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Bioaugmentation – It's Not Just for TCE Anymore!

Remediation Technologies Symposium East, Niagara Falls, May 30th – June 1st

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May 10, 2023

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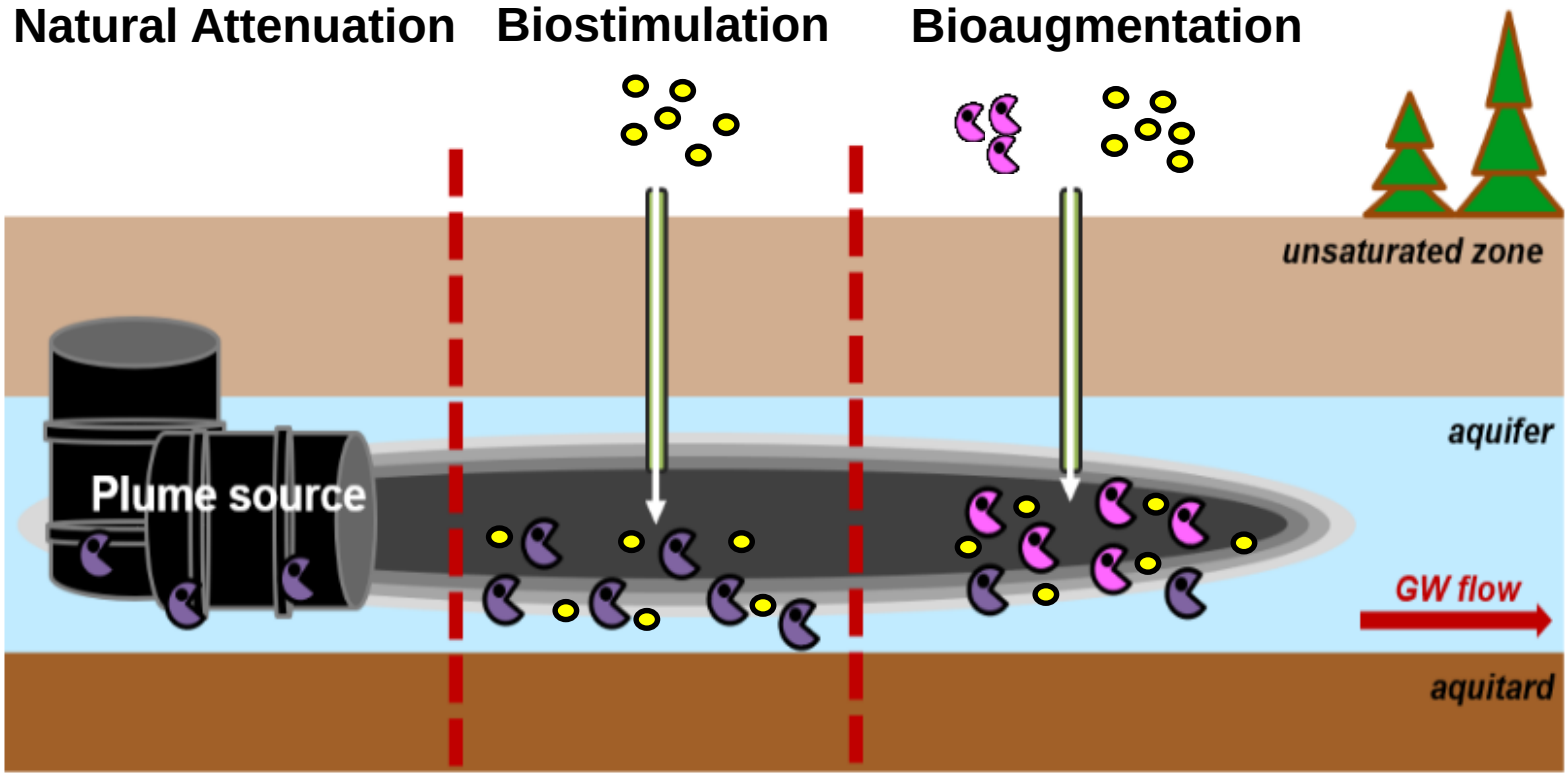
Review of Bioremediation



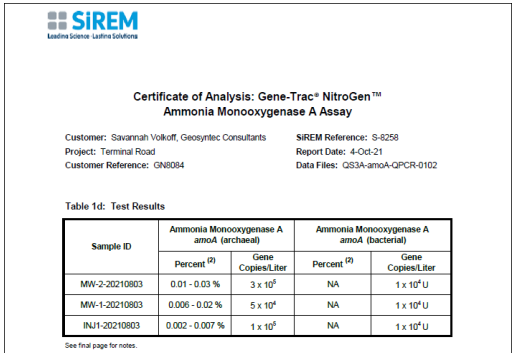
Does the Site have the right microbes?

gene trac®

Quantitative PCR

Quantify specific pre-selected targets:

- Microbial, e.g., *Dhc*, *Dhb*, *Dhg*
- Functional genes e.g., *tceA*, *bvcA*, *vcrA*



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Certificate of Analysis: Gene-Trac® NitroGen™
Ammonia Monooxygenase A Assay

Customer: Savannah Volkoff, Geosyntec Consultants
Project: Terminal Road
Customer Reference: GN8084

SiREM Reference: S-8258
Report Date: 4-Oct-21
Data Files: QS3A-amoA-QPCR-0102

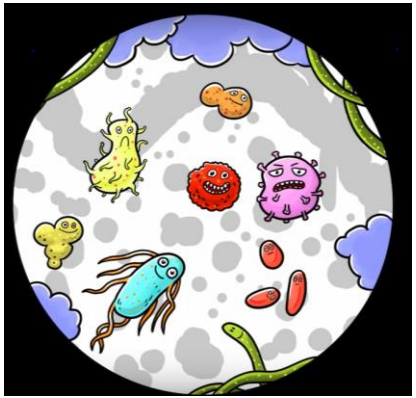
Table 1d: Test Results

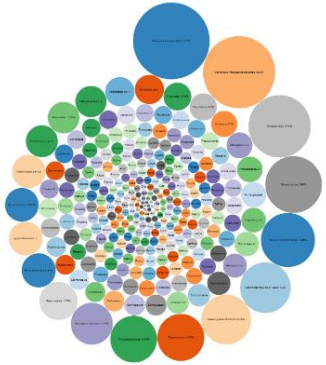
Sample ID	Ammonia Monooxygenase A *amoA* (archaeal)		Ammonia Monooxygenase A *amoA* (bacterial)	
	Percent [2]	Gene Copies/Liter	Percent [2]	Gene Copies/Liter
MW-2-20210803	0.01 - 0.03 %	3×10^5	NA	1×10^4 U
MW-1-20210803	0.006 - 0.02 %	5×10^4	NA	1×10^4 U
INJ1-20210803	0.002 - 0.007 %	1×10^5	NA	1×10^4 U

See final page for notes.

Next Generation Sequencing

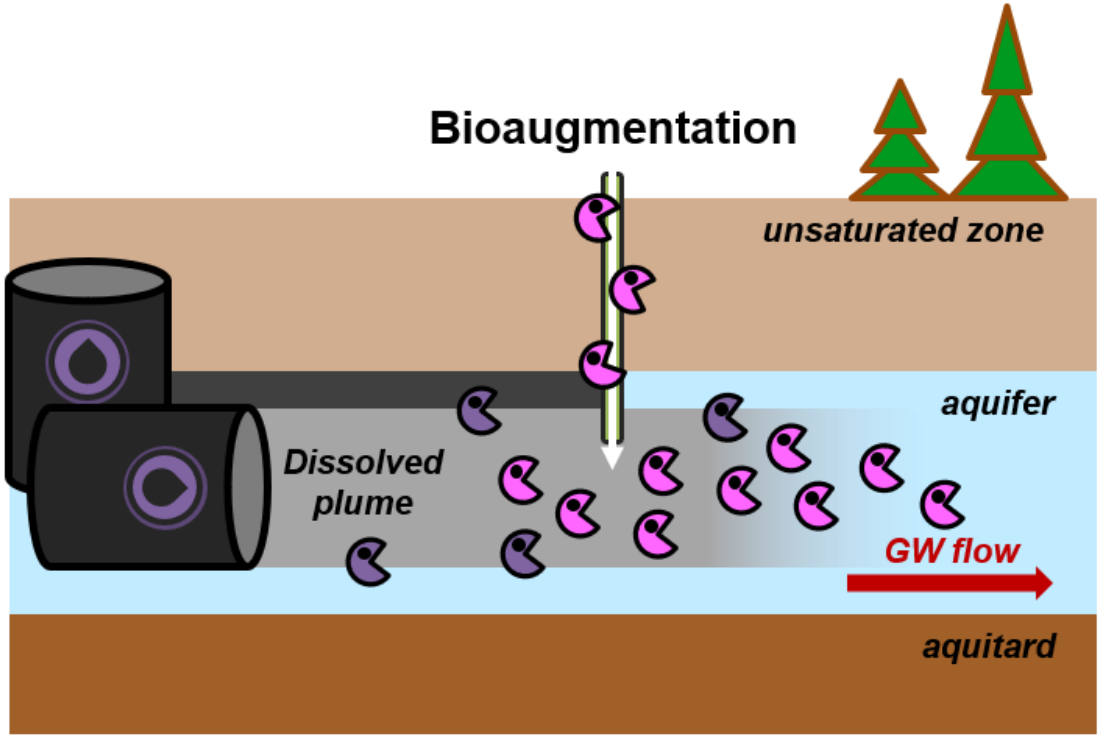
Characterize the entire microbial community





