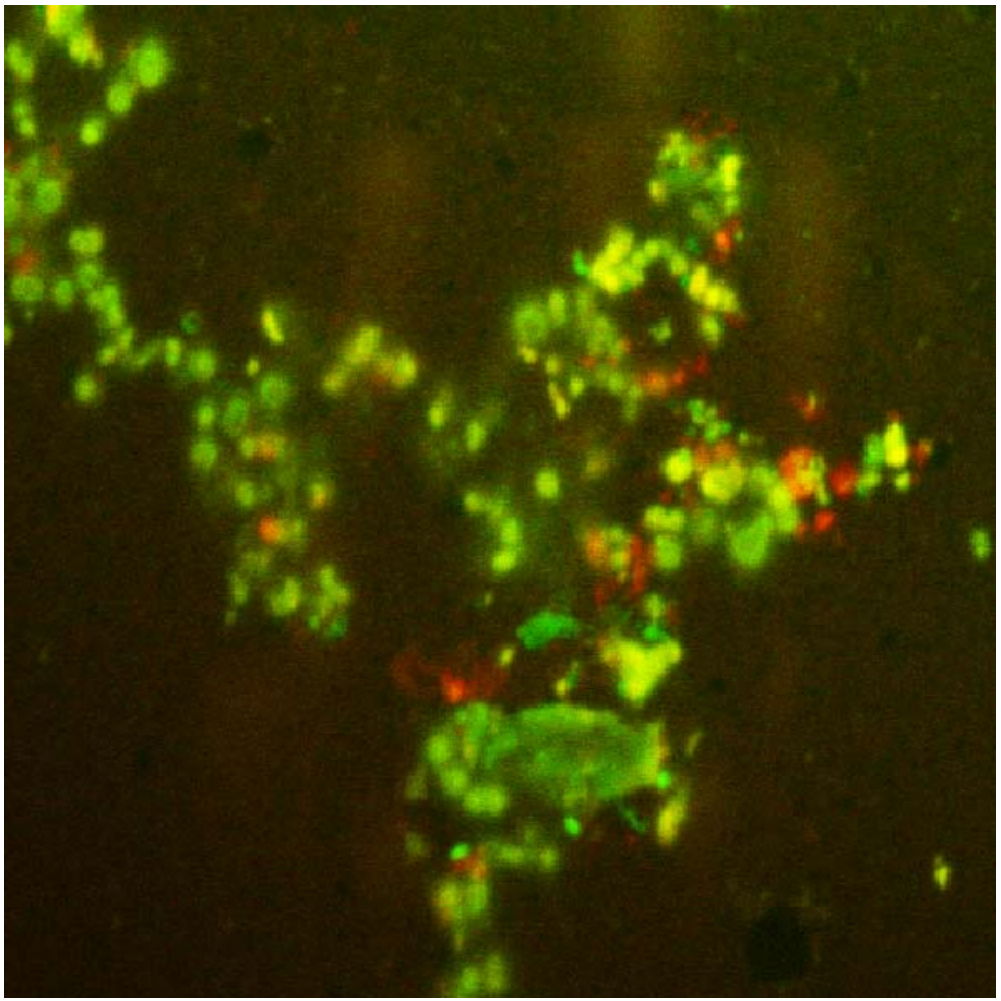




Interior

Biofilms Form Rapidly in Bio-Sep Beads

- High surface area
- Low shear
- Concentration of nutrients by PAC
- Rapid formation of pre-conditioning films



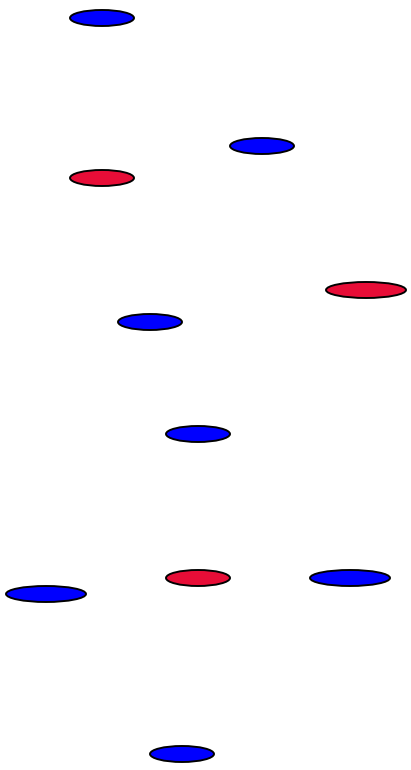
Live-dead stain of biofilm in Bio-Sep bead

What Do Bio-Sep Beads Collect?

- Bacteria have to enter the bead and grow there to be detected
- Slow-growing, non-growing, or dormant organisms are less likely to be collected in the beads unless the beads offer them a significant advantage
- Organisms collected in the beads are more likely to be the more active members of the sampled community

