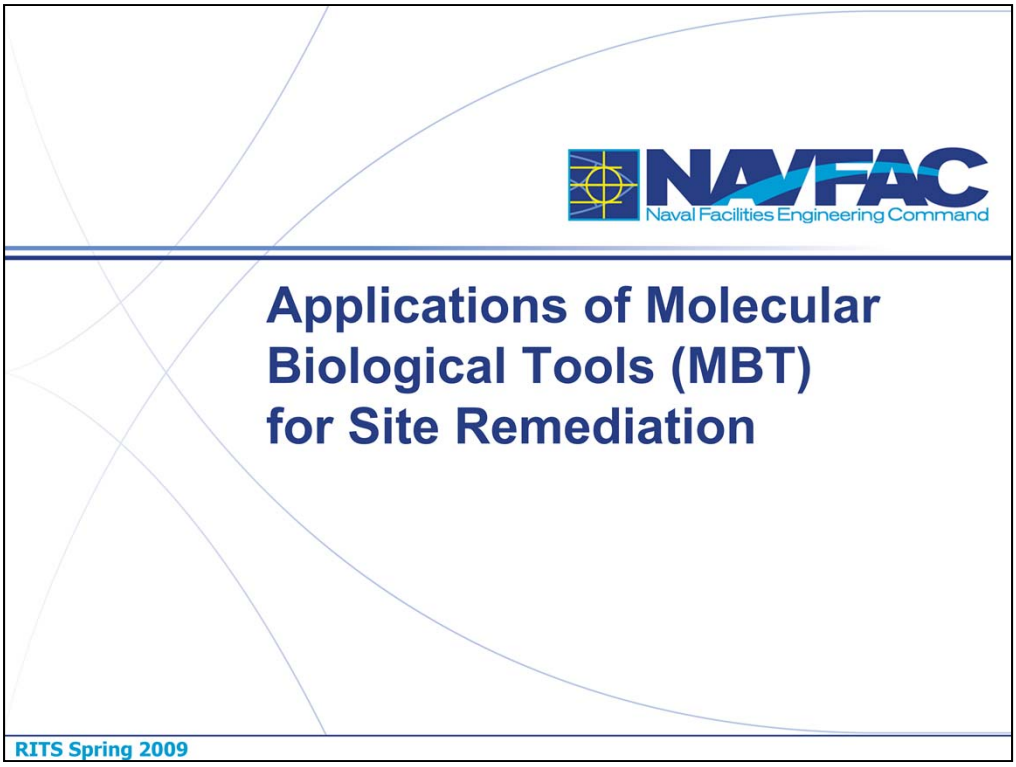


Goal today is to provide you with good science as well as great practical information on how to use these tools in your projects.

Presentation Overview

- Introduction
- MBT Applications
- Sampling
- Cost and Regulatory Concerns
- Case Studies
- Guidance Protocol
- Summary

- Start with an introduction and discuss some basics on bioremediation and MBTs.
- Review applications of MBTs for a variety of contaminants.
- Go into a lot of practical information that will help you on your projects – sampling, costs, regulatory issues.
- Several case studies to show that these tools work.
- Finish with review of guidance protocol.

Topic Context – Past RITS

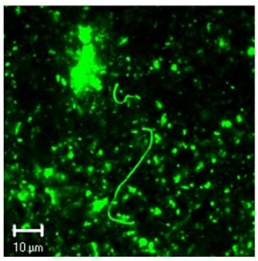
- Enhanced Bioremediation Technologies (Spring 2000)
- Advancements in In Situ Bioremediation (Spring 2001)
- DCE/VC Stall at Natural Attenuation Sites (Fall 2003)
- Anaerobic Bioremediation Using Biobarriers (Fall 2006)
- Bio – State of the Practice (Fall 2007)

3 Introduction RITS Spring 2009: Applications of MBTs for Site Remediation

- Various aspects of bioremediation have been covered in previous RITS seminars since 2000.
- You had a thorough “bio – state of the practice” review in 2007.
- I’ll be bringing you up to date on MBTs

Definition of MBT

- **Molecular Biological Tools (MBTs) are *"tools that target biomarkers (e.g., specific nucleic acid sequences, peptides, proteins, or lipids) to provide information about organisms and processes relevant to the assessment and or remediation of contaminants"* (SERDP, 2005)**



4 Introduction RITS Spring 2009: Applications of MBTs for Site Remediation

- Our first acronym…MBT…stands for molecular biological tools.
- They can be DNA, RNA, proteins or lipids that provide us info on biodegradation processes
- Here's an example where we can count the number of bacteria that have a specific DNA sequence using a fluorescent label

What's In It For Me?

- Learn the science behind MBTs
- Learn when to use MBTs
- Learn how to sample groundwater for MBTs
- Learn the "Rules of Thumb" for MBT data
- Learn how to save time and money with a smarter bioremediation design and operation – do I bioaugment?

5 Introduction RITS Spring 2009: Applications of MBTs for Site Remediation

- You'll learn the science, when to use MBTs, how to sample, the "Rules of Thumb" for the data and finally – how to work smarter when designing and reviewing performance data

Purpose of MBTs

- Reduce remediation costs and increase effectiveness by
  - Supporting sites where monitored natural attenuation (MNA) is being evaluated
  - Predicting sites where biostimulation will succeed
  - Identifying sites where bioaugmentation is required

- Why do we use MBTs?
- Three situations: supporting MNA evaluations, predicting sites where biostimulation is good enough, and identifying sites where we'll have to bioaugment

Biodegradation Review

- Subsurface environments consist of multiple electron accepting processes
  - Reduction of oxygen, nitrate, manganese, iron, sulfate, carbon dioxide
  - Can be stratified spatially – either horizontally or vertically
- Certain contaminant biotransformations are restricted to specific redox conditions

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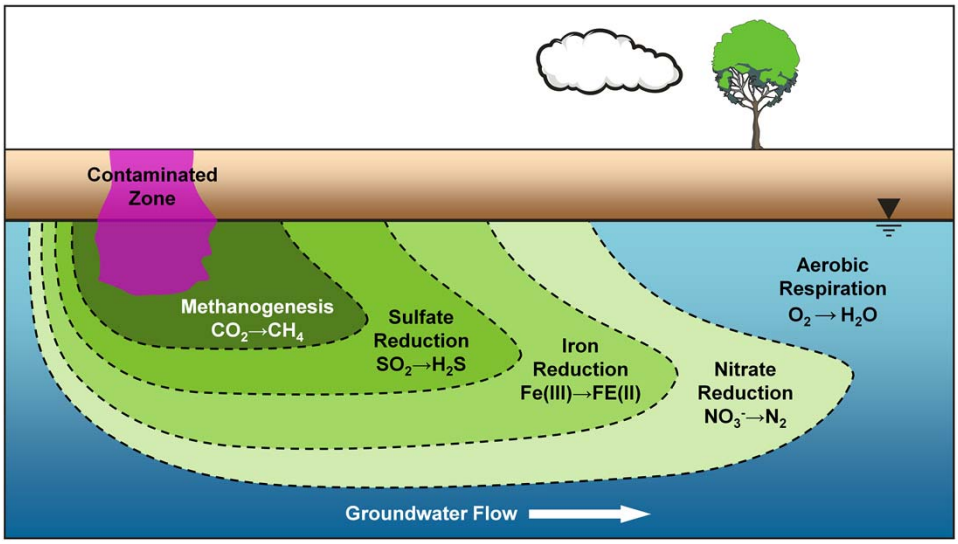
- Did anyone see in the news in April that scientists had found bacteria under a glacier in Antarctica?...could you survive in cold brine without light or oxygen?
- Just like we breathe oxygen, bacteria can use oxygen and a number of other electron acceptors, like nitrate, iron, sulfate or carbon dioxide
- Bacteria can live in these niches which can be stratified both horizontally or vertically

Biodegradation Review (cont.)



Carlton and Klug, 1990

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- Contaminant biodegradation can also be limited to certain conditions
- Here is a typical redox profile that would follow a release of an organic contaminant, like gasoline, or at a landfill
- The subsurface can be aerobic to begin with. At the release point, the organic is in high concentrations, so all of the electron acceptors are used up, leaving methanogenesis.
- Sulfate reduction is next, followed by iron and nitrate reduction as conditions are less reducing and approaching the aerobic background.

Biodegradation Review (cont.)

- **Aerobic microbial metabolism**
  - **Aerobic microorganisms use oxygen as an electron acceptor to oxidize organic compounds in order to obtain energy through aerobic respiration**

$$C_xH_x + O_2 \xrightarrow{\text{bacteria}} CO_2 + H_2O + \text{bacterial growth}$$

Carlton and Klug, 1990

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- In aerobic metabolism, an organic carbon source is oxidized to generate energy and more bacteria.

Biodegradation Review (cont.)

- **Anaerobic microbial metabolism**
  - Anaerobic microorganisms do not require oxygen for growth and may even die in the presence of oxygen
  - Electron acceptors such as nitrate, sulfate, or carbon dioxide can be used to oxidize organic compounds in order to obtain energy through various anaerobic respiration processes

$$C_xH_x + NO_3^- \rightarrow N_2 + CO_2 + H_2O + \text{bacterial growth}$$

$$C_xH_x + SO_4^{2-} + H^+ \rightarrow HS^- + CO_2 + H_2O + \text{bacterial growth}$$

$$C_xH_x + CO_2 \rightarrow CH_4 + H_2O + \text{bacterial growth}$$

- When oxygen has been depleted, bacteria can use other electron acceptors to generate energy…Nitrate…sulfate…carbon dioxide

Biodegradation Review (cont.)

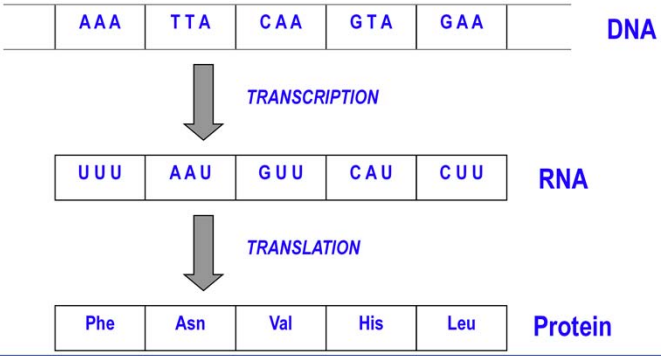
- **Halorespiration**
  - Organisms such as *Dehalococcoides* sp. can acquire energy by using halogenated compounds (e.g., TCE) as electron acceptors
  - This process can result in the reductive dechlorination of chlorinated solvents such as PCE and TCE to non-toxic degradation products (i.e., ethene)

$$C_2HCl_3\ (TCE) + H_2 \rightarrow C_2H_2Cl_2\ (DCE) + H^+ + Cl^- \rightarrow$$
$$C_2H_3Cl\ (VC) + H^+ + Cl^- \rightarrow C_2H_4\ (Ethene) + H^+ + Cl^-$$

- Halorespiration is a unique process where bacteria can breathe on chlorinated compounds, like TCE
- These bacteria have developed a unique niche – they can grow in contaminated zones

Biodegradation Review (cont.)