Review of Bioaugmentation

- **The use of high concentrations of cultured microorganisms to speed up degradation of specific (targeted) contaminants**



Injected microbes:

- Perform the **same/similar metabolic functions** as intrinsic pollutant-degrading microbes
- Are often **closely related** to intrinsic pollutant-degrading microbes

Anaerobic Injection Water

Benefits of KB-1 Primer

- Reducing conditions achieved within hours
- Fully dissolvable – no tank residues

Advantages of Enhanced In Situ Bioremediation (EISB)

- Cost Effective: As little as 1/3rd the cost of other in situ remediation options
- High Concentrations Treatable: Including DNAPL/LNAPL sites
- Sustainable: low carbon foot print/natural process
- Inobtrusive: no excavations or excavated soils that require treatment
- Compatible with remote sites: no utility or maintenance requirements
- Destroys Contaminants: doesn’t just move them
- Resistant to Rebound: Once down concentrations tend to stay down

NSN –why?

- The Canadian Environmental Protection Act, 1999 (CEPA 1999), promulgated in 1988 and amended in 1999, provides the federal government the authority to address pollution issues.
- It addresses substances ranging from chemicals to animate products of biotechnology (i.e., living organisms).
- The Act takes a preventative approach by requiring that substances be identified and assessed, prior to market introduction, to determine whether they are "toxic" or capable of becoming toxic.

NEW SUBSTANCES NOTIFICATION REGULATIONS (ORGANISMS)

To help protect the health of Canadians and the environment, the *New Substances Notification Regulations (Organisms)* were created to ensure the proper assessment of new living organisms introduced into the Canadian marketplace.

If you plan to manufacture or import a new living organism subject to notification under the Regulations, you are required to provide information to Environment and Climate Change Canada (ECCC).

DO YOU MANUFACTURE OR IMPORT LIVING ORGANISMS OR ANIMATE PRODUCTS OF BIOTECHNOLOGY?

A living organism is a substance that is an animate product of **biotechnology**. It can consist of micro-organisms like bacteria, fungi, yeasts, protozoa, algae, viruses, or eukaryotic cell culture. It can also consist of other organisms including animals and some plants, such as those that are not indigenous to Canada or are genetically modified.

Examples include:

- Naturally occurring micro-organisms, plants and animals used in biotechnology applications, such as bioremediation, industrial enzyme production and fermentation;
- All genetically modified or bio-adapted micro-organisms;
- All genetically modified, bio-adapted, and chimeric plants and animals, including vertebrates and invertebrates;
- Interspecies hybrids; and
- Animals derived from in-vitro culture.

Biotechnology is the application of science and engineering in the direct or indirect use of living organisms or parts of products of living organisms in their natural or modified forms.

Products derived from biotechnology can be used in a variety of sectors* including:

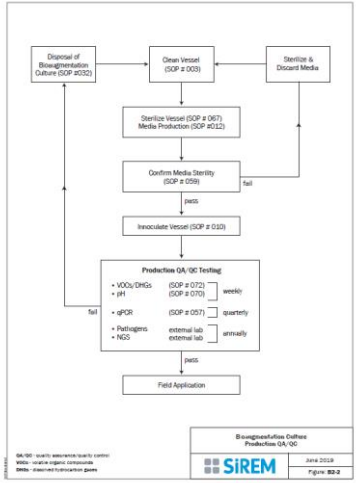
- Aquaculture
- Biocatalysis / Biosurfactant
- Biodegradation / Bioremediation
- Bioleaching / Biomining
- Biomass fuel
- Biodetergent / Degreaser
- Cosmetic
- Energy and Fuel
- Food
- Human health (vaccines, gene therapy etc.)
- Medical (diagnostic)
- Pharmaceutical
- Pulp and Paper
- Textile
- Wastewater treatment

*Please note that this list is not exhaustive

Environment and Climate Change Canada | Environnement et Changement climatique Canada | Canada

SiREM | siremlab.com

Lots of Information Required



Contents of bioaugmentation field injection kit, includes vessel in over pack, hose, regulator, scale fittings and miscellaneous tools

SiREM

Rev. No.: 1
Date: 25 April 2019
Page: 1 of 6

SAFETY DATA SHEET

1. CHEMICAL IDENTIFICATION AND COMPANY INFORMATION

Product Name: DGG-B
Company Info: SiREM
130 Stone Rd. W., Guelph, Ontario, Canada, N1G 3Z2
Phone: 519-822-2265
Toll Free, North America: 1-866-251-1747
Fax: 888-635-3470
www.siremlab.com

Emergency Phone Number: 519-822-2265 (for 24/7 assistance, contact poison center hotline in your jurisdiction).

Description: Microbial inoculum (non-pathogenic, non-hazardous) in growth media consisting of a dilute aqueous solution of mineral salts and nutrients.

Recommended Use: Bioremediation of contaminated groundwater.

Restrictions on Use: DGG-B product intended for laboratory research and field applications for cleanup of contaminated groundwater. Products are not intended to be used as human or animal therapeutics, cosmetics, agricultural or pesticide products, food additives, or as household chemicals.

2. HAZARDS IDENTIFICATION

GHS Classification: Not classified as "hazardous" per OSHA 29 CFR 1910.1200, "Hazard Communication".

GHS Label elements, including hazard and precautionary statements: Not Applicable.

HMIS Rating:	Health	Flammability	Physical Hazard	Personal Protection
	1	0	0	B*
NFPA Rating:	**Health**	**Flammability**	**Reactivity**	**Special Hazard**
	1	0	0	N/A

* B = Safety Glasses, Gloves.

A review of available data indicates minimal potential for health effects related to normal use of this product. Microbial components are non-pathogenic. The product is not expected to be a health hazard as a result of inhalation of mists, ingestion or skin contact. Eye contact may result in mild irritation/redness. Normal hygiene precautions should be observed, including eye protection, skin protection, and hand washing. The potential exists for individuals with hypersensitivity to biological materials to exhibit allergic sensitivity to biological components of this product (see Section 4, "First Aid Measures").

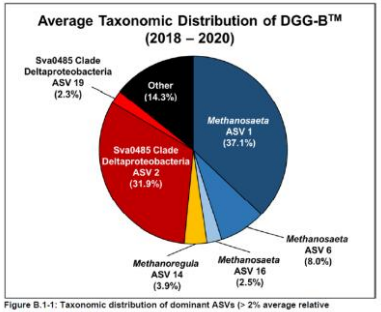


Figure B.1-1: Taxonomic distribution of dominant ASVs (> 2% average relative abundance) in DGG-B™.

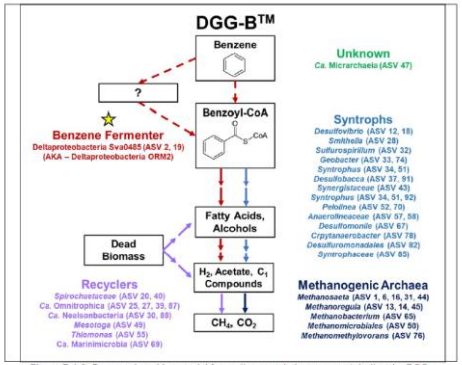


Figure B.1-3: Proposed working model for methanogenic benzene metabolism by DGG-B™

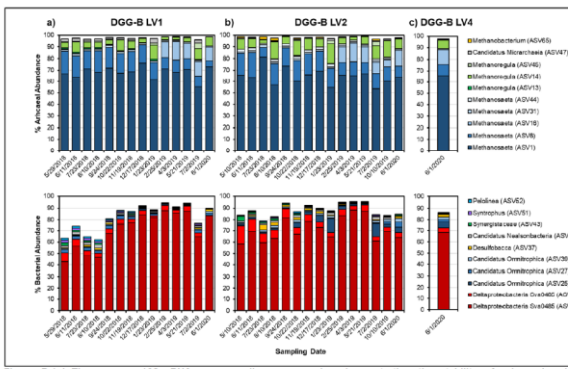


Figure B.1-4: Time course 16S rRNA gene amplicon sequencing demonstrating the stability of major archaeal bacterial ASVs in DGG-B™ (2018 – 2020)

DOMAIN ARCHAEA – GENUS: METHANOSAETA

Role in Benzene Degradation: *Methanosaeta* (88.4 ± 7.2% of Illumina archaea reads) are a genus of acetoclastic methanogens and the dominant Archaea in DGG-B™. Their role is to convert acetate (a chief benzene fermentation product) into methane and CO_2.