A Simple Example

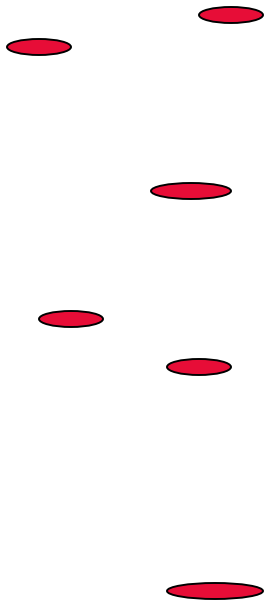
Groundwater

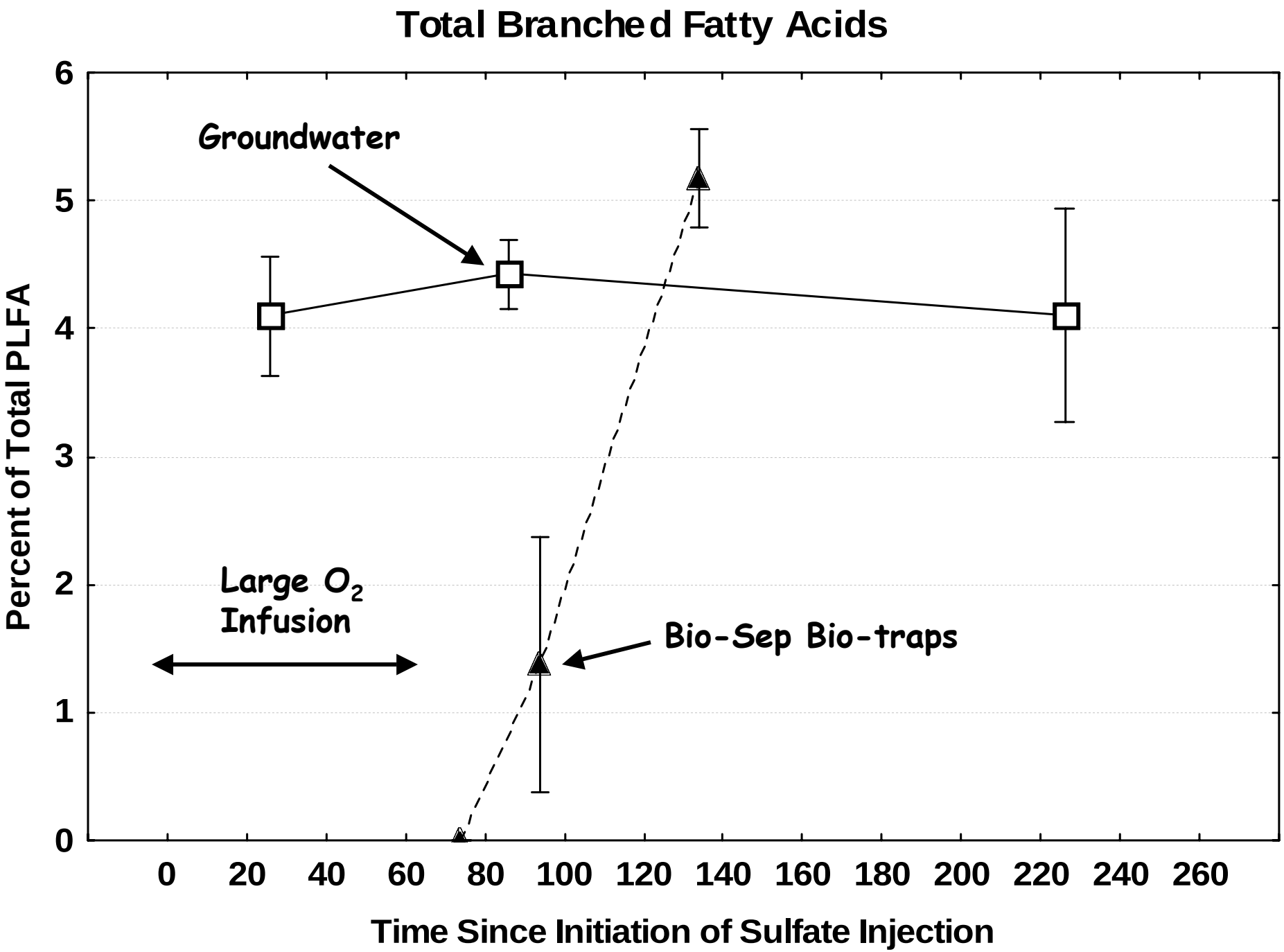


Blue = inactive

Red = active

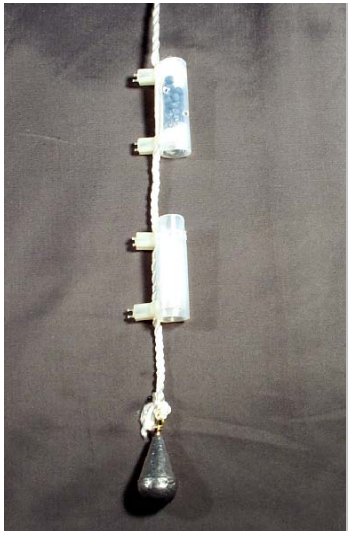
Beads



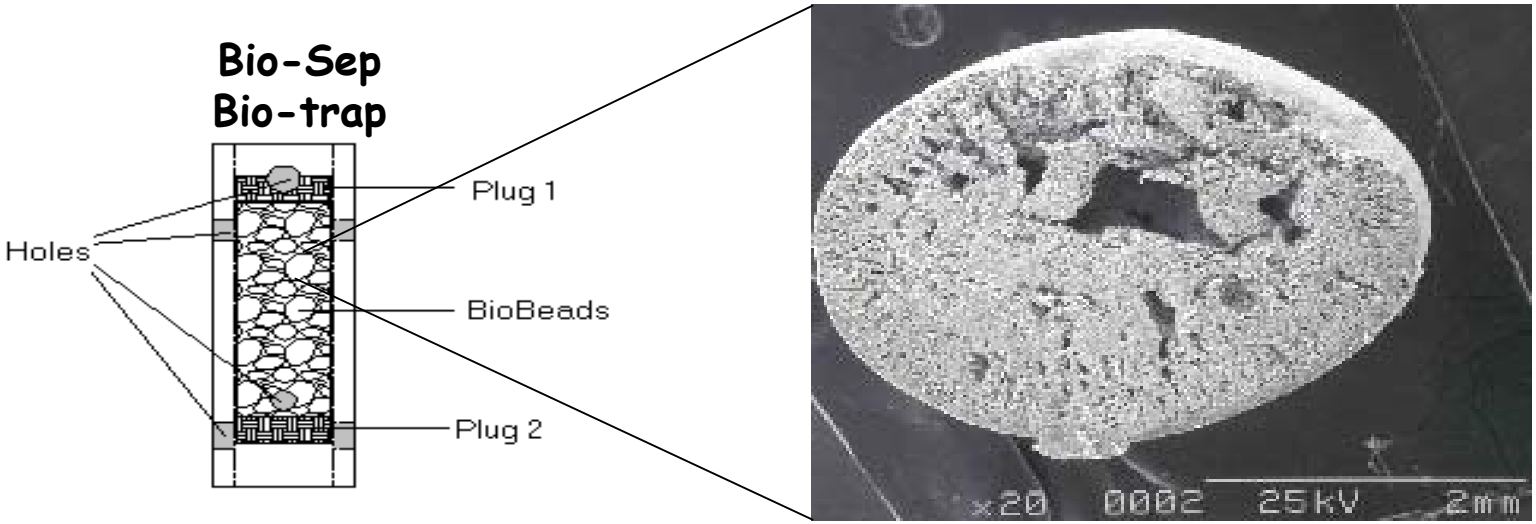

Total Branched Fatty Acids
Groundwater
Large O_2 Infusion
Bio-Sep Bio-traps
Percent of Total PLFA
Time Since Initiation of Sulfate Injection
0
1
2
3
4
5
6
0
20
40
60
80
100
120
140
160
180
200
220
240
260

Applications Bio-Sep Bead Samplers

- Drinking water systems
  - Measurable biofilms in 24 hrs
  - Detecting leaks in distribution lines
  - Pathogen tracking
  - Trouble shooting
- 70-m deep storage tank on offshore platform
  - Microbial ecology with depth
  - SRB and sulfide-oxidizers
- Aqueous phase of a solvent extraction system (pH < 2)
  - Cause of biological fouling
- Contaminated aquifers
  - PCE
  - Hydrocarbons
  - MTBE
- Stream monitoring
  - Source tracking for coliforms

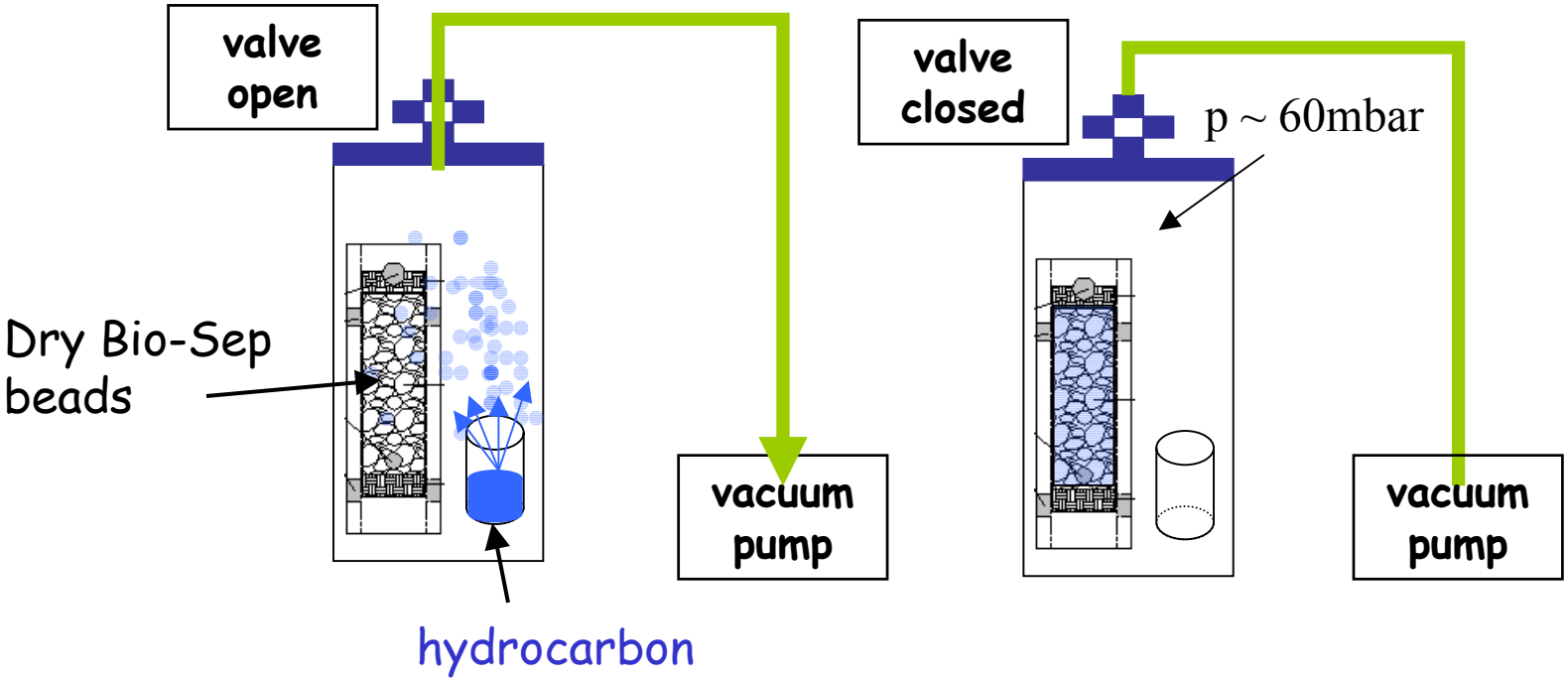


Hydrocarbon Biodegradation Assessment Using *In Situ* Microcosms



1. Load with hydrocarbon (^{13}C-labeled and/or non-labeled)
2. Expose under *in situ* conditions for 4-5 weeks
3. Analyze biomass, search biomarkers for the ^{13}C-labeling

Vapor Phase Loading of Hydrocarbon



Does Benzene Leach From the Beads?