- DNA to RNA to protein (enzyme)
  - Information contained within functional genes (DNA) lead to the synthesis of proteins (i.e., enzymes), which carry out metabolic processes for the cell
  - MBTs can be used for assessment of DNA, RNA, proteins, or lipids

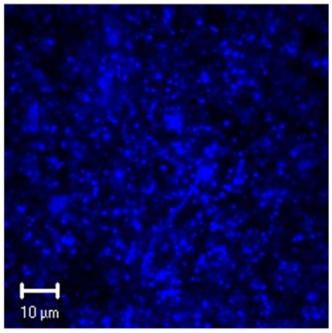


12 Introduction RITS Spring 2009: Applications of MBTs for Site Remediation

- Now let's move our discussion to molecular biology. I know it's been a while since bio 101
- Genes are a part of DNA. It's made up of two complementary double strands
- DNA gets unzipped and transcribed to make RNA which is single stranded
- The RNA is translated into a sequence of amino acids, which make up a protein. In the cell, proteins can twist and fold and become active enzymes which catalyze reactions.

Types of MBTs

- **Nucleic acid probes (DNA or RNA)**
  - **Various targets including**
    - 16S rRNA gene
    - Functional genes (e.g., RDase, Hydrogenase, Oxygenase, etc.)
- **Protein biomarkers**
- **Lipid biomarkers**



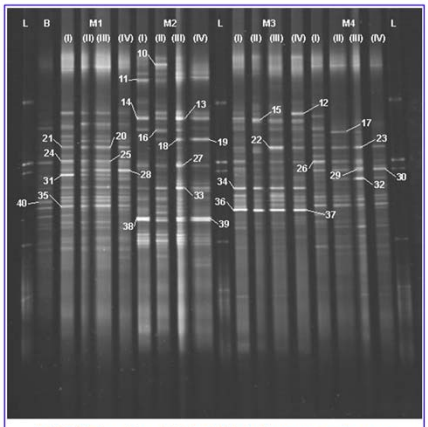
13 Introduction RITS Spring 2009: Applications of MBTs for Site Remediation

- MBTs are available for each step: DNA, RNA or protein
- Probes are available for a variety of targets that are general for a particular genera of bacteria, like the 16S rRNA gene…or specific for specific enzymes
- MBTs are also available for lipids that are in cell membranes to identify certain types of bacteria.

- Our focus today will be on DNA probes. They are commercially available and in use.

Types of MBTs (cont.)

- **Example Analytical Methods**
  - **Community profiling**
    - Denaturing Gradient Gel Electrophoresis (DGGE)
    - Temperature Gradient Gel Electrophoresis (TGGE)
    - Others
  - **Fluorescent *in situ* hybridization (FISH)**
  - **Quantitative real time Polymerase Chain Reaction (qPCR)**



DGGE gel – 16S rDNA fragments are separated into bands. Individual bands can be excised and sequenced.

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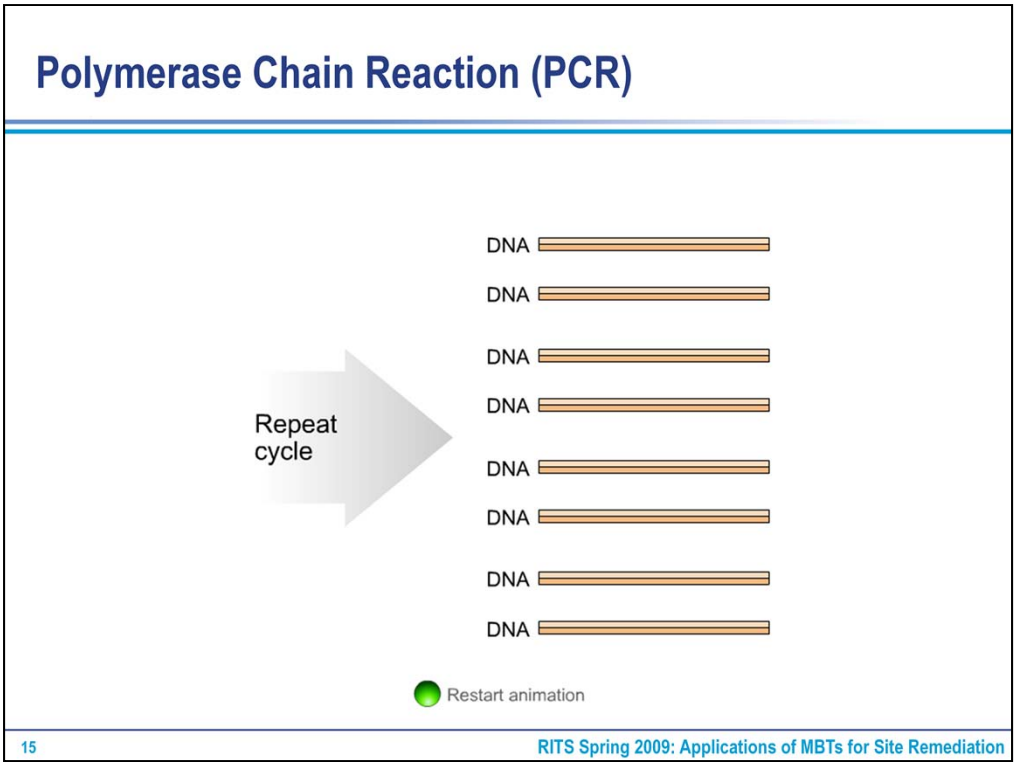
- There are a number of techniques available to analyze DNA. DGGE and TGGE are techniques used to separate fragments of DNA for further analysis.
- DGGE gel shows profiles from 4 microcosms (M1-M4) representing a mixed contaminant plume (BTEX and CE). Microcosms were enhanced with different electron donor amendments (M1-ORC, M2-Molasses, M3-HRC, M4-no amendment). Band positions indicate each amendment resulted in different community profiles.

- FISH uses fluorescence to identify genes of interest. You may recall the slides that I showed earlier with the blue or green bacteria.

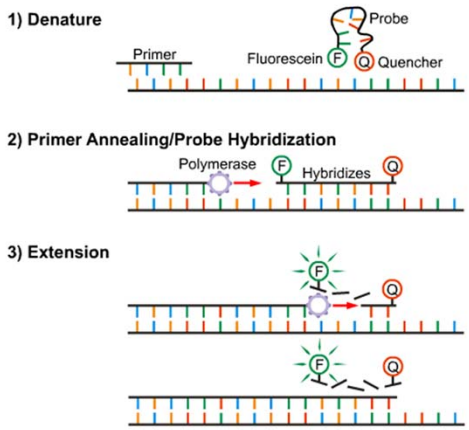
- The most common technique that we use is Quantitative real time polymerase chain reaction or qPCR. I'll be describing it in the next slide.

- DGGE gel shows profiles from 4 microcosms (M1-M4) representing a mixed contaminant plume (BTEX and CE). Microcosms were enhanced with different electron donor amendments (M1-ORC, M2-Molasses, M3-HRC, M4-no amendment). Band positions indicate each amendment resulted in different community profiles.



- In the PCR process, DNA is amplified through repeated cycles of denaturing and synthesis. The DNA unwinds, a specific gene of interest is synthesized and becomes a double helix again. This unwinding, and winding up again is done repeatedly to increase the number of copies of the gene of interest.

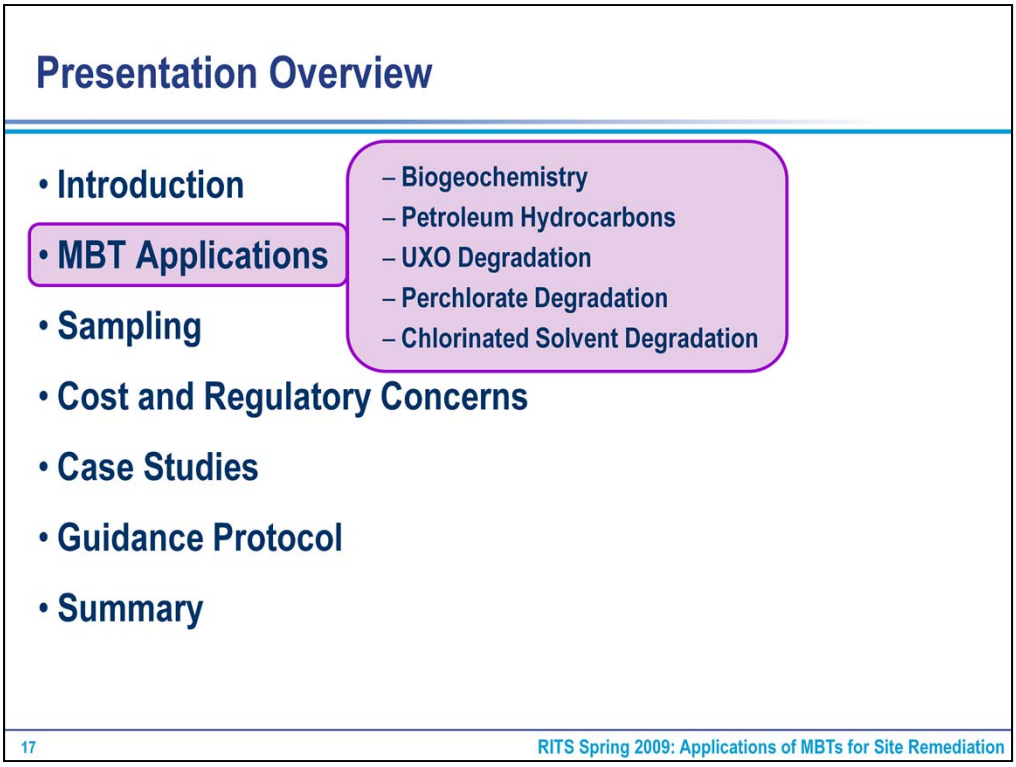
Quantitative Polymerase Chain Reaction (qPCR)



- A signal is detected in PCR by using a probe that has a fluorescent end and a quencher end. When the quencher is attached – no signal. When it's off – signal.

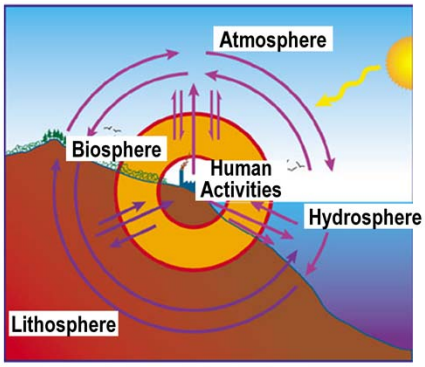
- In step 1, the DNA unwinds opening up the single strand
- In step 2, the probe finds its sequence on the DNA for hybridization
- In step 3, the DNA is synthesized, releasing the fluorescent signal

- The process is repeated to increase the signal. The signal is compared to a standard curve to identify the DNA concentration.