Overview: *Methanosaeta* spp. are Gram negative, obligate anaerobes that are non-motile, non-spore forming, and rod-shaped cells (2 – 6 µm in length) with flat ends (Patel and Sprott, 1990; Scholten and Stams, 2000). At high cell densities, cells may rearrange into long (>50 µm), flexible chains (Patel and Sprott, 1990). They are solely capable of using acetate for methanogenesis (Patel and Sprott, 1990; Scholten and Stams, 2000). Because no other acetoclastic methanogens exist in DGG-B™, *Methanosaeta* serve an unequivocal role in acetate transformation to methane and CO_2 (Ulrich and Edwards, 2003; Devine, 2013; Luo et al., 2016). This has been verified in numerous molecular and metagenomic surveys of the DGG-B™ culture lineage (Ulrich and Edwards, 2003; Devine, 2013; Luo et al., 2016). In nature, the *Methanosaeta* are among the most dominant methanogens on earth (Smith and Ingram-Smith, 2007). Isolates and 16S rRNA gene sequences have been retrieved from diverse anaerobic ecosystems such as rice paddies (Mizukami et al., 2006), contaminated aquifers (Struchtemeyer et al., 2005), sewage sludge (Patel, 1984), freshwater (Scholten and Stams, 2000) and marine sediments (Dhillon et al., 2005), oil reservoirs (Grabowski et al., 2005), and reactors (Ma et al., 2006) among others (Holmes and Smith, 2016). There are 2 complete, published genomes of *Methanosaeta*, available here. There is no evidence that any methanogenic archaea including *Methanosaeta* are toxic or pathogenic.

Stability: Clone and pyrosequencing archives suggest that the relative abundance of *Methanosaeta* in OR-1b and OR-1bBa was < 50% (Ulrich and Edwards, 2003; Devine, 2013; Luo, 2016). From December 2012 (OR-1bBa) to May 2017 (DGG-100), *Methanosaeta* steadily increased to ~90% of archaeal reads, where they have remained stable ever since.

Phylogeny: *Methanosaeta* belong to the family *Methanosarcinaceae*, as do the *Methanomethylovorans*. They can be distinguished by their 16S rRNA gene sequences, cell morphology, and growth substrates. OTU 1 is the only microorganism in DGG-B™ classified as *Methanosaeta*. OTU 1 shares 100% sequence identity to *M. concilii* strain GP6, the type species of this genus (Table B.1-1 and Figure B.1-7). Strain GP6 was first isolated from a mixed cultured enriched from a wastewater treatment plant in Ottawa, Ontario, and is only known to metabolize acetate (Patel, 1984).

SiREM Bioaugmentation Cultures

SiREM has bioaugmentation capabilities for the following compounds:

Commercially Available in Canada

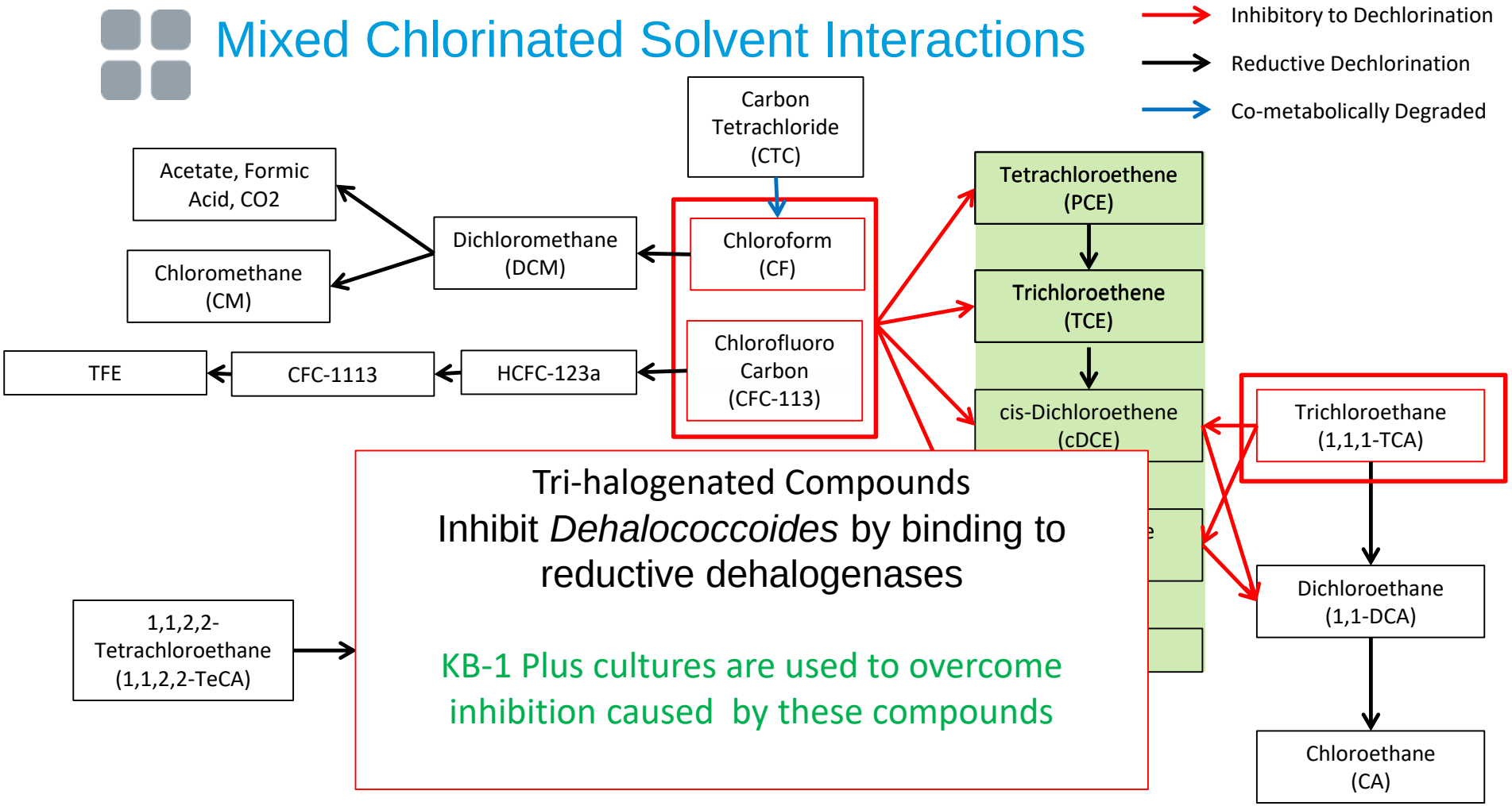
- Chlorinated ethenes (PCE, TCE, DCE, VC)
- Benzene, Toluene and Xylene (anaerobic pathway)

Coming Soon:

- Chlorinated ethanes (1,2-DCA, 1,1,1-TCA, TeCA)
- Chlorinated methanes (CF, DCM)

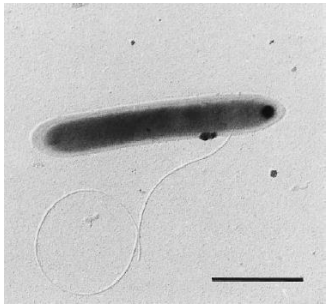
KB-1® DGG PLUS™ KB-1 plus®

Mixed Chlorinated Solvent Interactions

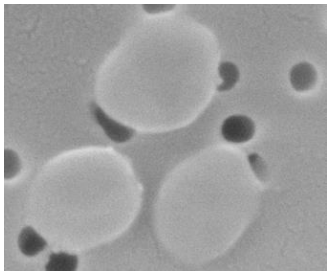


Dehalobacter (*Dhb*) & *Dehalogenimonas* (*Dhgm*)

- 1,1,1-TCA degradation to CA (*Dhb*)
  (Grostern and Edwards, 2006)
- Chloroform to Dichloromethane *(cfrA*)
  (Grostern, Edwards, Duhamel and Dworatzek, 2010)
- DCM to acetate
  (Justicia-Leon et al., 2011)
- 1,1,2,2-TeCA to ethene (*Dhgm*)
  (Manchester et al., 2012)