Leaching experiment:

- 25-mL VOA vials with 50 Bio-Sep beads in each, set up in triplicate
- 10 mM sodium azide to prevent microbial growth
- Solution replaced after each sampling to avoid vapor space
- Samples analyzed quantitatively by GC-MS

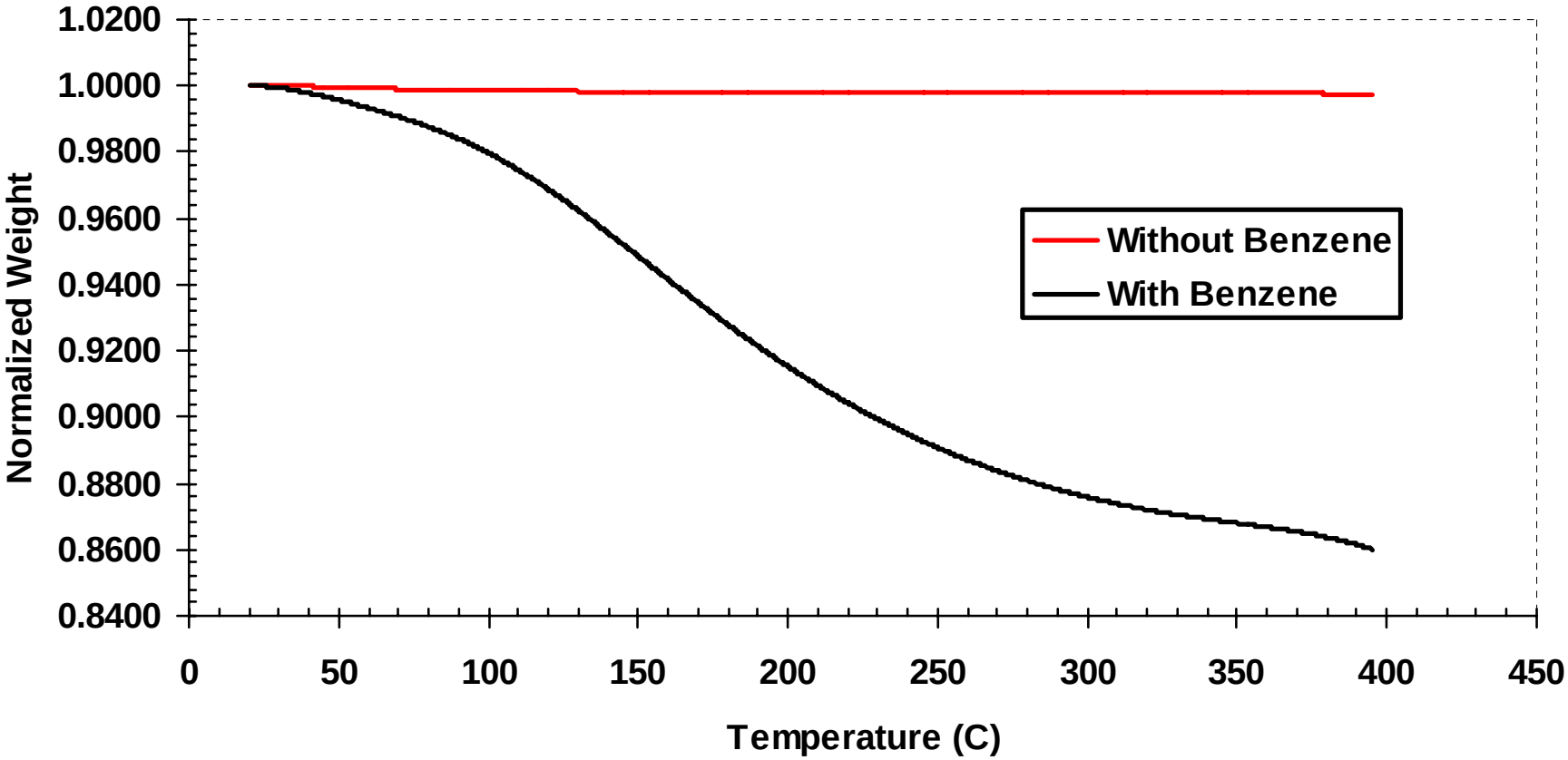
Benzene Leaching in Sterile Water

Incubation time	Avg. benzene concentration in leachate (mg/L)
Day 0	9.93E-04
Day 1	9.70E-04
Day 2	9.76E-04
Day 3	9.82E-04
Day 4	9.78E-04
Day 5	9.75E-04
Day 6	9.74E-04
Day 7	9.74E-04
Day 8	BDL
Day 9	9.74E-04
Day 10	9.74E-04
Day 11	9.74E-04
Day 12	9.75E-04
Day 13	BDL
Day 14	BDL
Day 15	9.78E-04

Incubation time	Benzene extracted from beads (mg/bead ± std. dev) N=3
t_0	1.05 ± 0.04
Day 15	0.99 ± 0.02
Day 30	0.97 ± 0.03

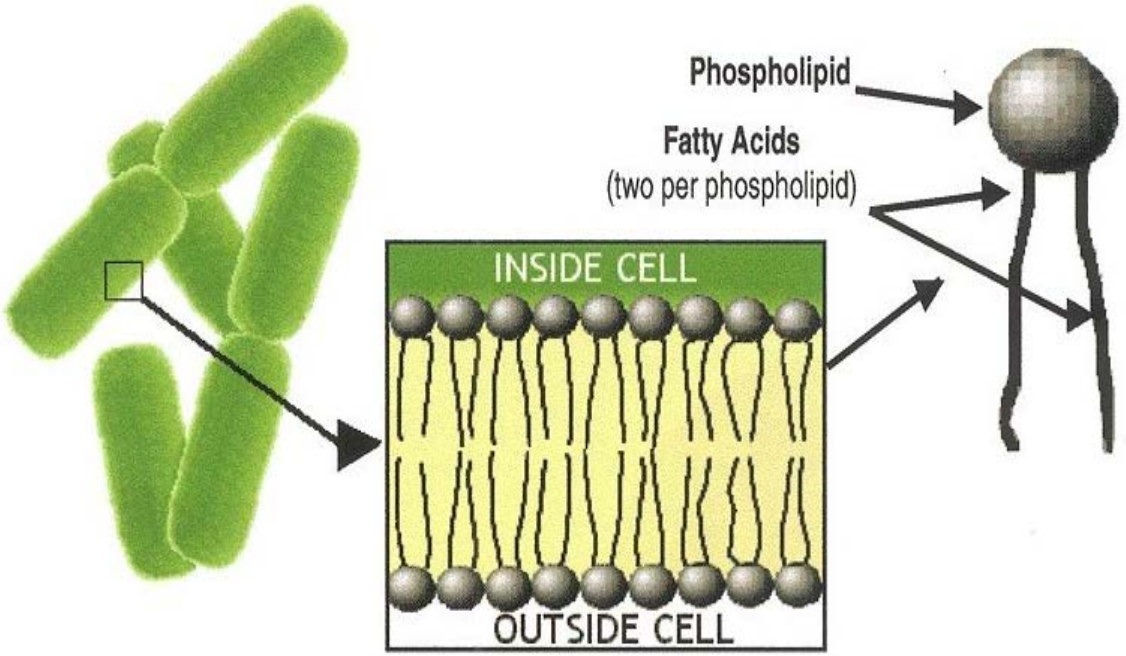
Benzene does not easily **leach** from the beads!