- Next, I will discuss the various applications of MBTs, including biogeochemical and various contaminants – BTEX, RDX, perchlorate. The focus of the presentation will be on chlorinated solvents.

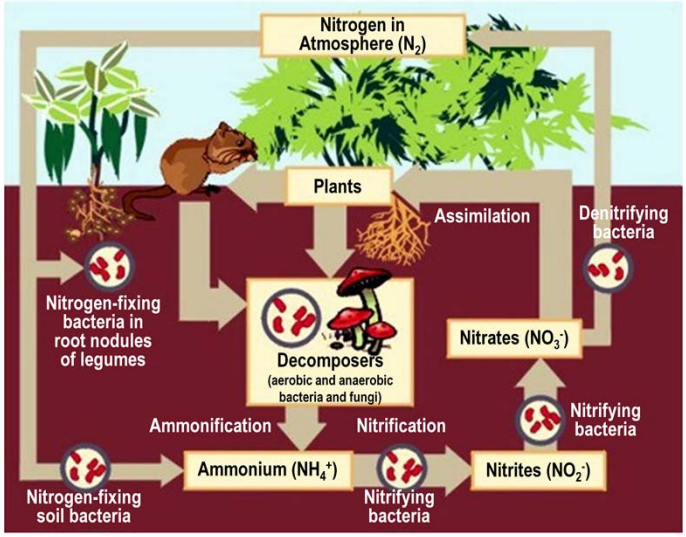
Definition of "*biogeochemistry*"



Biogeochemistry deals with how biological, geological, and chemical processes affect element cycles in Nature

- Many of you may be familiar with the nitrogen cycle. Nitrogen in the atmosphere is fixed by bacteria into ammonia and nitrate. Bacteria also breakdown organic matter and produce ammonia. Nitrate can be assimilated by plants or converted to nitrogen gas by denitrifying bacteria.

Biogeochemistry: Nitrogen Cycling



U.S. EPA

19 MBT Applications – Biogeochemistry RITS Spring 2009: Applications of MBTs for Site Remediation

- Many of you may be familiar with the nitrogen cycle. Nitrogen in the atmosphere is fixed by bacteria into ammonia and nitrate. Bacteria also breakdown organic matter and produce ammonia. Nitrate can be assimilated by plants or converted to nitrogen gas by denitrifying bacteria.

Nitrogen Cycling (cont.)

- **Nitrate reduction is widespread among unrelated groups**
- **Functional genes are known for nitrogen cycling**
  - **Respiratory nitrate reduction and dissimilatory nitrate reduction to ammonia**
    - *narGHJI and/or napABC* operons
    - Nitrite reductases - *nirK* and *nirS*
    - Nitrous oxide reductase - *nosZ*
  - **Ammonia oxidation**
    - Ammonia monoxygenase – *amoA*
    - Bacterial and Archaeal populations involved
- **Nitrate is often co-contaminant with uranium (U(VI))**
  - **Nitrate reduction near U(VI)**
  - **Complete nitrate reduction prior to U(VI) reduction**

- Genes for enzymes that are important in nitrogen cycling, such as nitrate reductases and ammonia monooxygenase, have been identifed and MBTs are available to assess the presence of these bacteria.

- One aspect that is important in environmental cleanup is that nitrate is often a cocontaminant with uranium 6. By assessing the state of nitrate reduction with MBTs, one can predict the likelihood of uranium reduction.

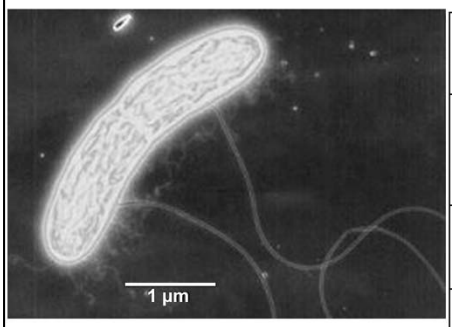
Biogeochemistry: Iron Reduction

- **Functional gene for iron reduction has not yet been identified**
  - **Multiple iron-reducing populations to track**
  - **Iron reducing populations also reduce alternative electron acceptors**
    - Includes environmental contaminants
    - Changes in population may not be due to process of interest (iron reduction)
    - Parallel increases in Fe(II) production should also be monitored to indicate activity

21 MBT Applications – Biogeochemistry RITS Spring 2009: Applications of MBTs for Site Remediation

- Iron reduction is another important geochemical process. Iron reducers generally have quite diverse metabolic capabilities. We typically conduct additional chemical analyses because of confounding factors.

Iron Reduction (cont.)



Target Population	Electron Acceptors
Geobacter	NO_3^-, Mn(IV) and Fe(III) oxides, S^0, U(VI), choroethenes
Shewanella	O_2, NO_3^-, NO_2^-, Mn(IV), $S_2O_3^-$, Fe(III), U(VI), Cr(VI)
Anaeromyxobacter	O_2, NO_3^-, NO_2^-, Mn(IV), soluble and amorphous Fe(III), *ortho*-halophenols, U(VI)

22 MBT Applications – Biogeochemistry RITS Spring 2009: Applications of MBTs for Site Remediation

- Three well known genera of iron reducing bacteria include Geobacter, Shewanella and Anaeromyxobacter. What's incredible about these bacteria is their ability to use a wide range of electron acceptors.

- Here's a photomicrograph of *Geobacter metallidreducens,* an organism with wide metabolic capabilities. It's an organism that is being studied for use in microbial fuel cells.

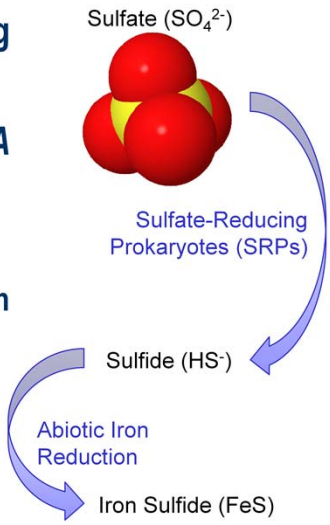
- Shewanella is another interesting organism – by the way – fish spoilage is primarily due to this bacteria.

- Shewanella can live in aerobic or anaerobic environmental because of its ability to use oxygen to nitrate/nitrite to manganese to sulfite to uranium and chromium. Interestingly, their ability to degrade organic pollutants is somewhat limited.

Biogeochemistry: Sulfate Reduction

- **Functional genes for sulfate-reducing prokaryotes are known**
- **Dissimilatory sulfite reductase – *dsrA***
  - **Conserved among sulfate reducing organisms**
  - **Catalyzes final step in sulfate reduction**

    HSO_3^- to HS^-
- **Sulfate reducers are very diverse**
  - **>130 different species**
  - **5 bacterial and 2 archaeal phyla**



23 MBT Applications – Biogeochemistry RITS Spring 2009: Applications of MBTs for Site Remediation

- Sulfate reducing bacteria are widespread and convert sulfate to sulfide. When you smell hydrogen sulfide in salt marshes or mudflats, it due to the activity of these bacteria.
- The mining industry has long been interested in the use of sulfate reducing bacteria for treating acid mine drainage and precipitating metals.
- Sulfate reducers are very diverse.

Biogeochemistry: Capped Sediment Site (Lab Scale)

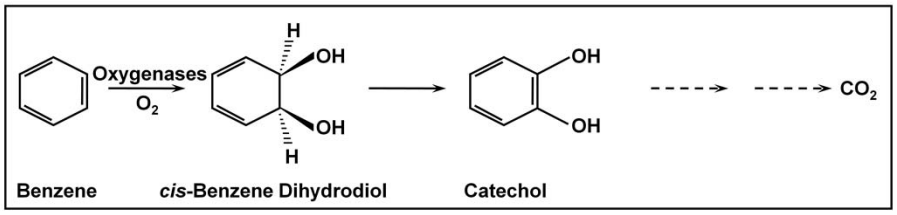
- Geochemical monitoring demonstrated spatial and temporal evolution of redox zones within sediment cap
- Bacterial results mirrored geochemical data
  - Iron-reducing populations were highest at locations of elevated Fe^{2+}
  - Sulfate-reducers were most prevalent in sediment below iron-reducing zone
  - Methanogens were focused in lower cap and in sediment below other processes

24 MBT Applications – Biogeochemistry RITS Spring 2009: Applications of MBTs for Site Remediation

- Sediment remediation is a growing area. As capping is one of the few technologies used for sediments, researchers are interested in the microbial colonization of a cap.

Aerobic Petroleum Biotransformation

- **Functional marker genes for aerobic petroleum biotransformation**
  - **Many aerobic oxygenases genes have been identified for various contaminants (e.g., BTEX compounds and naphthalene)**

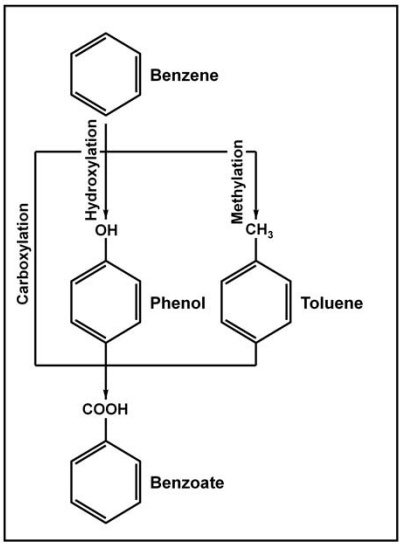


- Let's move now to contaminants. We'll look first at petroleum hydrocarbons. Aerobic biodegradation of BTEX is well–characterized and the genes for oxygenases that can catalyze the ring cleavage of aromatic hydrocarbons have been sequenced. So, we can certainly assess the potential for aerobic BTEX degradation using MBTs.

Anaerobic Petroleum Biotransformation

- **Functional marker genes for anaerobic petroleum biotransformation**
  - ***bssA* – benzylsuccinate synthase**
    - First step of anaerobic toluene and xylene degradation
    - Expressed under variety of anaerobic conditions

BTEX degradation can be tracked with an MBT

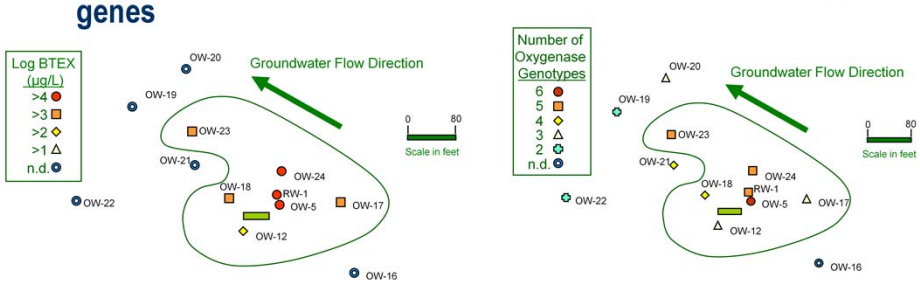


26 MBT Applications – Petroleum Hydrocarbons RITS Spring 2009: Applications of MBTs for Site Remediation

- Genes involved in the anaerobic biodegradation of BTEX have been identified. For example, the first step in anaerobic toluene and xylene degradation involves the bssA gene. It has been sequenced and probes are available. It's a great target for assessing anaerobic degradation of gasoline. Other organisms are being identified, like a Desulfobacterium involved in benzene degradation.