Dehalobacter



Dehalogenimonas

Abiotic & Biotic Degradation of Trihalogenated Compounds

- 1,1,1-TCA

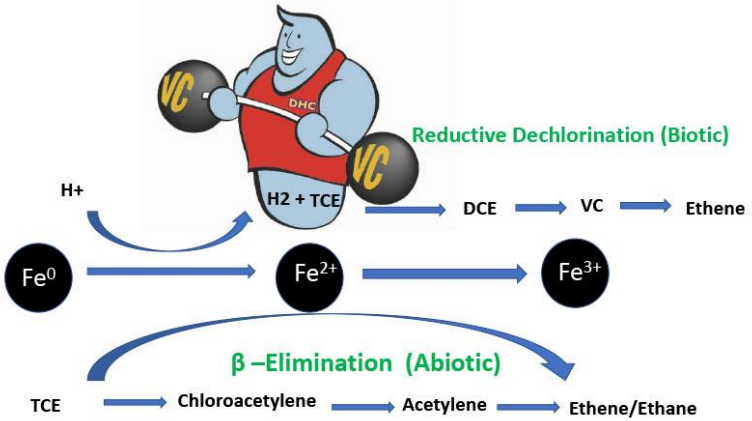
  Metal sulfides can degrade 1,1,1-TCA (Scheutz et al., 2011)

- CFC-113

  Abiotic dechlorination of CFC-113 and CFC-11 by ZVI (Philips et al, 2020)

- Chloroform

  CF degradation was 8X-14X faster when a *Dhb* culture was combined with ZVI compared with ZVI alone. (Lee et al., 2015)



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CASE STUDY 1: CHLORINATED METHANES AND ETHENES

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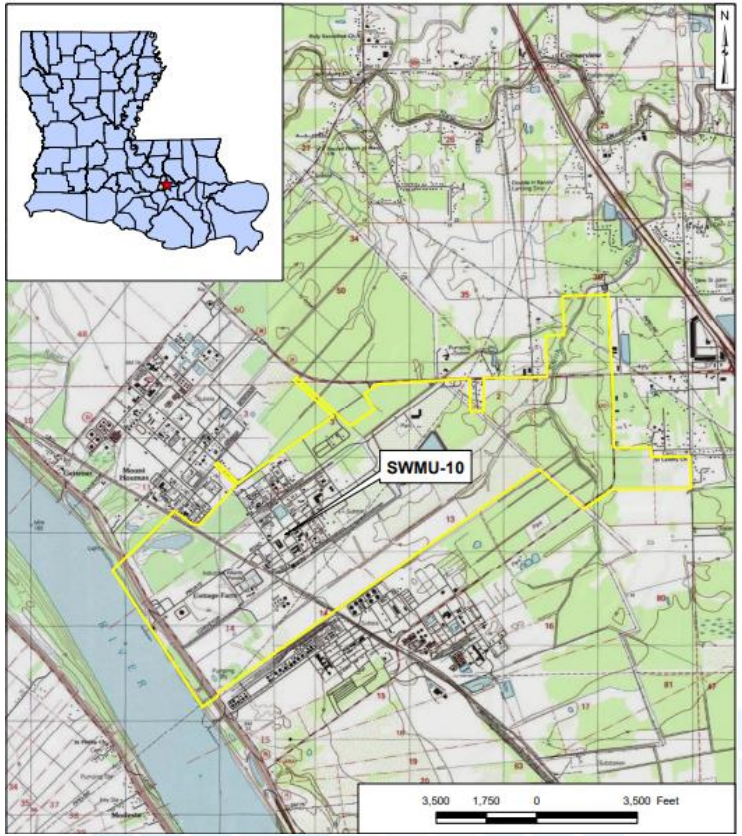
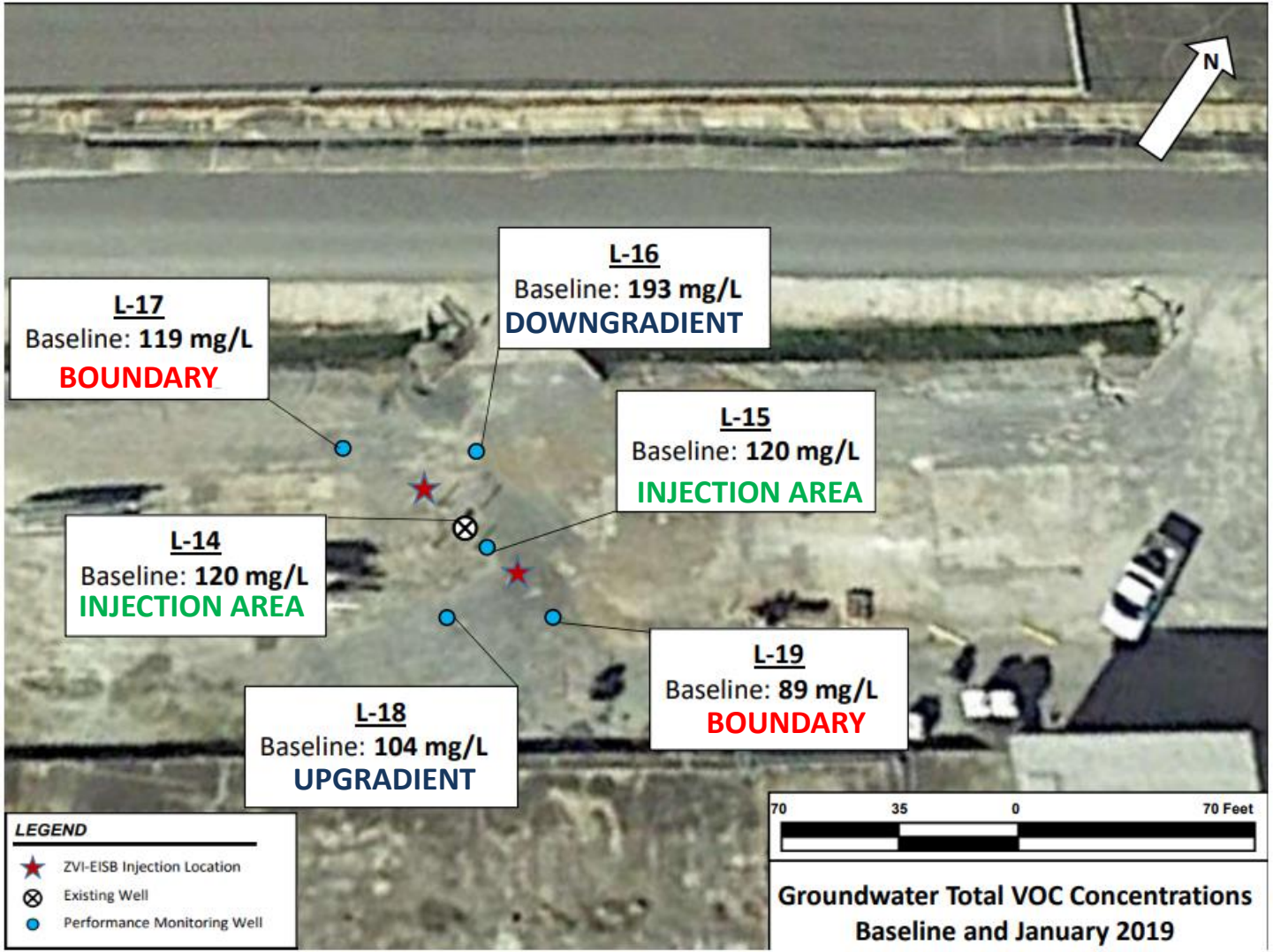
Site Overview

- Manufacturing facility located in Louisiana
- Contaminants include: PCE, TCE, CTC, CF

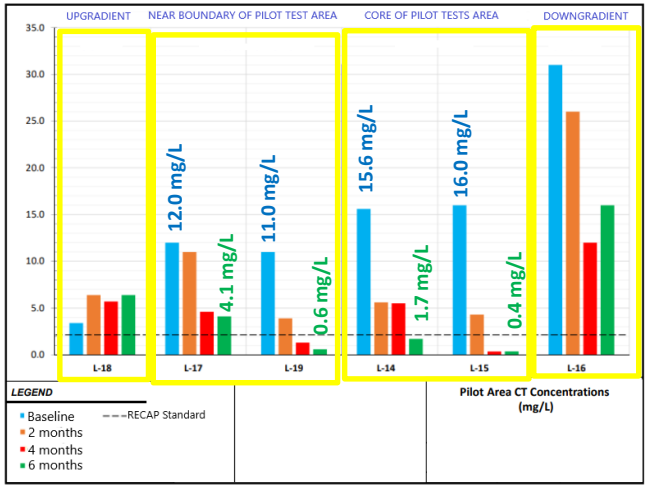
- Treatability Study in 2016
  - ➢ Is anaerobic biodegradation a viable remedial option?
  - ➢ Can ZVI optimize EISB?
  - ❖ Conclusion: The best treatment strategy was observed with the addition of ZVI combined with KB-1 Plus and electron donor addition.