TGA Analysis of Benzene-loaded Bio-Sep Beads



Biomarker of Choice: PLFA

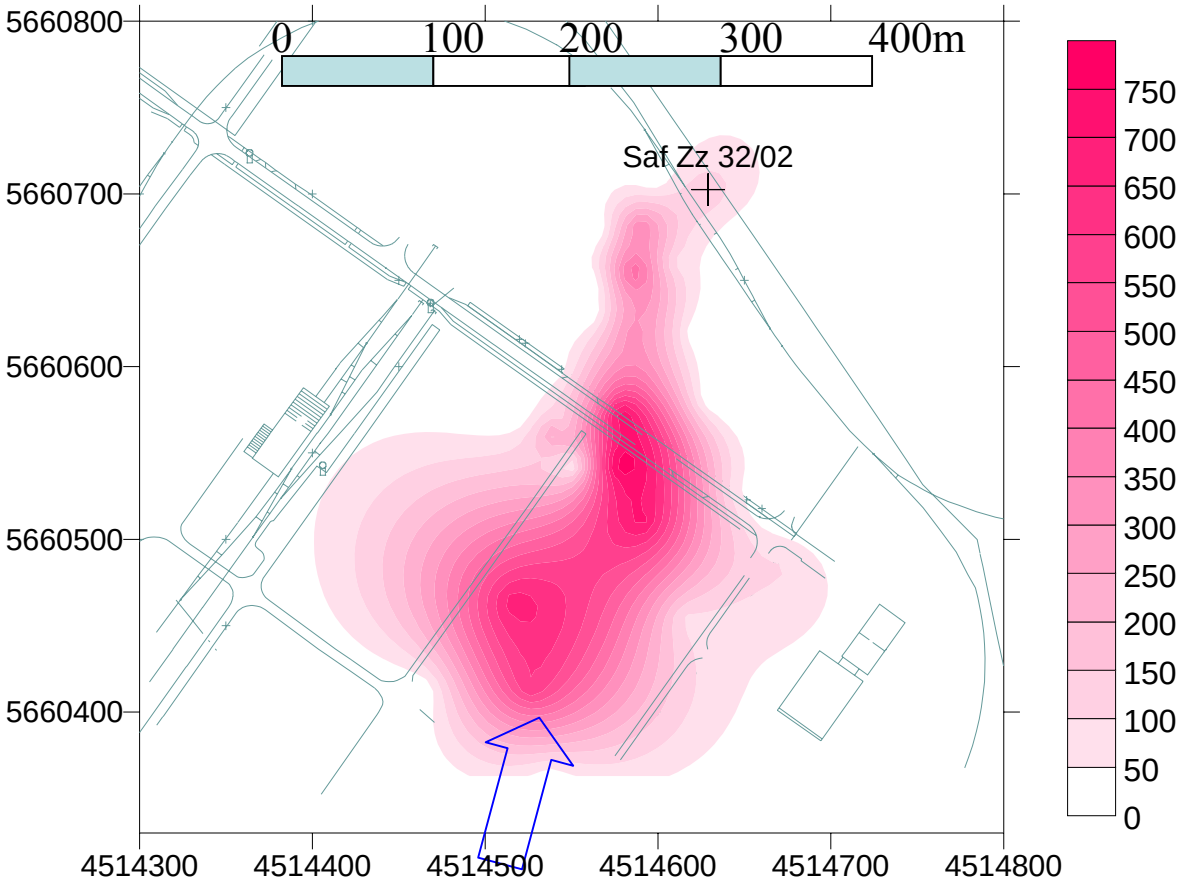
Lipids with ^{13}C incorporated into the phospholipid bilayer indicate utilization of the ^{13}C-labeled compound and incorporation into biomass



Two Case Studies

- BTEX impacted aquifer in Germany
- LUST site in southern California

Germany



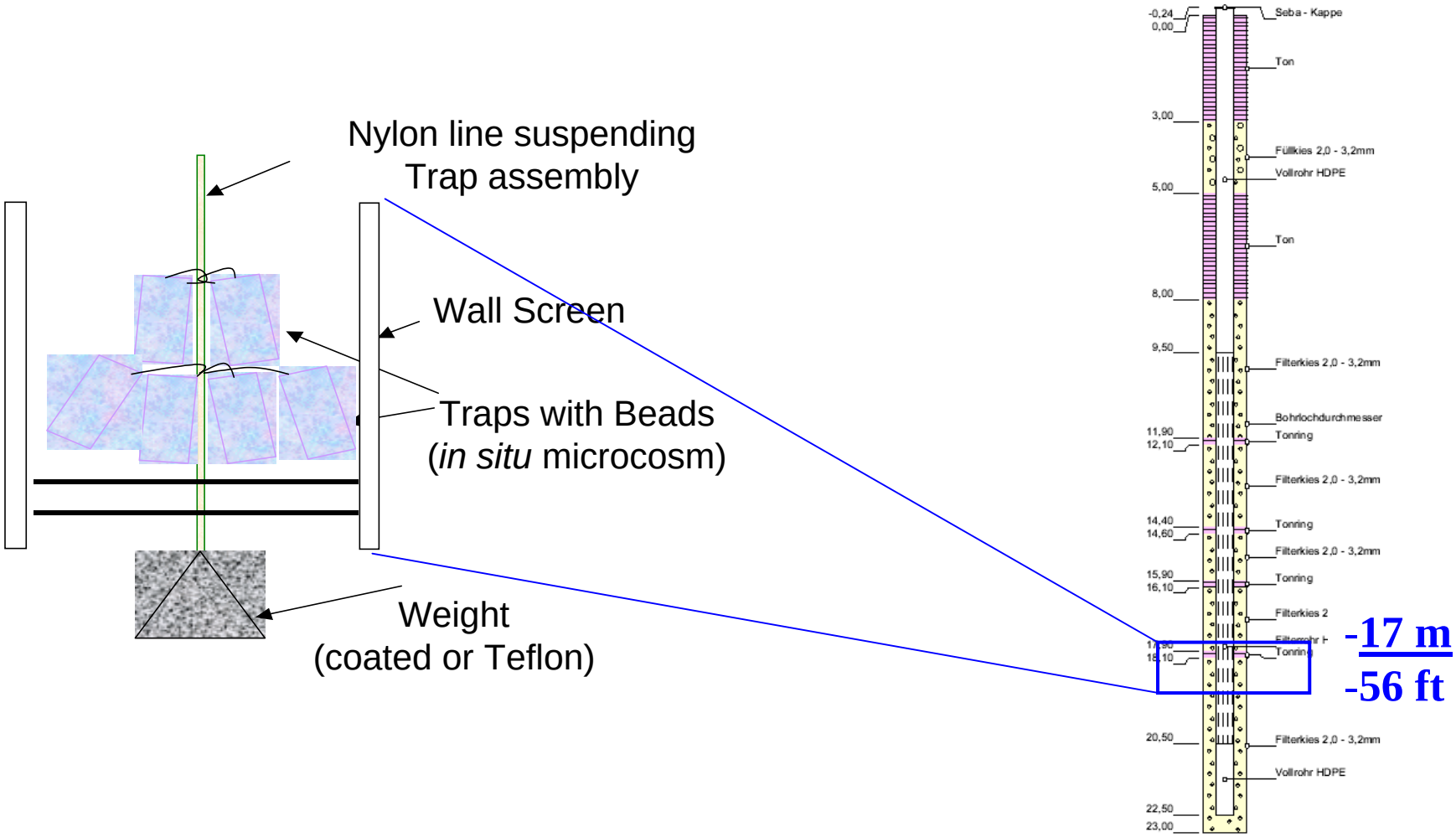
[Benzene] up to 850 mg/L

Aquifer is virtually free of dissolved oxygen

groundwater flow

Exposure of '*In Situ* Microcosms'