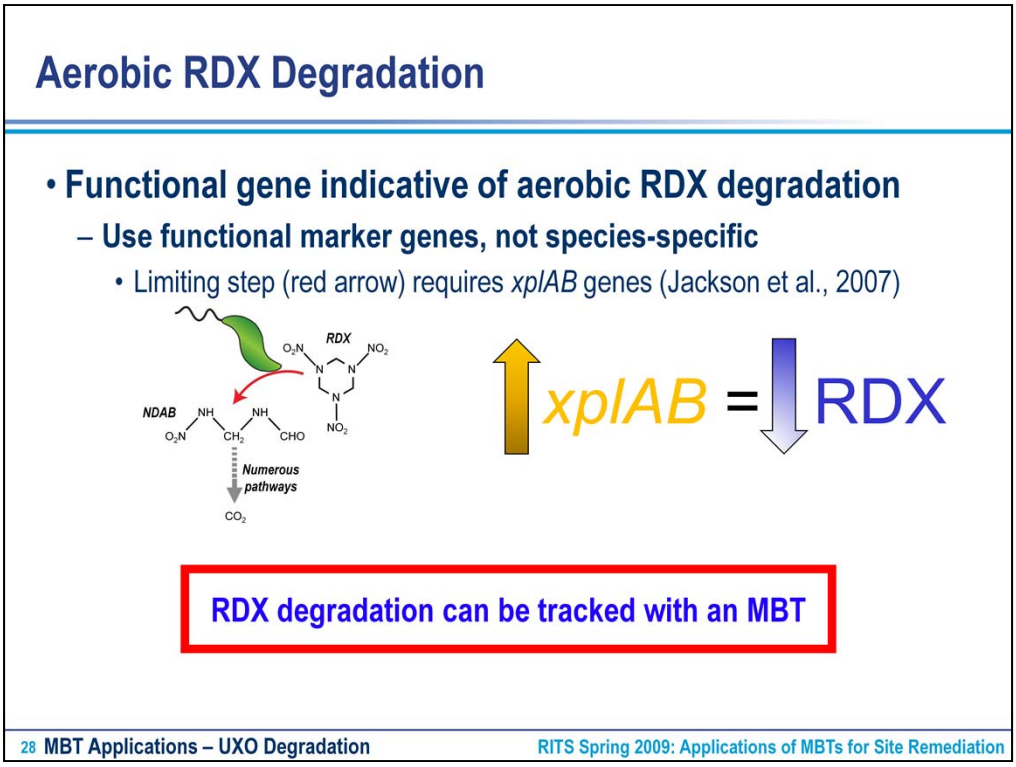
BTEX Site Remediation (Field Scale)

- **MBT for assessing aeration remediation technologies and natural attenuation at BTEX sites**
  - **Target oxygenase genes identified and primers designed**
  - **Microcosm studies link community structure to gene function**
  - **Field scale correlation between BTEX attenuation and oxygenase genes**



27 MBT Applications – Petroleum Hydrocarbons RITS Spring 2009: Applications of MBTs for Site Remediation

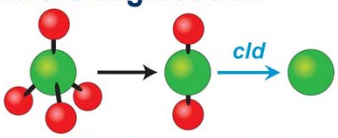
- Here's an example of using MBTs for assessing BTEX degradation in the field. This is a gasoline UST release site where oxygen-releasing compounds have been injected to stimulate biodegradation. You'll note where BTEX concentrations are the highest, the greatest number of types of oxygenase genes were also identified.



- Now let's move to explosives. A key gene has been identified involved in the aerobic biodegradation or RDX. Correlations have been identified linking the presence of xplAB to reduced concentrations of RDX in the field.

Anaerobic Perchlorate Degradation

- Dissimilatory (Per)chlorate-Reducing Bacteria (DPRB) linked to anaerobic perchlorate degradation



- DPRBs associated with nitrate-reducing microbes
- Widely distributed, commonly-found species; requires functional gene marker

Perchlorate degradation can be tracked with an MBT

29 MBT Applications – Perchlorate Degradation RITS Spring 2009: Applications of MBTs for Site Remediation

- Perchlorate biodegradation is well understood and widely distributed in the environment. The chlorite dismutase gene that catalyzes the last step in degradation has been isolated and probes are available.

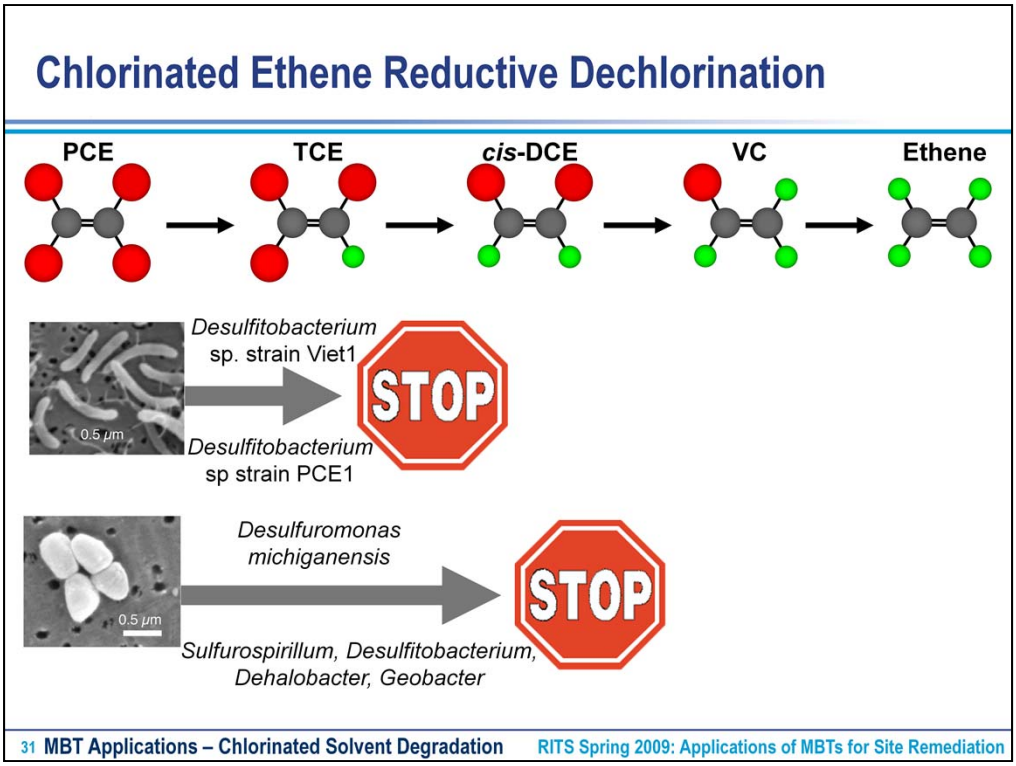
MBT Applications Summary

- MBTs can be used in a variety of applications
  - Biogeochemistry
  - Petroleum hydrocarbons
  - RDX
  - Perchlorate

ORNL

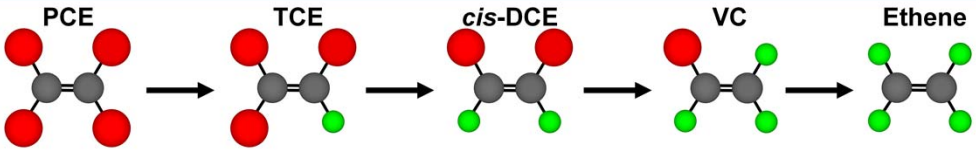
30 MBT Applications RITS Spring 2009: Applications of MBTs for Site Remediation

- So, I have shown you that MBTs are available to assess a variety of microbial processes, from geochemical cycling to contaminant degradation. Now, we will move on to chlorinated solvents.



- The step wise reductive dechlorination of PCE and TCE to cis-DCE and VC can be catalyzed by a wide variety of organisms. The endpoint for these organisms is cis-DCE or VC. Hence, the “DCE/VC stall” observed at sites that have been biostimulated.

Dehalococcoides (Dhc) Involved in Reductive Dechlorination



32 MBT Applications – Chlorinated Solvent Degradation RITS Spring 2009: Applications of MBTs for Site Remediation

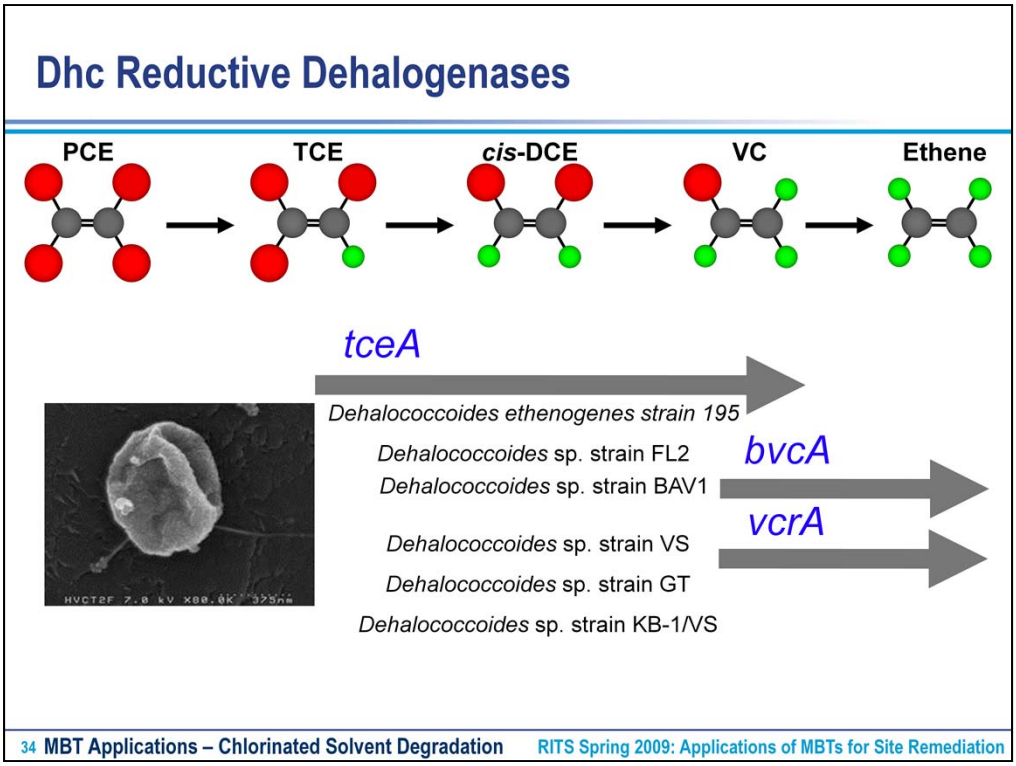
- The bacteria that we are interested in are of the genus Dehalococcoides or Dhc.
- Dhc have been identified at sites where complete dechlorination to ethene is oberved
- However, not all Dhc are alike! Some organisms can catalyze this last step cometabolically. This reaction is slow – hence the yield sign
- Other species of Dhc can use VC to generate energy. This reaction is fast. These are the Dhc that we want to use in remediation