- Pilot Test in 2018
  - ❖ ZVI was injected into the “60 foot zone” – consisting of silts, sandy silts, and silty clays
  - ❖ Two injection wells in SWMU-10 area – injected with ZVI,, KB-1 Plus, and electron donor targeted an ROI of 15’

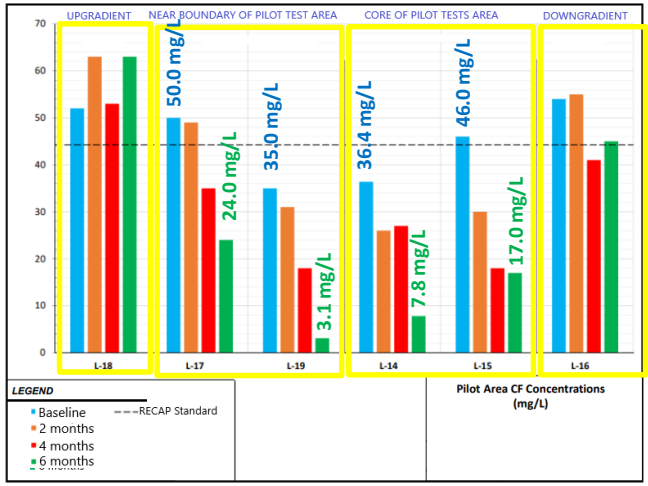



N
L-16
Baseline: 193 mg/L
DOWNGRADIENT
L-17
Baseline: 119 mg/L
BOUNDARY
L-15
Baseline: 120 mg/L
INJECTION AREA
L-14
Baseline: 120 mg/L
INJECTION AREA
L-19
Baseline: 89 mg/L
BOUNDARY
L-18
Baseline: 104 mg/L
UPGRADIENT
LEGEND
ZVI-EISB Injection Location
Existing Well
Performance Monitoring Well
70
35
0
70 Feet
Groundwater Total VOC Concentrations
Baseline and January 2019

CT Concentration (mg/L)

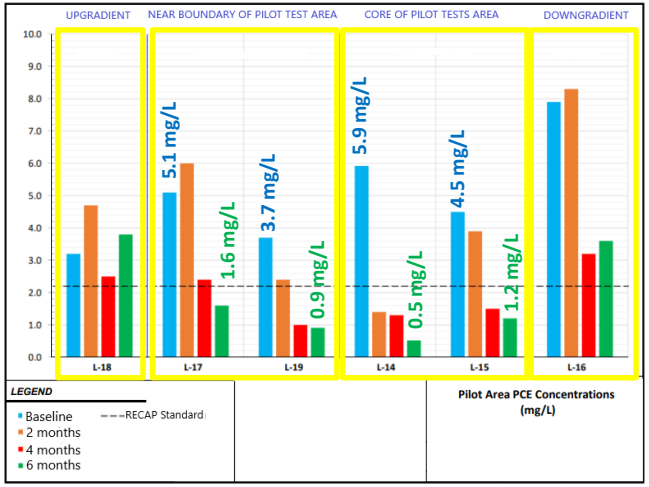


CF Concentration (mg/L)

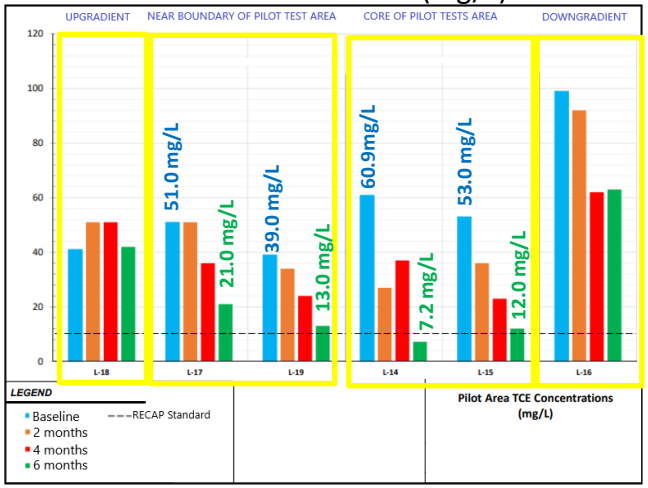


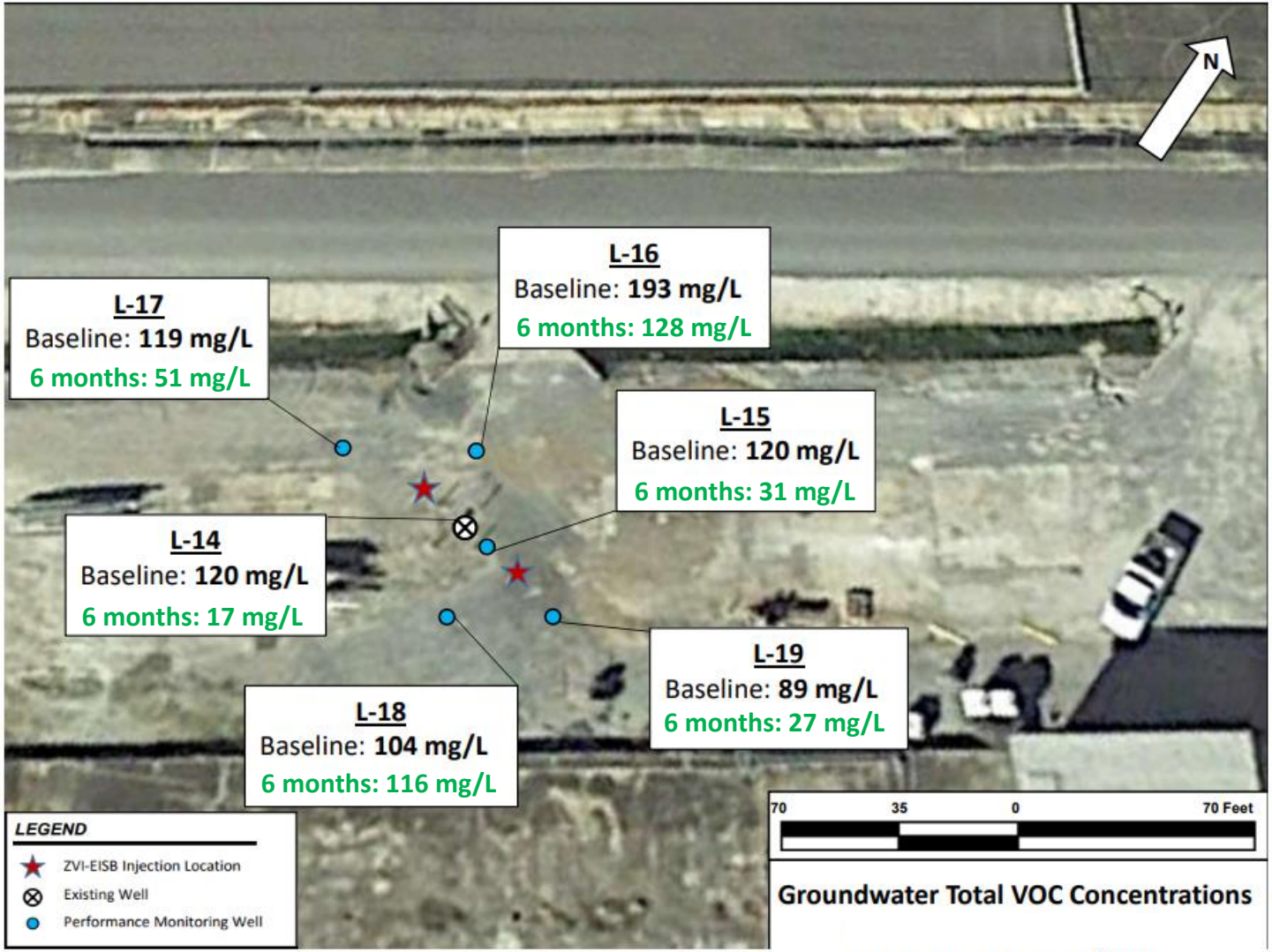
Baseline
2 Months
4 Months
6 Months
RECAP Standard (LA State Standard)

PCE Concentration (mg/L)



TCE Concentration (mg/L)




N
L-16
Baseline: 193 mg/L
6 months: 128 mg/L
L-17
Baseline: 119 mg/L
6 months: 51 mg/L
L-15
Baseline: 120 mg/L
6 months: 31 mg/L
L-14
Baseline: 120 mg/L
6 months: 17 mg/L
L-19
Baseline: 89 mg/L
6 months: 27 mg/L
L-18
Baseline: 104 mg/L
6 months: 116 mg/L
LEGEND
ZVI-EISB Injection Location
Existing Well
Performance Monitoring Well
70
35
0
70 Feet
Groundwater Total VOC Concentrations