depth profile of well Saf Zz 32/02



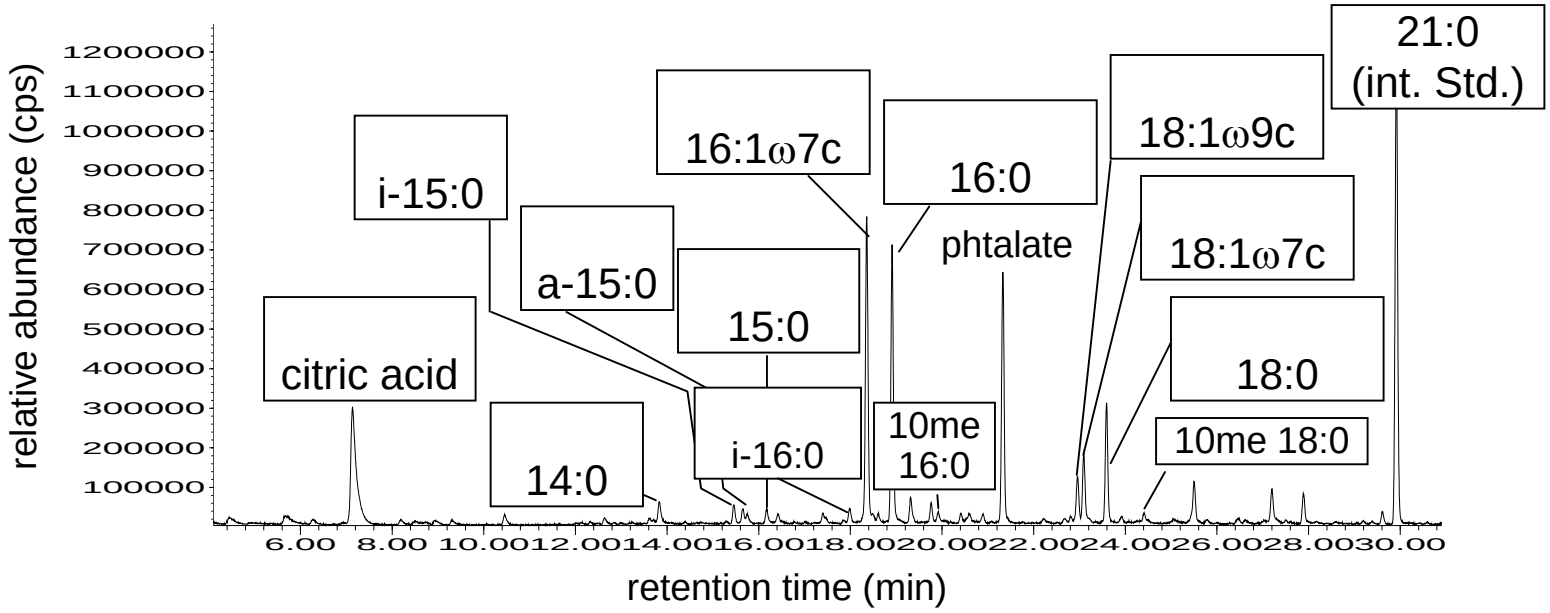
Analysis of Microcosm BTEX Loading After 4 Weeks *In situ* Exposure

	^{13}C abundance At%	Residual hydrocarbon (mg/trap)	Loss (%)
natural benzene	0.1	7.9 ± 0.1	**82**
$^{13}C_6$-benzene	98.0	7.9 ± 0.1	**82**
natural toluene	0.1	8.0 ± 0.7	**85**
$^{13}C_1$-toluene	14.0	8.9 ± 0.7	**84**
blank	0.1	0.23 benzene	N/A

- **isotopic composition of contaminants unchanged**
  - **no significant exchange with aquifer**
  - **no crosstalk between traps**

- **80 % decrease in contaminant concentration (biodegradation)**

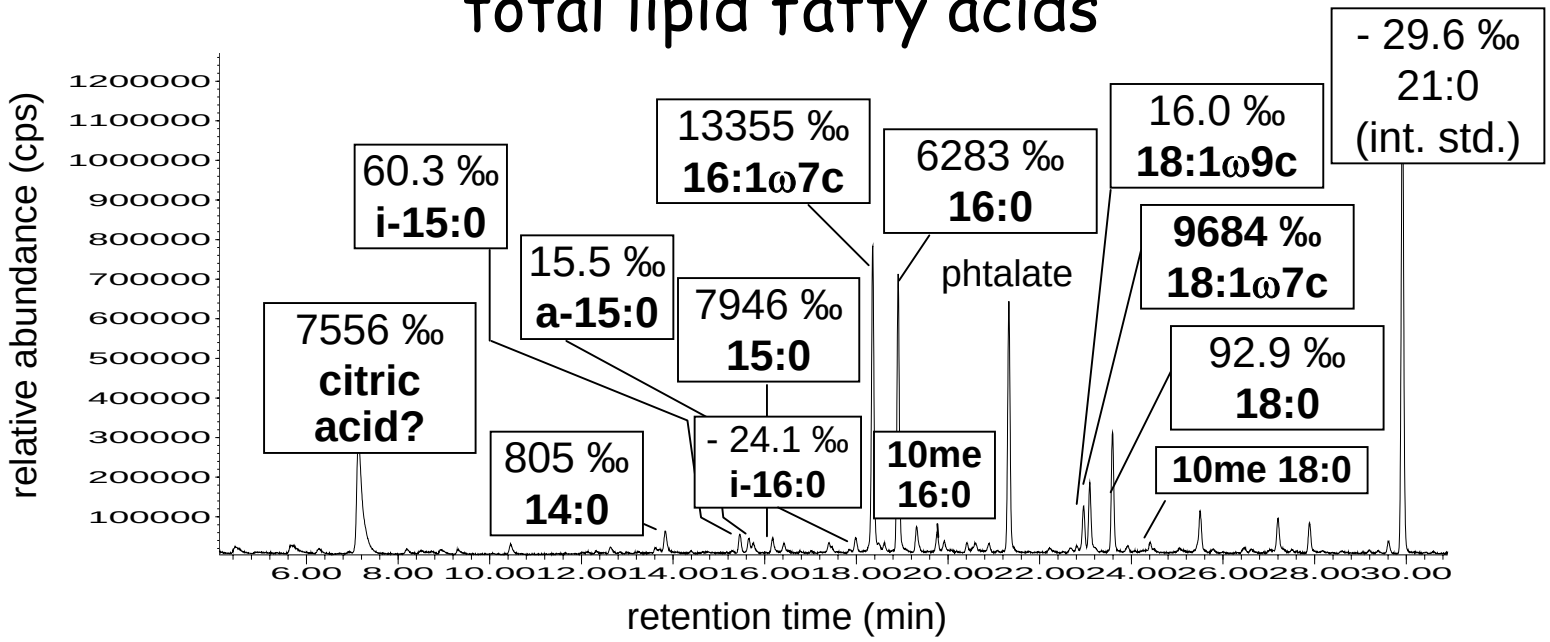
Analysis of Microcosm Biomass After *In Situ* Exposure