16S rRNA Gene Targets for Dhc

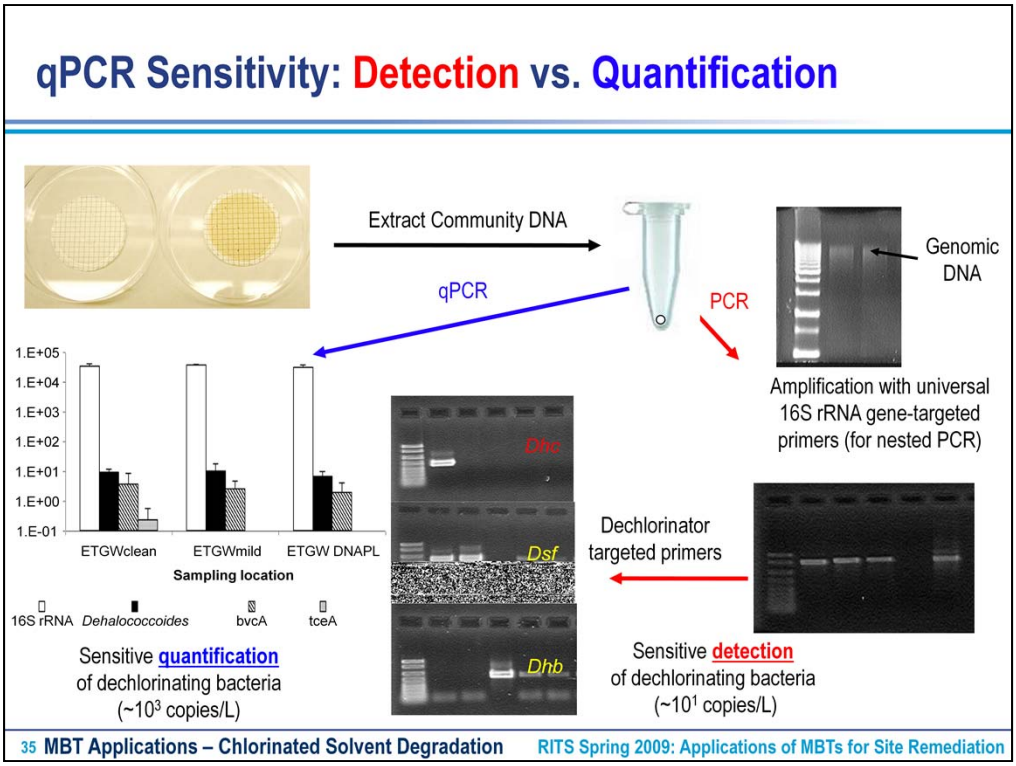
- 16S rRNA found in all bacteria
- rRNA part of the ribosome; critical for protein biosynthesis
- Contains variable regions which allows for bacterial identification
- *Dhc* has one 16S rRNA gene per cell
- *Dhc* 16S rRNA gene count = number of *Dhc* cells

The 16S rRNA molecule has insufficient information to infer physiological traits

- One way to track Dhc are by looking for the 16 ribosomal RNA gene…we'll call it 16S for short.
- rRNA is part of the ribosome
- Some regions are constant, some are variable, which can be used to identify bacteria
- Dhc has one copy of the 16S gene – so, we can quantify the number of Dhc in the sample.
- When you get a lab report, this is technique that is used – the number of 16S gene copies are quantified in the sample
- However, since not all Dhc are alike, this 16S count doesn't tell us about the ability to degrade VC quickly



- To answer this question, we can go beyond the 16S analysis and look for the actual genes that code for reductive dehalogenases…"reductive dechlorination" is the reaction…"ases" for enzymes
- tceA codes for a reductase that can degrade a variety of substrates, but VC is cometabolic
- bvcA and vcrA are the genes we're most interested in, because this last step generates energy and is fast



- So we have these great targets…what is their sensitivity?
- Frankly, these methods are incredibly sensitive.
- In the lab, we extract total DNA from a filtered sample. Using the most sensitive PCR method, we can detect one gene copy in a liter of groundwater. We run gels in the lab to get a yes/no answer…yes, down to one copy in a liter of water.
- Using quantitative PCR, we can detect 1000 copies in a liter of groundwater

Dhc Rules of Thumb in the Field

Dhc 16S rRNA gene copies per L	Interpretation
10^3 or lower	Suboptimal *Dhc* to sustain dechlorination rates
$10^4 – 10^6$	May sustain appreciable dechlorination rates
10^7 or greater	Often associated with high rates of dechlorination and ethene production

- So what do the numbers mean?
- Here's one of the big take home messages of the day.
- We can detect 10^e per liter…which is very sensitive. However, at 10^3, little dechlorination is observed. In the 10^4 to 10^6 range, we're in the gray zone…you may observe appreciable dechloriantion at these titers. We are really shooting for Dhc in the 10^7 range. At these titetrs, we observe high rates of dechlorination and production of ethene.

Presentation Overview

- Introduction
- MBT Applications
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- So, we're through MBT applications with a particular focus on RD. Next, we'll discuss sampling, which is the most important part of the analysis.

MBT Sampling

- Groundwater sampling preferred
  - Difficulties with repetitive soil sampling
  - Spatial variability in soil
- Sampling method influences results
- Use SOP that complements VOC sampling methods

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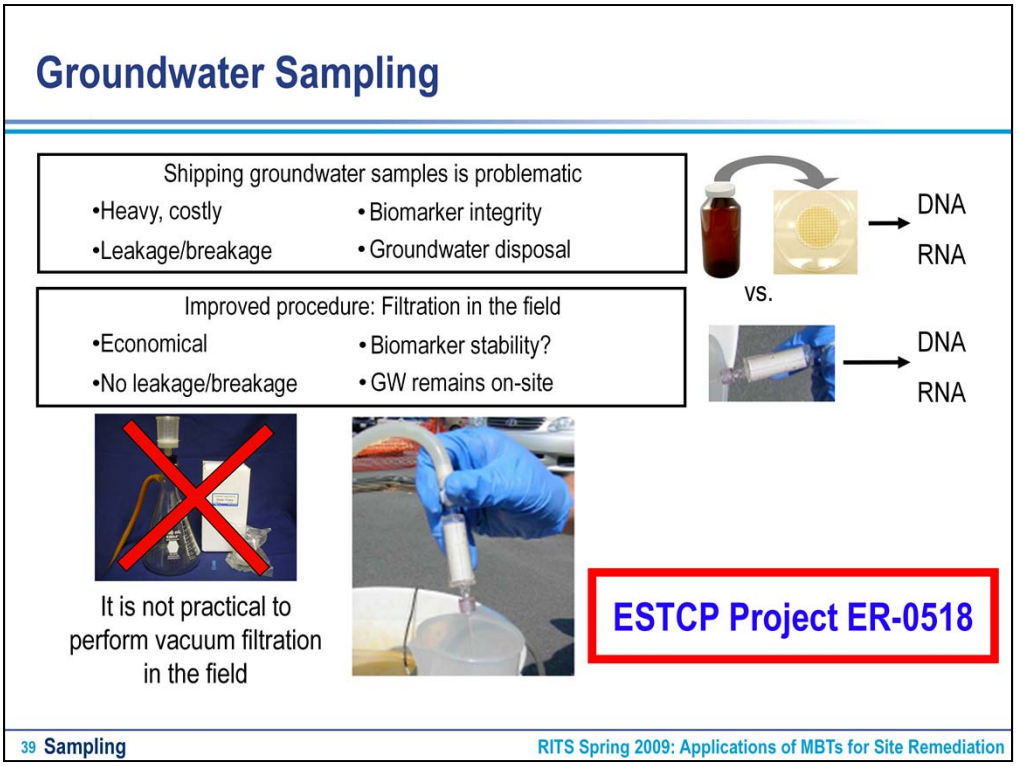
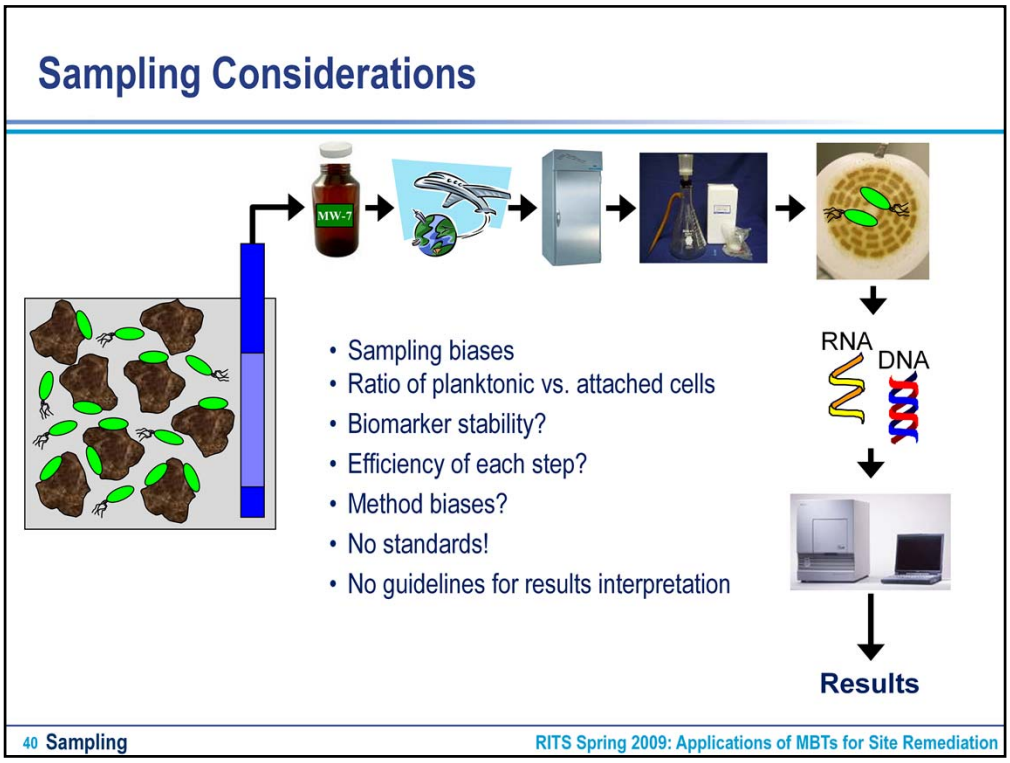
- We prefer to sample groundwater. Lot of variability cm to cm in soil
- However, the sampling method has a great influence on the results.
- Choices are low flow or bailing



- Shipping groundwater can be problematic – shipping lot of bottles, they leak, break and the lab has to deal with them
- Filtration in the field is the answer
- We showed in our ESTCP project that field filtration has great precision. DNA recoveries have been about 30%, but now approaching 90%. However, false negatives are not really an issue, with such sensitive techniques. Whether you have 1x10^3 or 3x10^3, the answer is the same…no activity.