Optimize Bioremedation at Mixed Contaminant Sites

- Treatability studies provide proof of concept and information to optimize the remedial strategy
- Molecular Gene-Trac testing can be used to determine if key degrading bacteria are present and at sufficient concentrations
- Bioaugment to introduce key degrading bacteria

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CASE STUDY 2: CHLORINATED METHANES AND 1,4-DIOXANE

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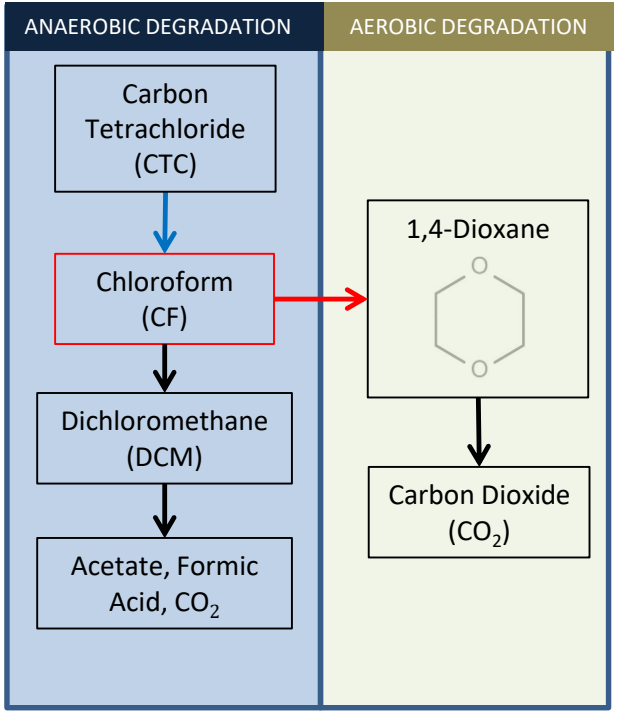
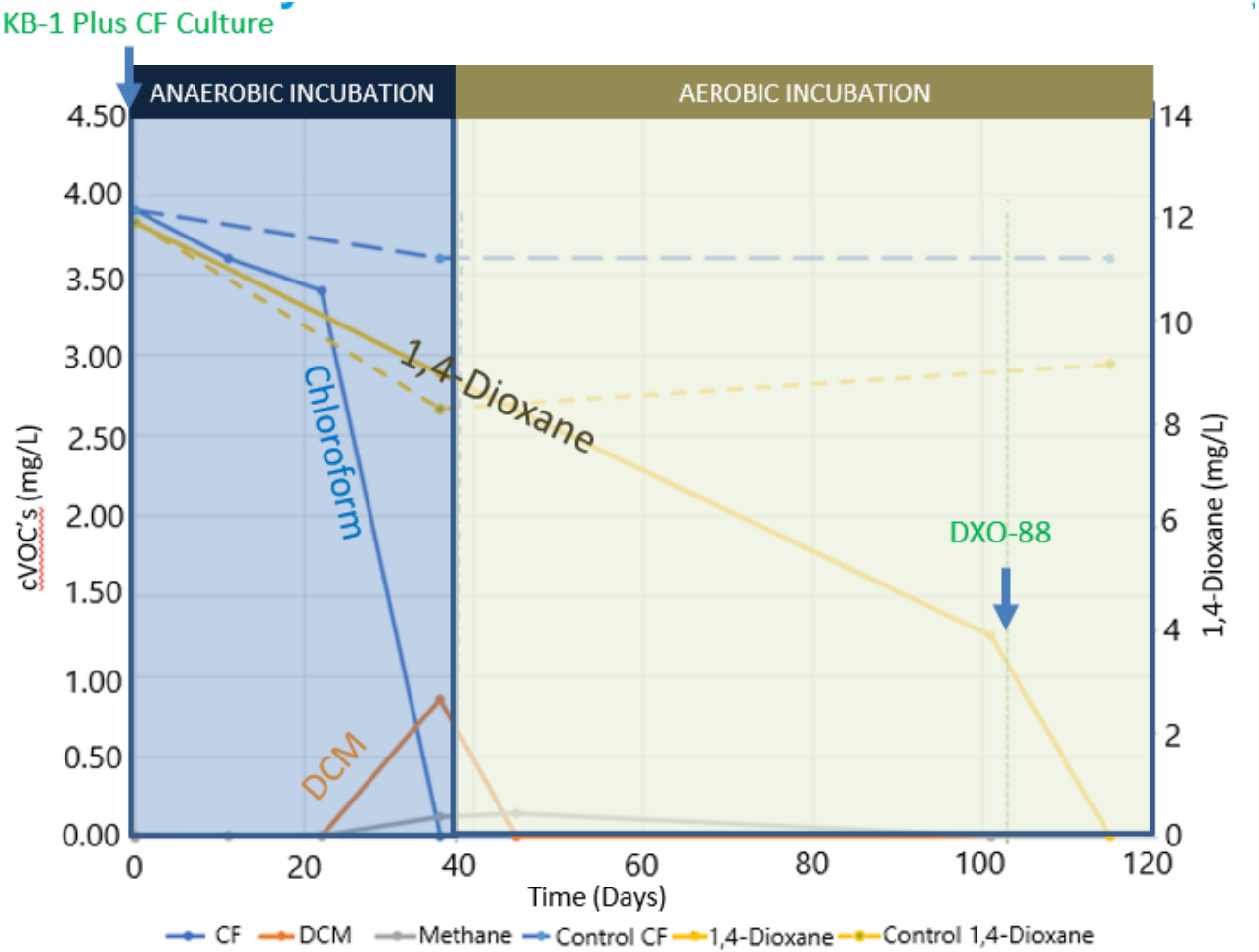
1,4-D and CF Treatability Study (Confidential Site)

- Problem: Chloroform more readily degrades under anaerobic reductive conditions and 1,4-D under aerobic conditions, CF inhibits aerobic 1,4-D degradation

Solution?: Phased anaerobic/aerobic bioaugmentation

- KB-1® Plus – CF Anaerobic Culture
- DXO-88™ – 1,4-Dioxane Aerobic Culture




KB-1 Plus CF Culture
ANAEROBIC INCUBATION
AEROBIC INCUBATION
cVOC's (mg/L)
1,4-Dioxane (mg/L)
Time (Days)
Chloroform
1,4-Dioxane
DCM
DXO-88
CF
DCM
Methane
Control CF
1,4-Dioxane
Control 1,4-Dioxane

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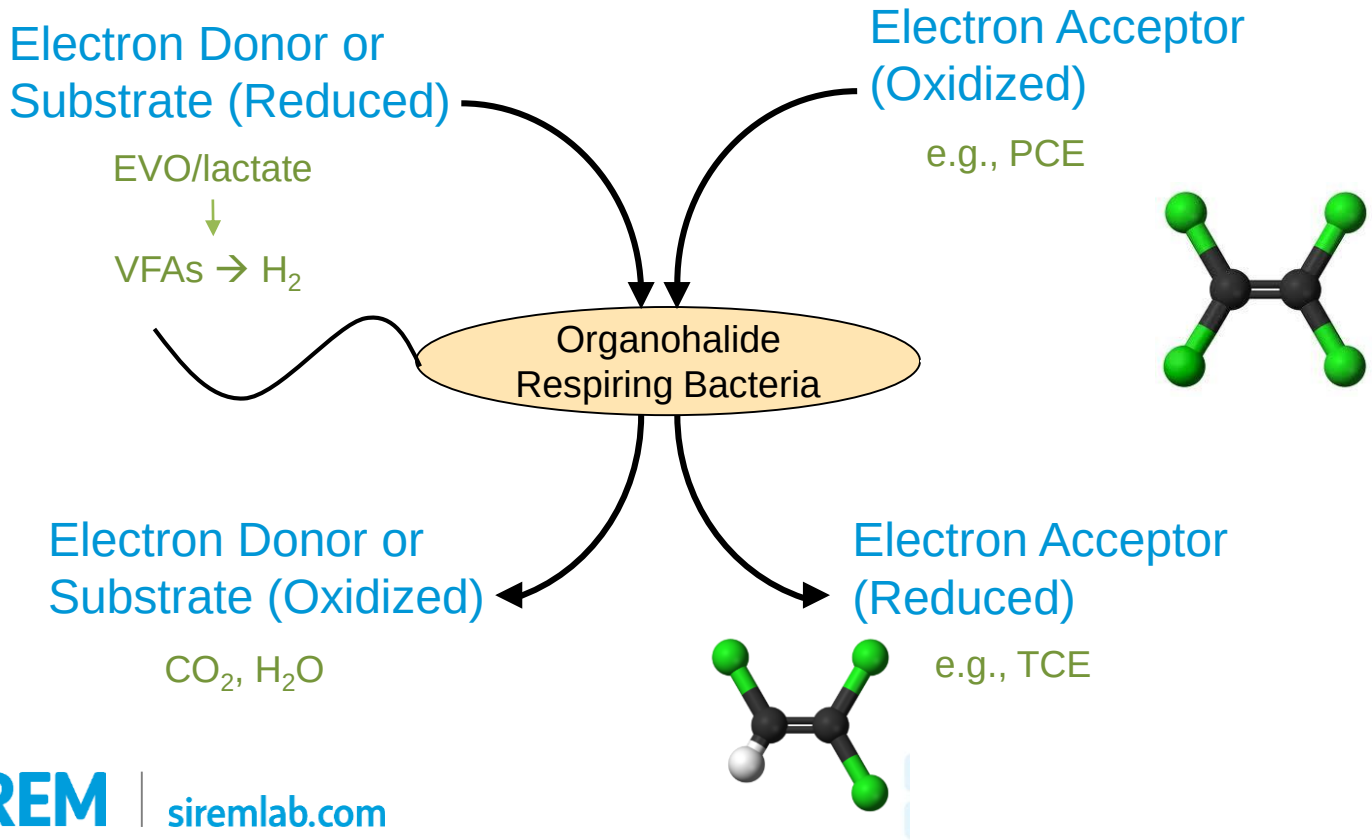
CASE STUDY 3: CHLORINATED ETHENES & CHLORINATED ETHANES, & PETROLEUM HYDROCARBONS (ANAEROBICALLY)