Total lipid fatty acids profile from ^{13}C-toluene loaded microcosm or bio-trap

Analysis of Microcosm Biomass After *In Situ* Exposure

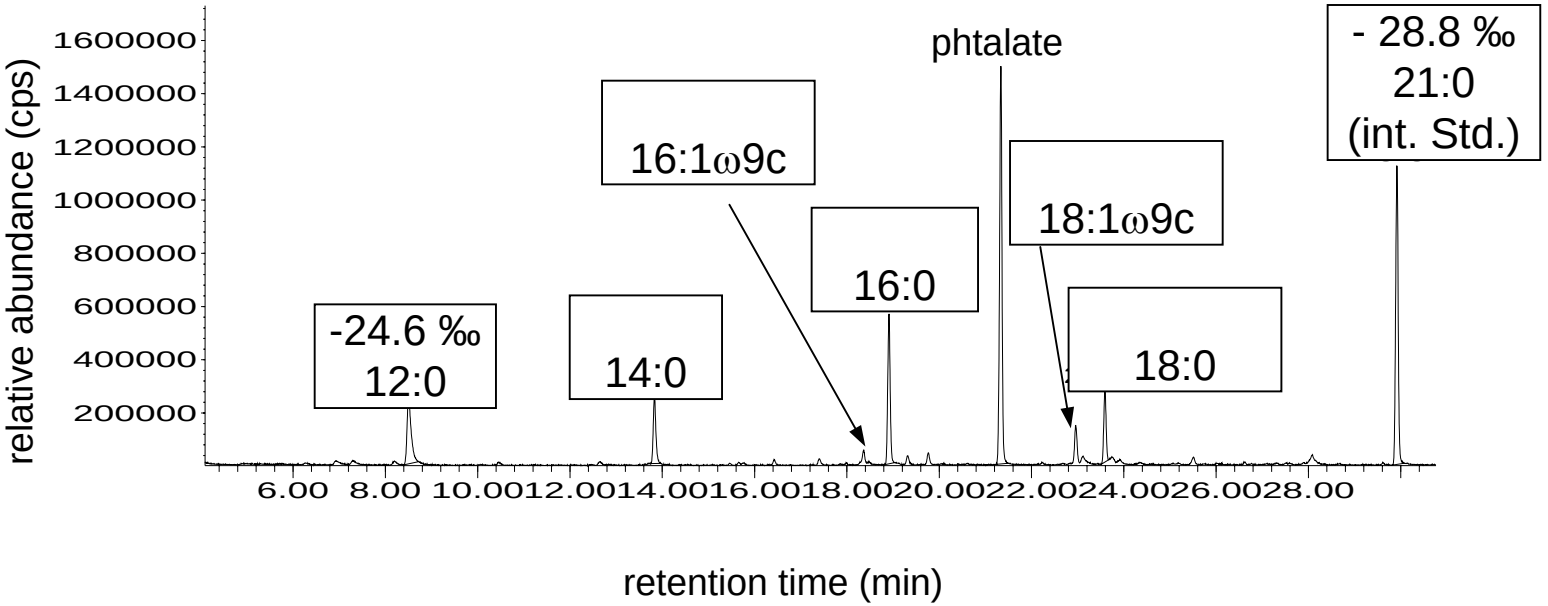
Toluene ^{13}C label detected in total lipid fatty acids



Stabile isotope analysis proves toluene biodegradation and growth of microbial biomass under *in situ* conditions

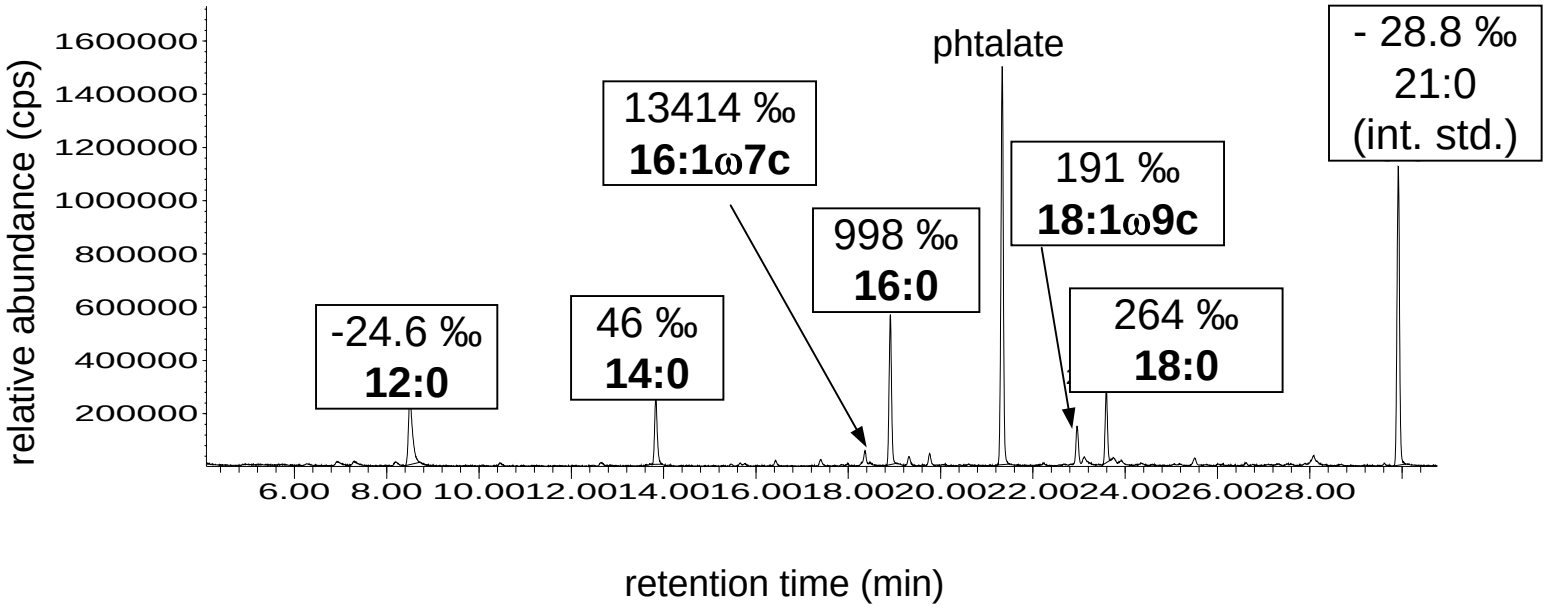
Analysis of Microcosm Biomass After *In Situ* Exposure

Total lipid fatty acids profile from
^{13}C benzene loaded bio-trap



Analyze Microcosms Biomass After *In Situ* Exposure

Benzene ^{13}C label detected in total lipid fatty acids



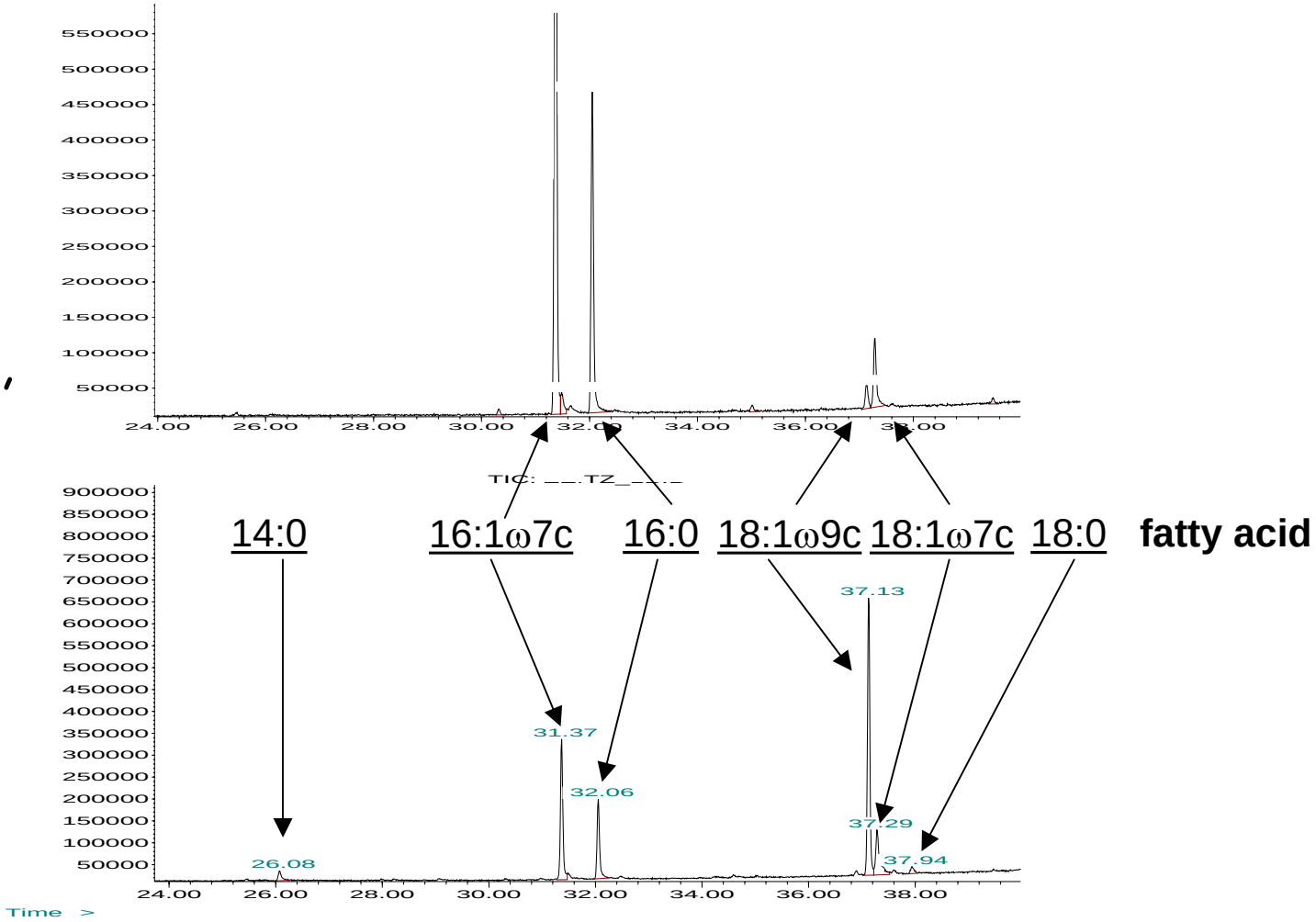
Stabile isotope analysis proves benzene biodegradation and growth of microbial biomass under *in situ* conditions