- When sampling MBTs, there are a number of things to consider that can introduce bias

Groundwater Sampling SOP (ER-0518)

- Low-flow purge
  - Wait for stabilization of geochemical parameters to obtain a sample representative of formation groundwater
- Surging
  - Increases particulate matter in sample for recovery of associated (i.e., attached) biomass
- Field filtration
  - Filters for biomass collection in the field
    - Economical, no leakage/breakage, groundwater remains onsite
- Shipping
  - Secure samples for overnight shipment to laboratory
  - Maintain samples at 4°C

Sampling protocol should be defined and maintained for the duration of the monitoring effort for a particular site

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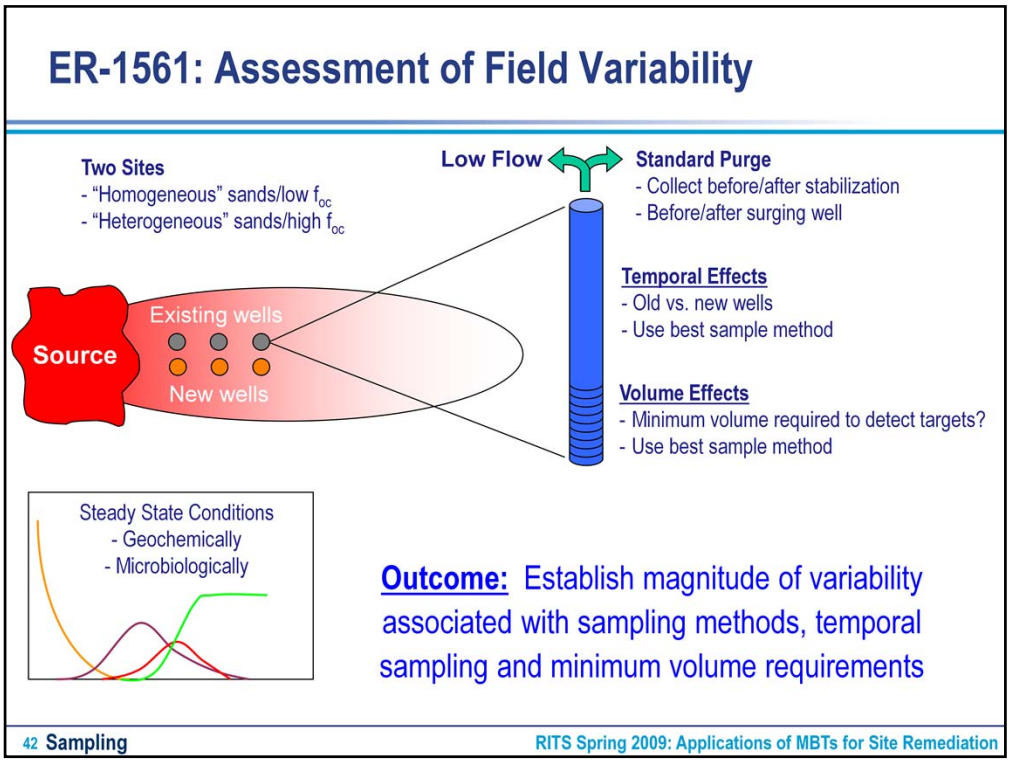
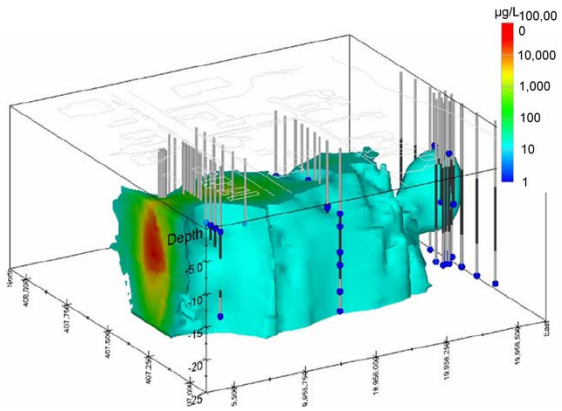
- We've attempted to address these biases by developing an SOP for groundwater sampling.
- Since most sites use low-flow sampling for VOCs, MBT sampling can be piggy backed afterwards
- Wait for parameters to stabilize
- You may surge the well to recover more attached biomass
- Ship it overnight.
- Bottom line…whatever method you choose, keep it consistent throughout the work



- To further evaluate this issue of field variability, we have a SERDP project to develop a standard lab and sampling method for MBTs. Much like we use low-flow sampling and EPA Method 8260 for VOCs, we'll have something for MBTs.
- We will be looking at the following variables – geology, the age of a well, the sampling methods, volume effects

Sampling Locations

- **Key sampling locations should include**
  - **Source area(s)**
  - **Downgradient plume**
  - **Vertical stratification**



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- The number of sample depends on a number of site specific factors, like dimensions of the plume, remedial goals, the VOC and geochem data
- Here's a picture of the Bachman Road site, where we recently injected veg oil and KB-1 in the source area and a downgradient plume biobarrier.
- The red area is the hottest zone in the source. We collected samples here, downgradient of the source to montior migration of the culture and in the biobarrier – a minimal amount

Sampling Frequency

- **Seasonal variability**
  - Geochemical conditions and biomarker abundance can be affected by seasonal changes (e.g., rain events, temperature changes, etc.)…be aware!
- **Bioremediation field implementation**
  - Baseline and 1-2 months after injections
  - Quarterly in first 12-18 months
  - Collect with VOC, geochemical and TOC data

- MBT data can be impacted by seasonal issues
- When implementing bioremediation, MBT samples should be collected at the baseline and in the first few months following injections and quarterly for 12-18 months.
- Collect with VOC and TOC data to evaluate performance

Presentation Overview

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- Done with sampling and on to cost and regulatory concerns

Cost

- **Field labor**
  - **Biomass collection can be performed concurrently with sampling events planned for assessment of contaminants**
  - **Minimal additional time is needed for collection of samples for biomarker analysis**
- **Laboratory**
  - **Microbiology labs specializing in biomarker analysis are typically independent from chemical laboratories used for other analyses**
  - **Typical cost for quantification of *Dehalococcoides* in a sample of groundwater is approximately $250**

- The cost for collecting MBT data is relatively low. Minimal additional field time. Labs specializing in MBTs are different from typical analytical labs. Costs are about $250/sample

Cost for Not Using MBTs

- Unnecessary bench tests
- Poorly designed pilot tests
- Inefficient full-scale treatment
  - Application of bioaugmentation and/or biostimulation when MNA would be appropriate
  - Bioaugmentation when sufficient Dhc are present to meet remediation goals
  - Failure to bioaugment when Dhc populations are insufficient

47 Cost and Regulatory Concerns RITS Spring 2009: Applications of MBTs for Site Remediation

- Rather than focus on the $250/crack number, let's discuss the cost of not having MBT data
- You may run bench tests that really aren't needed
- The pilot test design may not be very good
- At full-scale, MBT data are critical in evaluating performance
- You may bioaugment when there are sufficient DFC or you may fail to bioaugment when you should have
- All of these scenarios cost time and money

Regulatory Concerns

- Lack of awareness
  - Many federal, state and local regulators have had limited exposure to MBTs
  - It may be necessary to educate regulators on the benefits of MBTs for particular applications
  - ITRC training proposed for 2010
- Supporting data
  - Limited data from laboratory research and field validation of MBTs have been published
  - It is important for industry practitioners and regulators to familiarize themselves (and each other) with currently available guidance and SOPs

Presentation Overview

- Introduction
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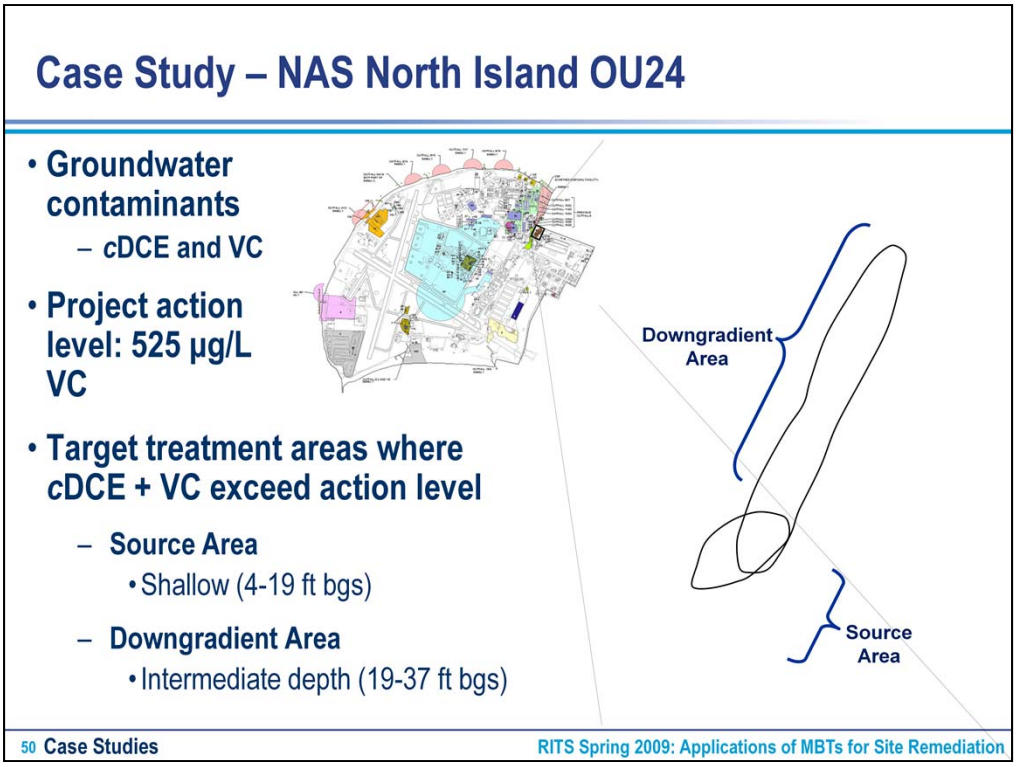
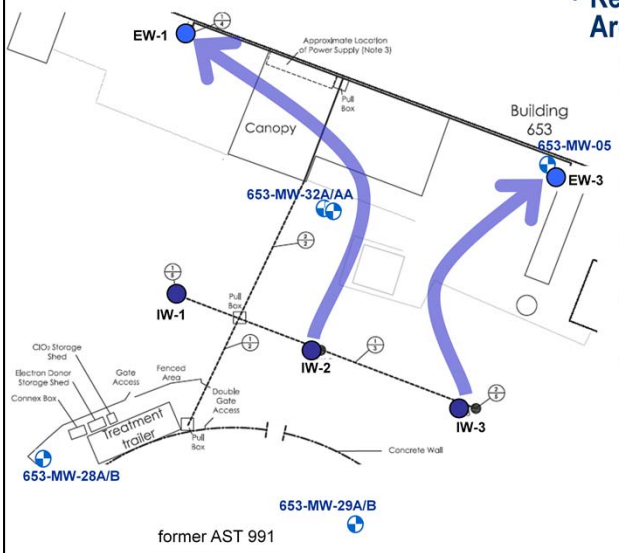
- Case Studies
  - NAS North Island OU24
  - NAS Cecil Field Site 59
  - NASA Mobile Launch Platform/ Vehicle Assembly Building (MLP/VAB) Site
  - NAWC Trenton
  - Milledgeville
- Guidance Protocol
- Summary