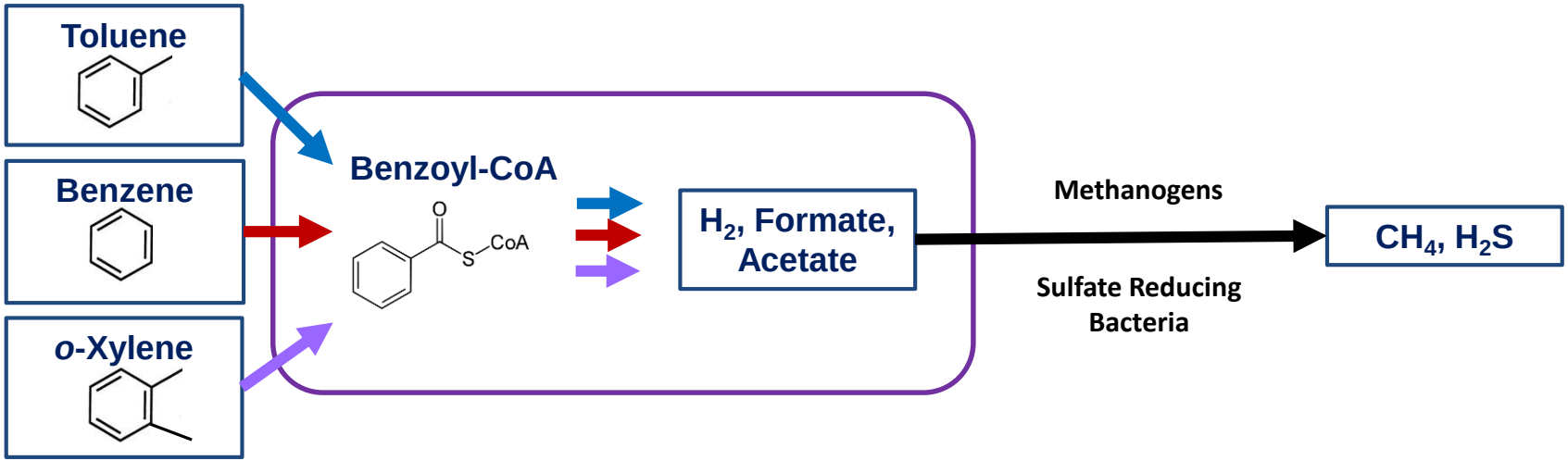
Chlorinated Solvents as Electron Acceptor



Key Difference Between Bioremediation of Chlorinated Solvents vs Hydrocarbons

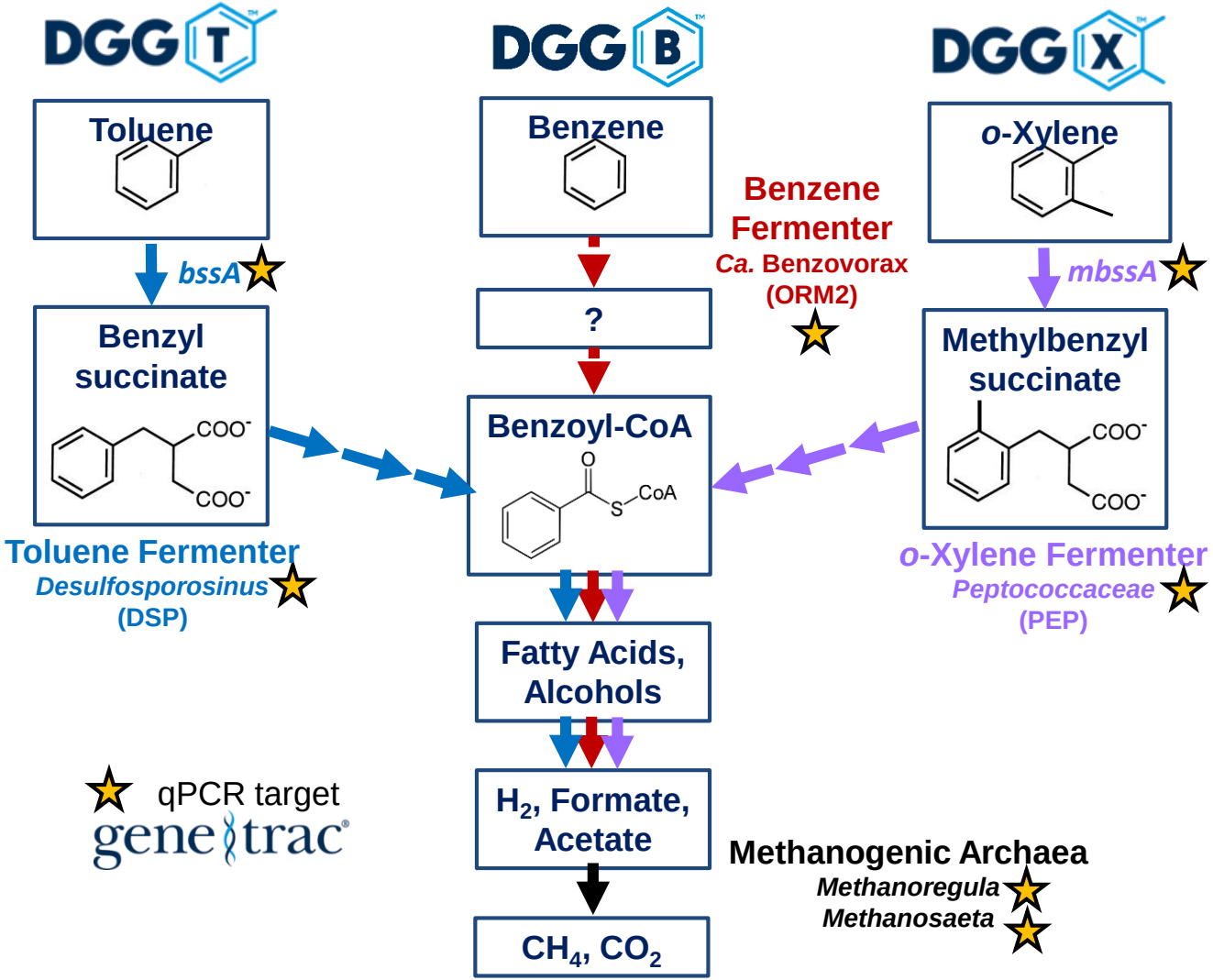
Hydrocarbons are *electron donors* rather than electron acceptors

- Adding carbon (sugars, VFAs, yeast extract) may not enhance bioremediation performance