Polar Lipid Fatty Acid (PLFA) Profiles of Viable Microorganisms Enriched in Bio-traps

toluene baited

same PLFAs, different abundance

benzene baited



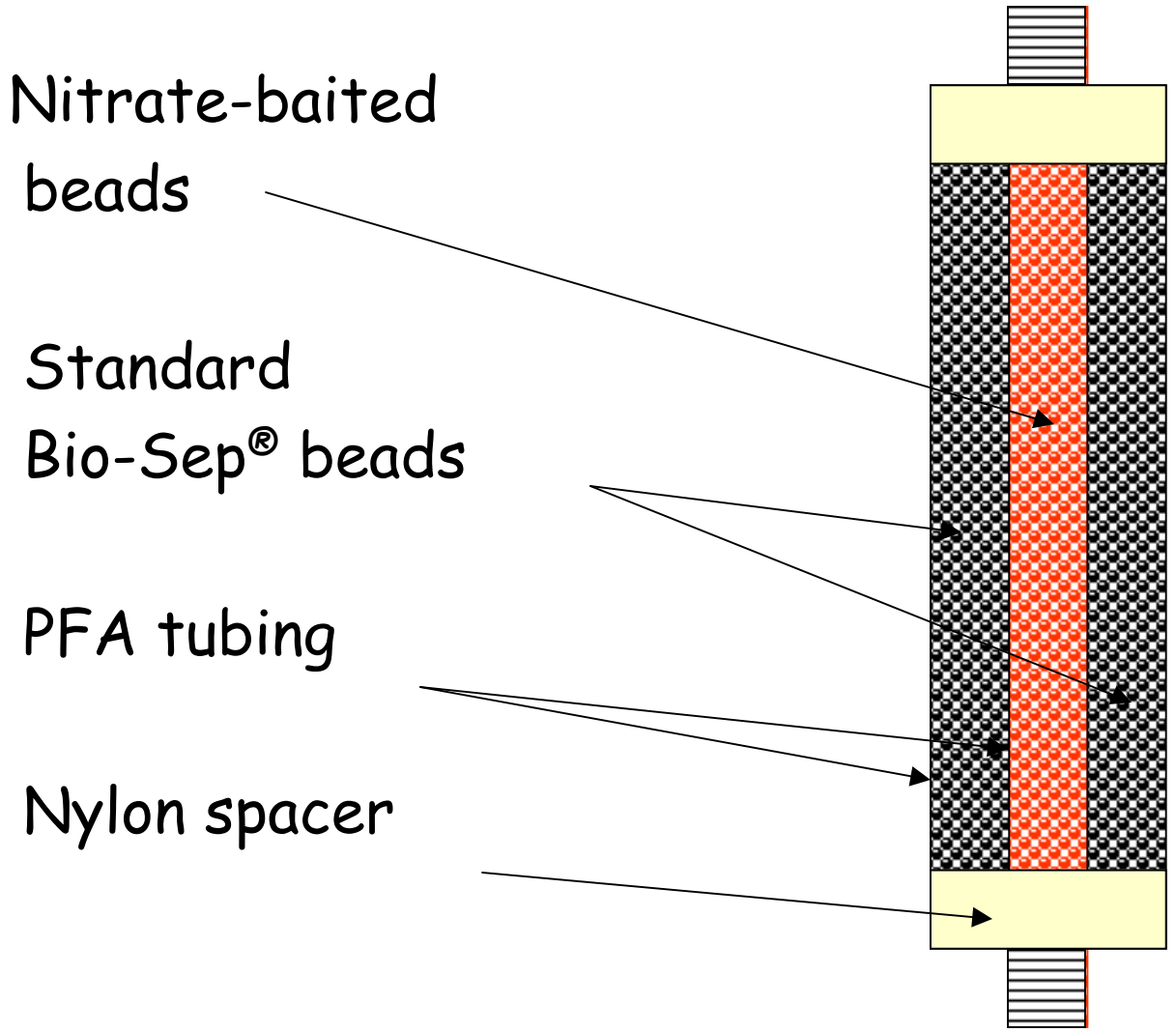
Yucaipa, CA

- Site of gasoline LUST
- Is there intrinsic bioremediation of benzene at this site?
- Will nitrate stimulate the intrinsic bioremediation of benzene?
- Bio-Sep *in situ* microcosms (non-baited and nitrate baited), both with ^{13}C-benzene, installed in triplicate into MW20 suspended 30 cm below water table with float; incubated for 45 days
- Specific activity of ^{13}C-benzene only about 8%

Geochemistry of MW20 and Unimpacted Background Well

Parameter	Background well	MW20
Benzene	ND	6.6 μg/L
TEX	ND	ND
MTBE	ND	1.2 μg/L
pH	7.4	7.5
Nitrate-N	9.4 mg/L	8.6 mg/L
Sulfate	23 mg/L	19 mg/L
Alkalinity	140 mg/L	130 mg/L
TDS	280 mg/L	250 mg/L

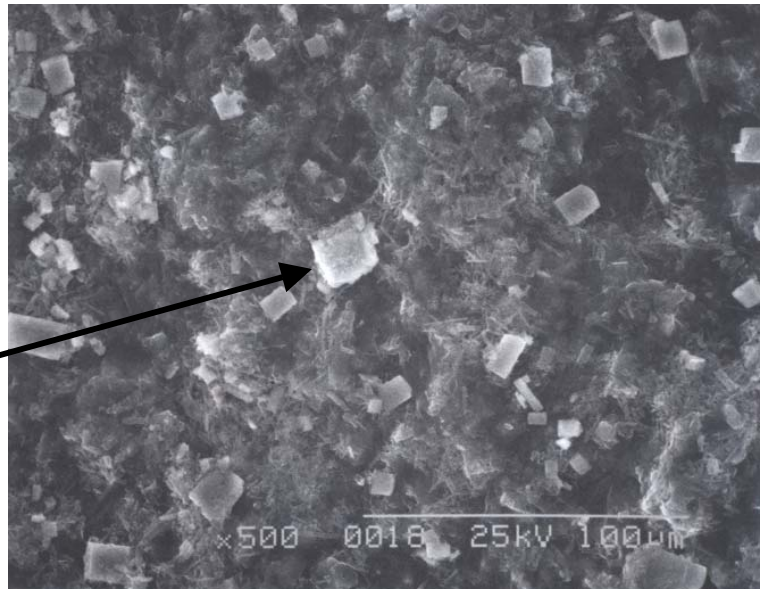
Bio-Sep® Tube-in-Tube Nitrate-baited Sampler