- Our first case study is the Naval Air Station at North Island, OU24.
- A bioremediation remedy has been implemented in the source area and five downgradient biobarriers

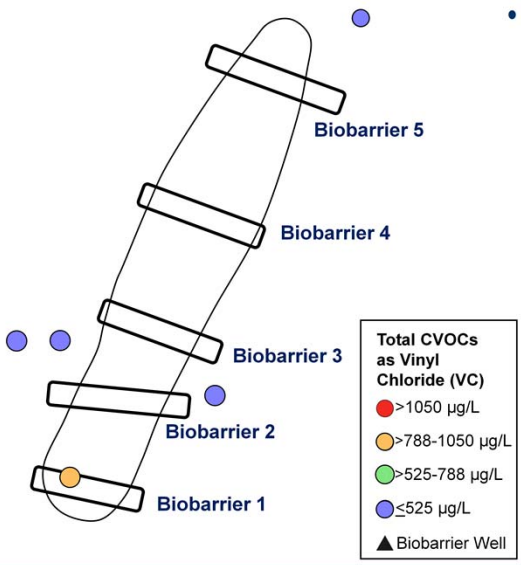
Source Area Treatment



- Recirculation System in Source Area
  - Start-up May 2007
  - Weekly dosing of lactate through 2008
  - Bioaugmentation June 2007
  - Anaerobic conditions established and bacteria growing
  - Total CVOC mass reducing and ethene generated
  - CVOCs persist in certain locations
  - 2009 system optimization (recirculation configuration shown in figure): target "hot-spots" in source area and switching to ethanol and benzoate as electron donors

51 Case Study – NAS North Island OU24 RITS Spring 2009: Applications of MBTs for Site Remediation

Downgradient Plume Treatment



- Downgradient biobarriers
  - Temporary recirculation of groundwater amended Sept-Nov 2007
  - Bioactive zones established
  - Dechlorination within certain biobarriers is occurring
  - CVOCs persisting elsewhere due to low groundwater flow
  - 2009 system optimization: re-dose biobarriers

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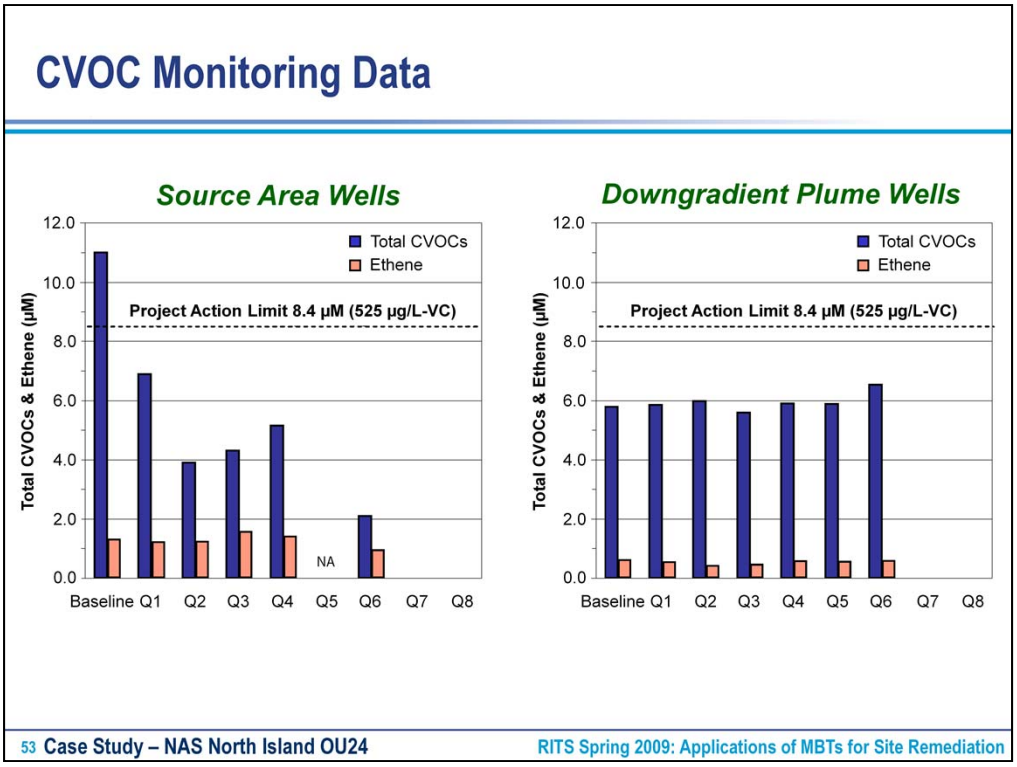
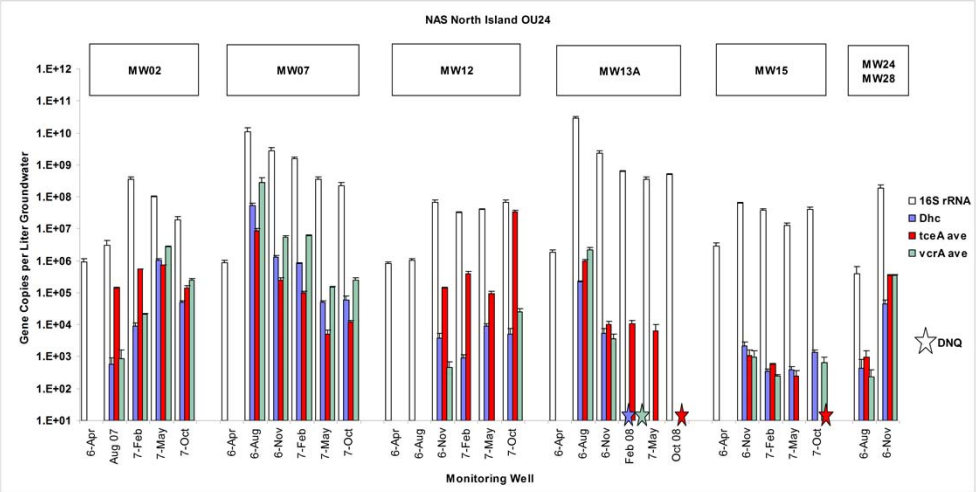
- See slide



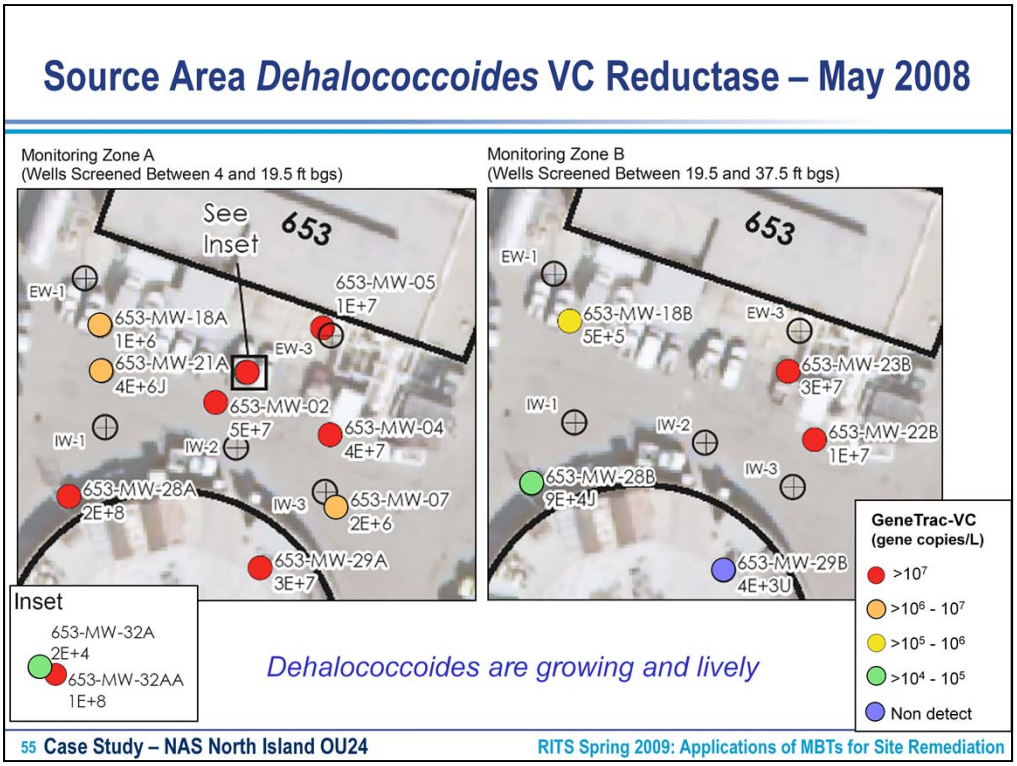
- Average total VOC data in source area wells and downgradient plume wells
- 2 years of data post injection

MBT Monitoring Data

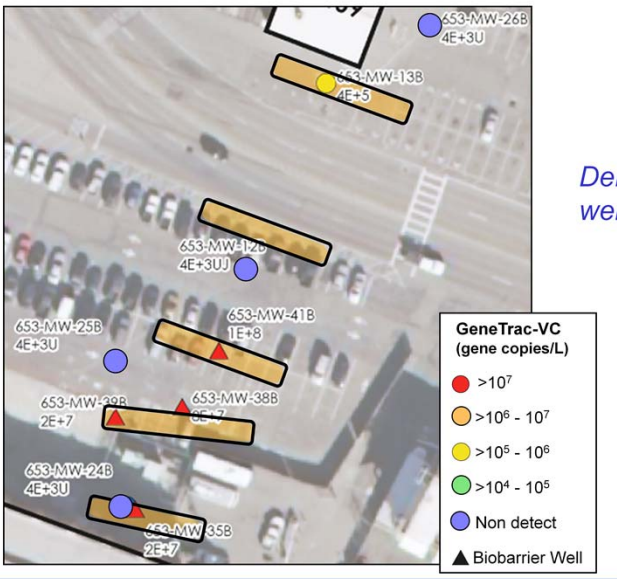


54 Case Study – NAS North Island OU24 RITS Spring 2009: Applications of MBTs for Site Remediation

- Source: MW-2, 7
- Downgradient: 12, 13A, 24, 28
- Quay: 15


Source Area *Dehalococcoides* VC Reductase – May 2008
Monitoring Zone A
(Wells Screened Between 4 and 19.5 ft bgs)
Monitoring Zone B
(Wells Screened Between 19.5 and 37.5 ft bgs)
See Inset
653
EW-1
653-MW-18A
1E+6
653-MW-21A
4E+6J
653-MW-05
1E+7
EW-3
653-MW-02
5E+7
653-MW-04
4E+7
IW-1
IW-2
IW-3
653-MW-28A
2E+8
653-MW-07
2E+6
653-MW-29A
3E+7
653-MW-18B
5E+5
653-MW-23B
3E+7
653-MW-22B
1E+7
653-MW-28B
9E+4J
653-MW-29B
4E+3U
Inset
653-MW-32A
2E+4
653-MW-32AA
1E+8
Dehalococcoides are growing and lively
GeneTrac-VC
(gene copies/L)
>10^7
>10^6 - 10^7
>10^5 - 10^6
>10^4 - 10^5
Non detect
55 Case Study – NAS North Island OU24
RITS Spring 2009: Applications of MBTs for Site Remediation