DGG PLUS™

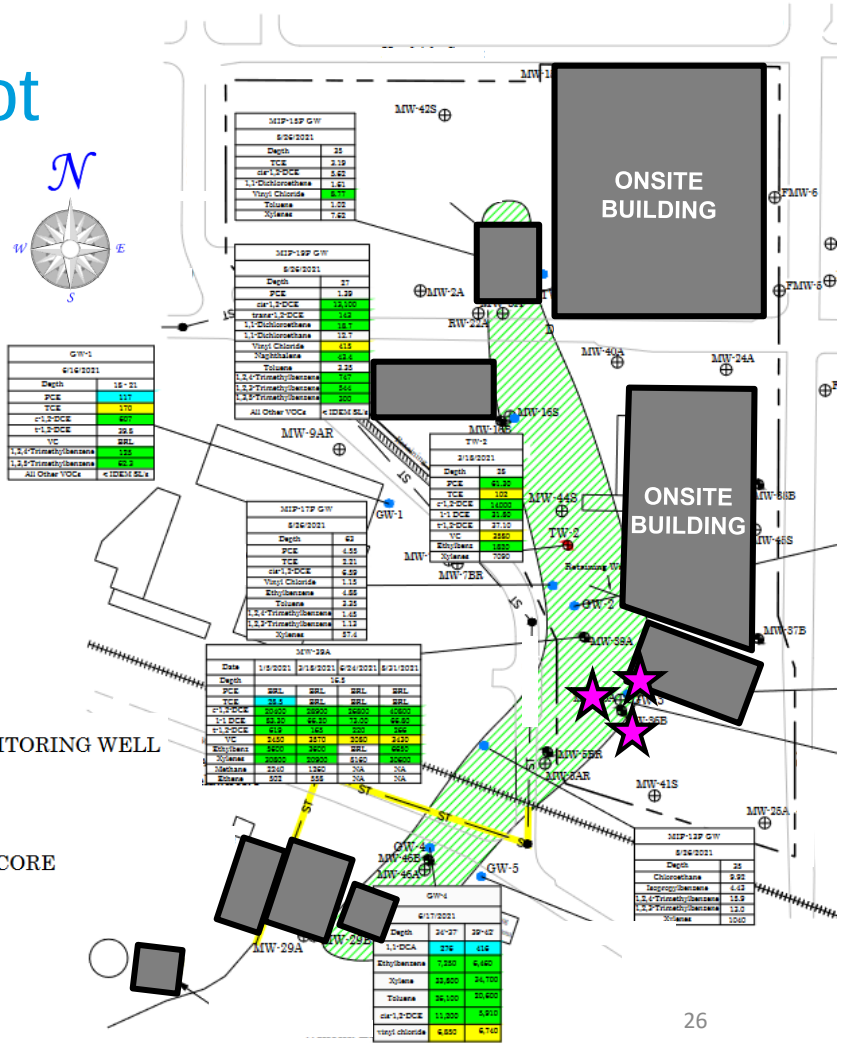
- Anaerobic culture for benzene, toluene and xylene
- The key microbes in each culture include **hydrocarbon fermenters** and **methanogens**
- Key microbes & functional genes can be monitored by **qPCR** and/or **NGS**



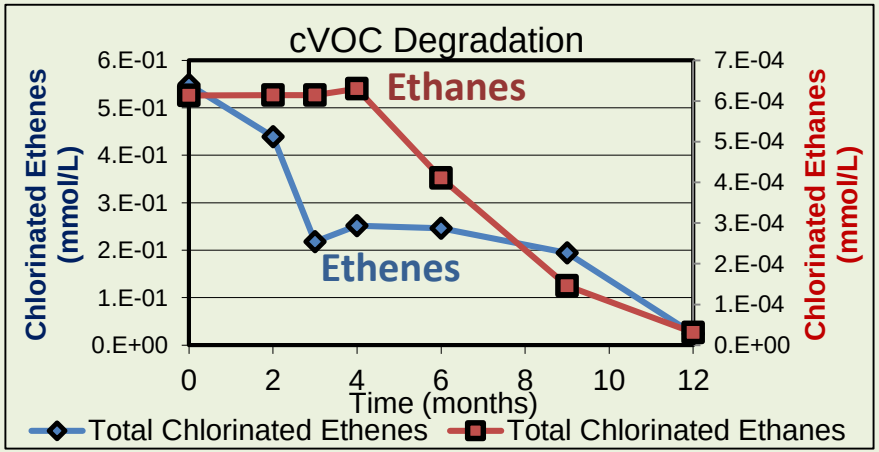
Results from a Field Pilot

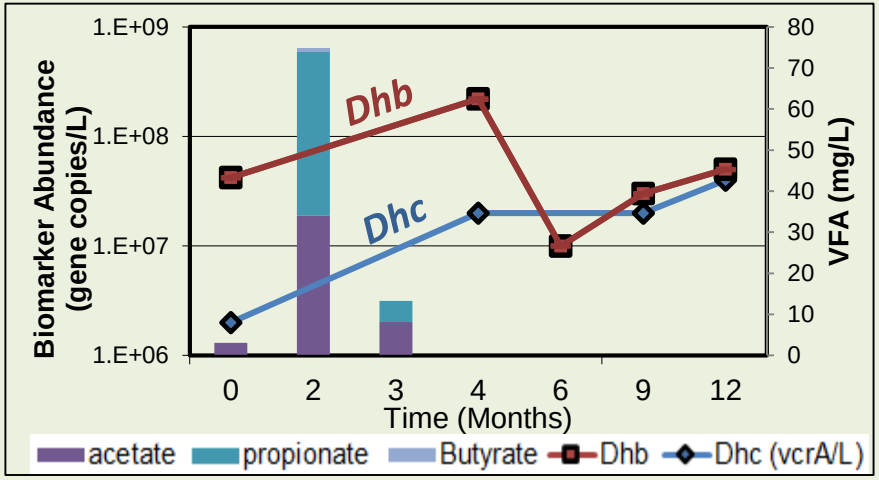
- US chemical manufacturing site, groundwater contaminated with chlorinated ethenes, chlorinated ethanes, and TEX
  - Green = exceeds drinking water limits
  - Blue = exceeds residential vapor limits
  - Yellow = exceeds industrial vapor limits

- In Sept 2020, a blend of KB-1® Plus and DGG Plus™ was injected at 3 points (★) near the center of the plume core

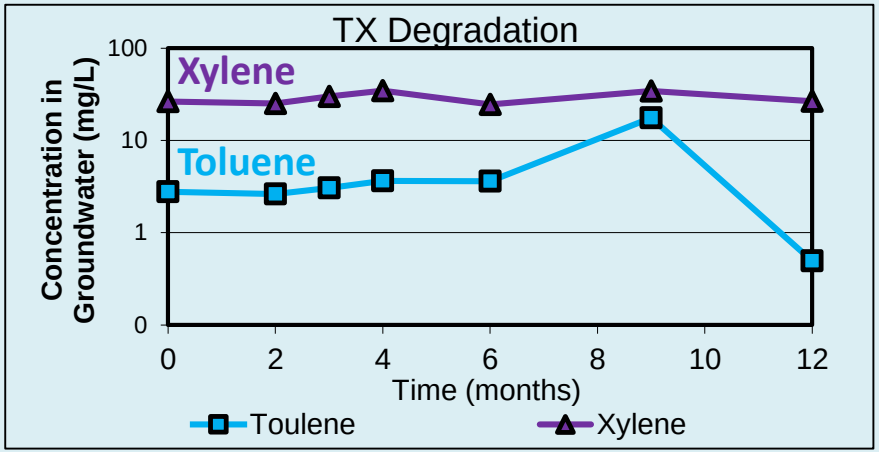


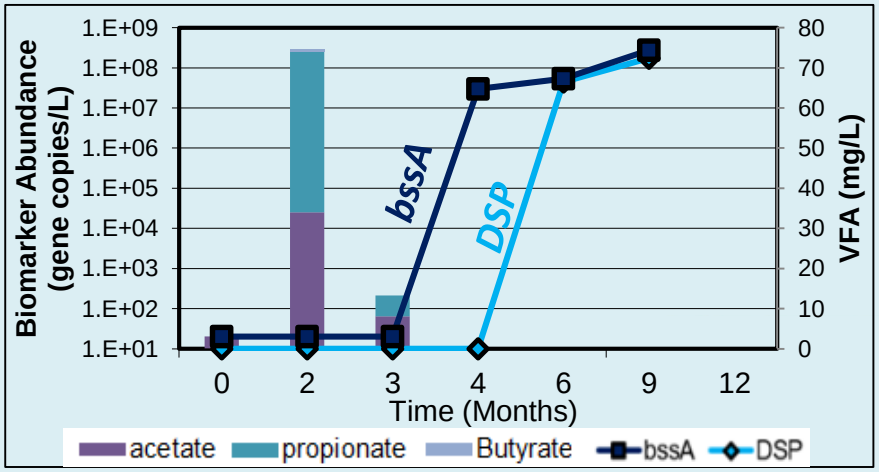
CHLORINATED ETHENES & ETHANES





TOLUENE & XYLENE





To Wrap up

1. Know your microbes;
   - *Dhc* for chlorinated ethene degradation (KB-1)
   - *Dhb* and *Dhg* for chlorinated methane and ethane degradation (KB-1 Plus)

2. Optimize Degradation of mixed chlorinated solvents by promoting chemical reduction and biotic degradation

3. Environment Canada NSN approval for KB-1 Plus cultures target date is August 2023

Questions?

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