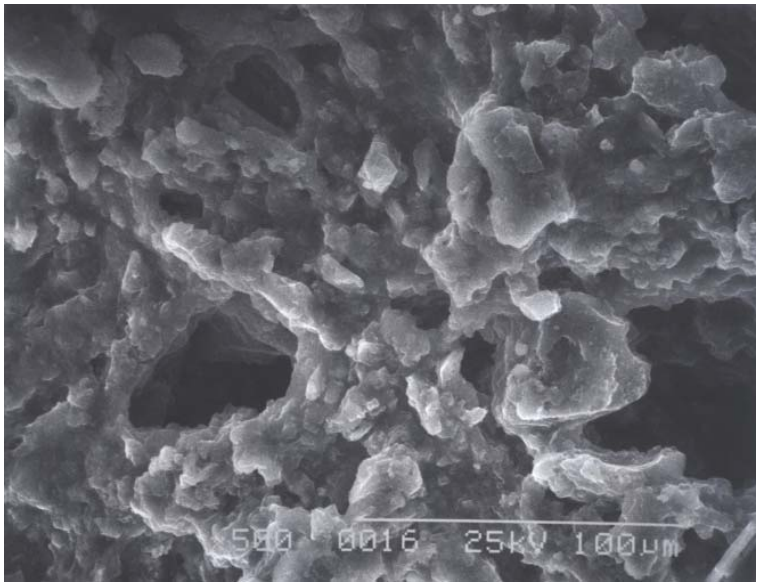
SEM of Nitrate-baited Bio-Sep Beads

Surface

KNO_3 crystals



Interior



Relative Abundances of Phospholipid Fatty Acids Structural Groups and Key Fatty Acids in Non-baited and Nitrate-baited Bio-traps

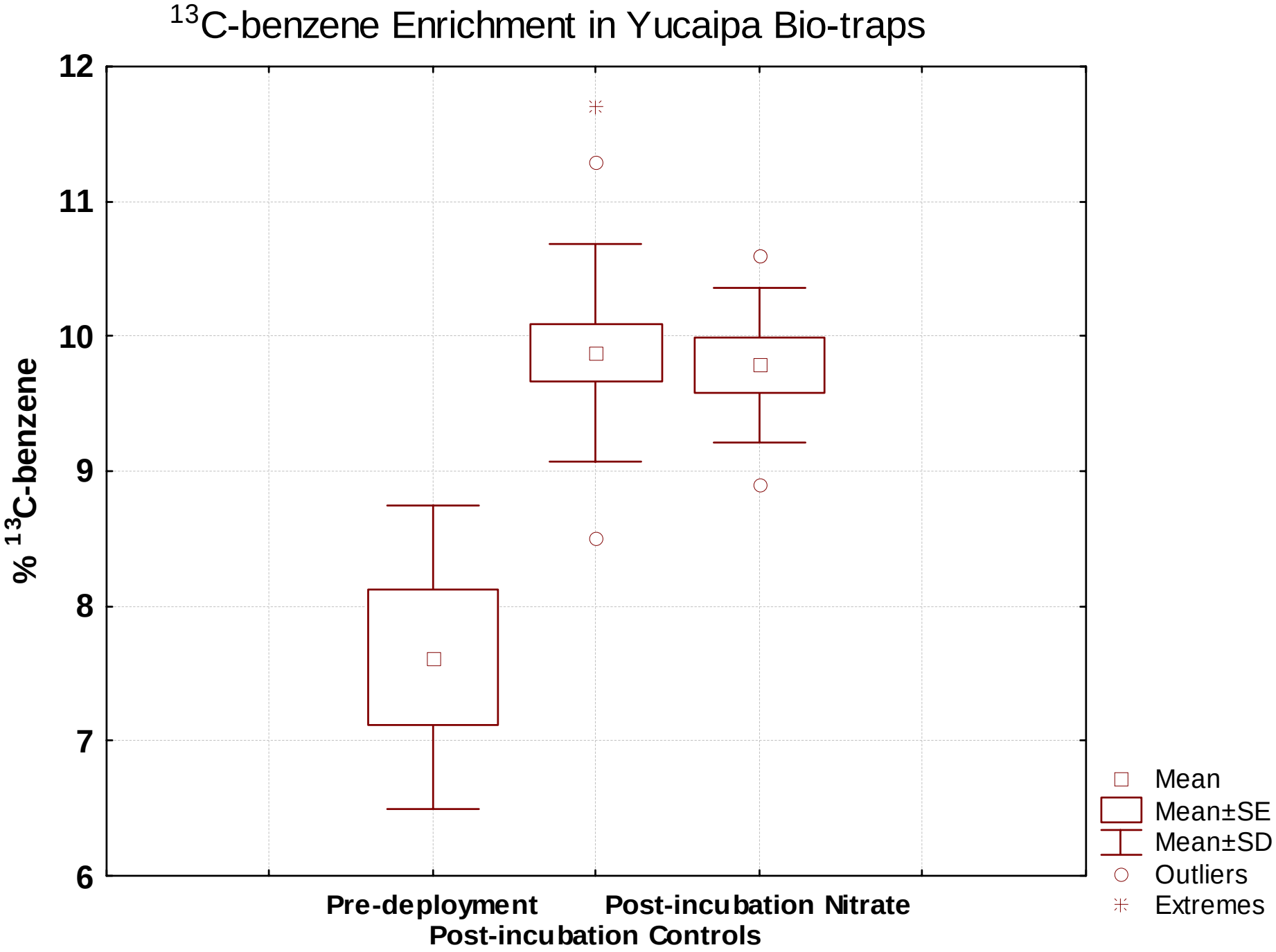
Phospholipid structural group or fatty acid	Non-baited Bio-traps*	Nitrate-baited Bio-traps*
Terminally branched	1.33 ± 1.12	2.00 ± 0.17
Monoenoics	72.2 ± 2.8	73.9 ± 1.1
Branched Monos	0.1 ± 0.17	0.4 ± 0
Mid-branched Sats	0.6 ± 0.17	0.6 ± 0.17
n-Sats	23.0 ± 1.89	21.5 ± 0.45
16:1ω7c	32.0 ± 3.5	35.5 ± 1.9
16:0	20.5 ± 2.1	19.7 ± 0.70
18:1ω7c	22.3 ± 4.0	24.7 ± 2.5
18:1ω9c	9.7 ± 3.1	5.9 ± 3.6
cy19:0	2.0 ± 0.55	2.0 ± 0.15

*Mean ± std.dev. (n=3)

$\delta^{13}C$ Values of Individual Fatty Acids Derived from Phospholipids From the Non-baited and Nitrate-baited Bio-traps.

Phospholipid fatty acid	Non-baited Bio-traps	Nitrate-baited Bio-traps
16:1ω7c	+5699 ± 161*	+6095 (n=2)***
16:0	+5342 ± 240	+5762 (n=2)
18:1ω7c	+3514 ± 756	+4037 ± 251
18:1ω9c	+754 (n=1)**	+1137 (n=1)
cy19:0	+1055 (n=1)	

The δ ^{13}C of natural benzene is about -26 $^{o}/_{oo}$. Values more positive reflect increasing enrichment with ^{13}C.
*** Mean ± std. dev. (n=3)**
****One observation**
***** Avg. of two observations**


13C-benzene Enrichment in Yucaipa Bio-traps
% 13C-benzene
12
11
10
9
8
7
6
Pre-deployment
Post-incubation Nitrate
Post-incubation Controls
Mean
Mean±SE
Mean±SD
Outliers
Extremes

Yucaipa Conclusions

- Bacteria indigenous to the aquifer at Yucaipa are capable of biodegradation of benzene under aquifer conditions.
- No evidence that nitrate stimulated benzene biodegradation or had a significant effect on the subsurface microbial community structure.
- Incorporation of ^{13}C-benzene into PLFA was easily detected with low specific activity in loaded beads

What's Next?

- Deployment of *in situ* microcosms in sediments and soil
- ^{13}C-fuel oxygenates
- ^{13}C incorporation into DNA – from labeled 16S rDNA we can potentially identify degraders

Acknowledgement

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