Downgradient *Dehalococcoides* VC Reductase – May 2008

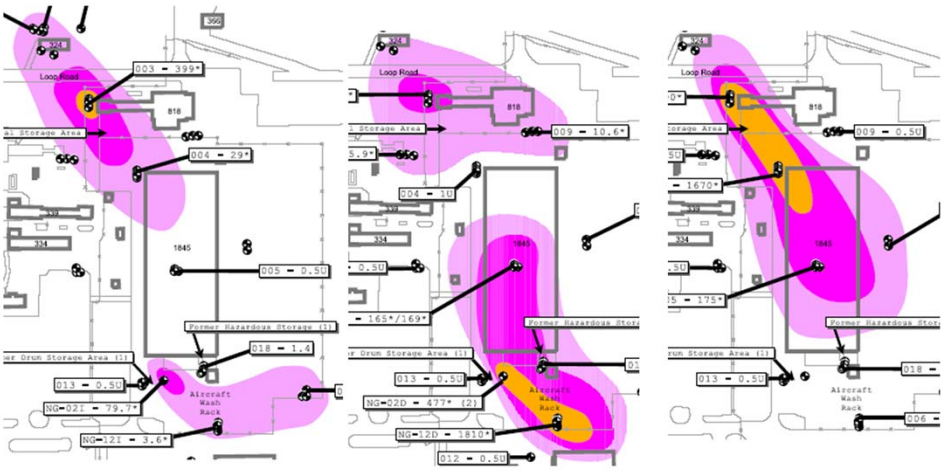


Dehalococcoides populations well established in biobarriers

56 Case Study – NAS North Island OU24 RITS Spring 2009: Applications of MBTs for Site Remediation

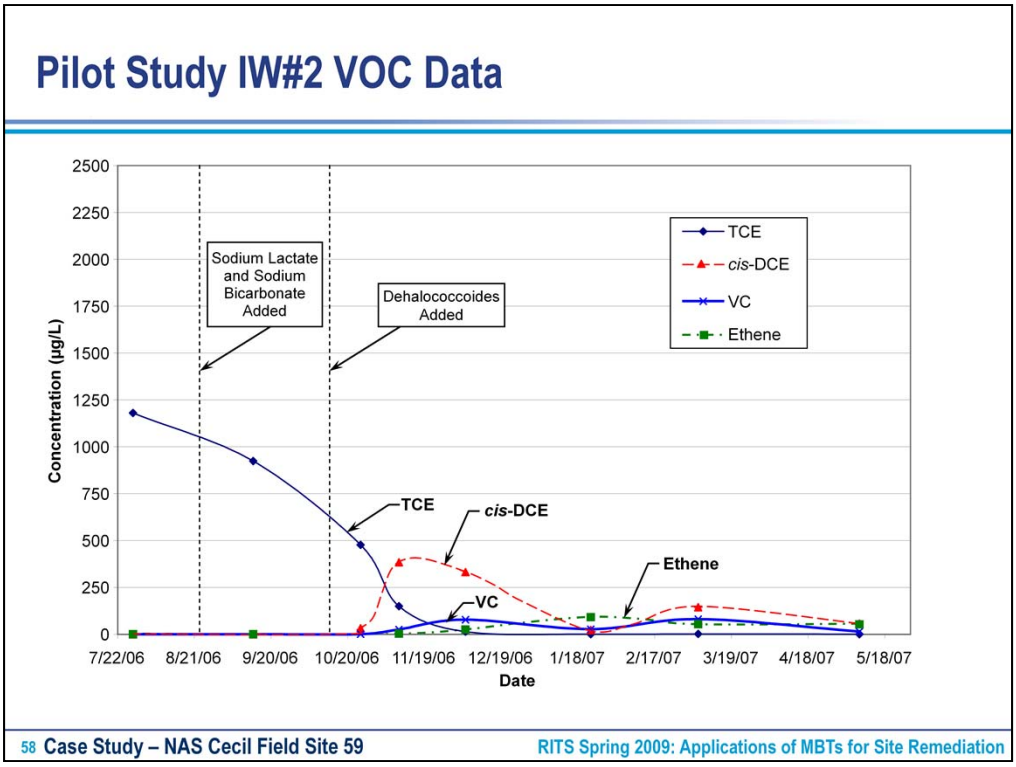
- Hitting 10^7 in several biobarriers

Case Study – NAS Cecil Field Site 59



57 Case Studies RITS Spring 2009: Applications of MBTs for Site Remediation

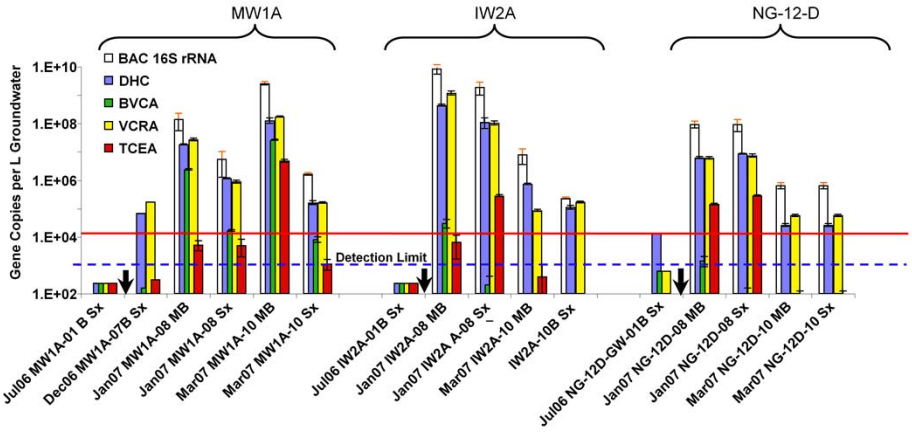
- NAS Cecil Field Site 59. Former haz waste storage areas- Bioremediation pilot test – recirculation of lactate



- Classic pattern of TCE deg, followed by DCE, VC and ethene following bioaug

Dhc and RDase Data

- Three wells sampled in July 2006, December 2006 (MW 1A only), January 2007, and March 2007
- Biostimulation with sodium bicarbonate, lactate August 2005
- Bioaugmentation October 2006



59 Case Study – NAS Cecil Field Site 59 RITS Spring 2009: Applications of MBTs for Site Remediation

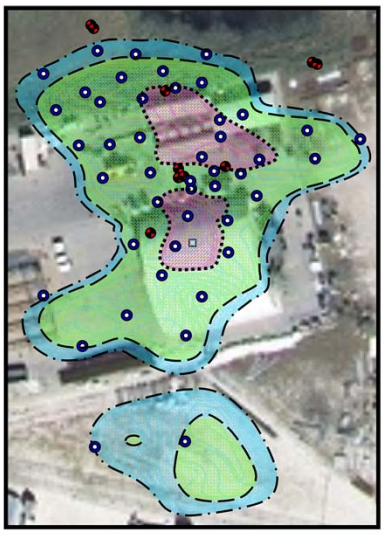
- Note tceA drop as TCE gone

Case Study – NASA MLP/VAB Site

- TCE Source Area
  - (~4,000 gal release 1960's)
- Biostimulation
  - Ethyl lactate
- Performance monitoring
  - Every other month
  - TCE, cDCE, VC, ethene
  - Dhc and vcrA
  - ~6 data points from each well

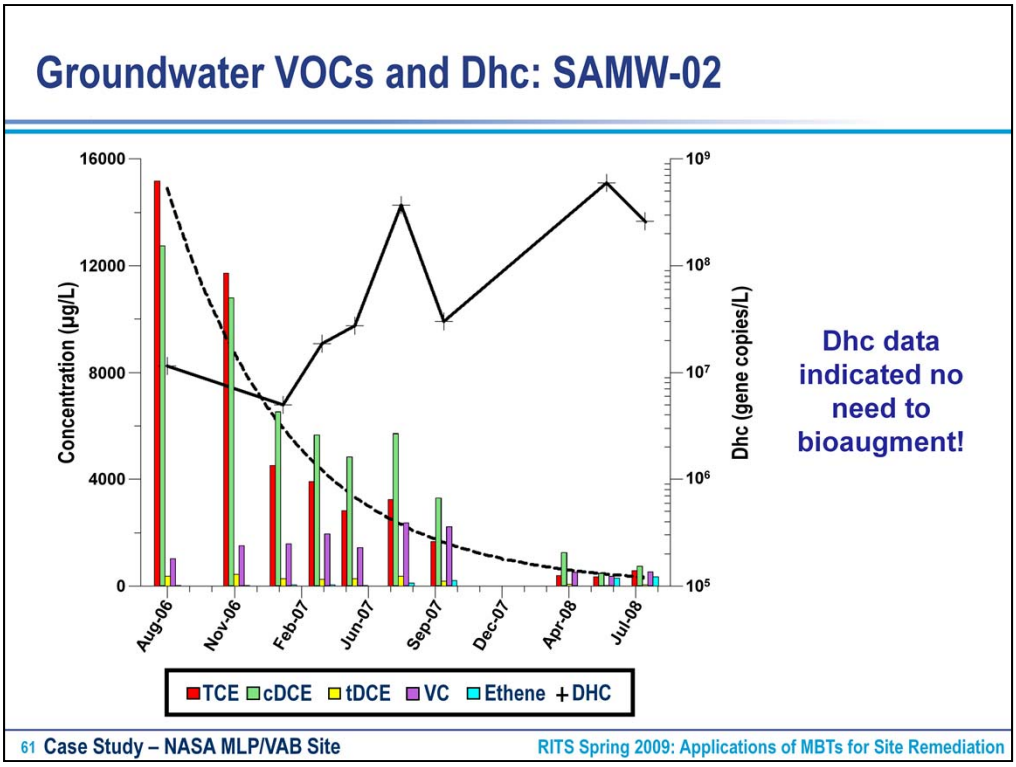
Monitoring Well
Injection Well

30,000 µg/L TCE
3,000 µg/L TCE
1,000 µg/L TCE

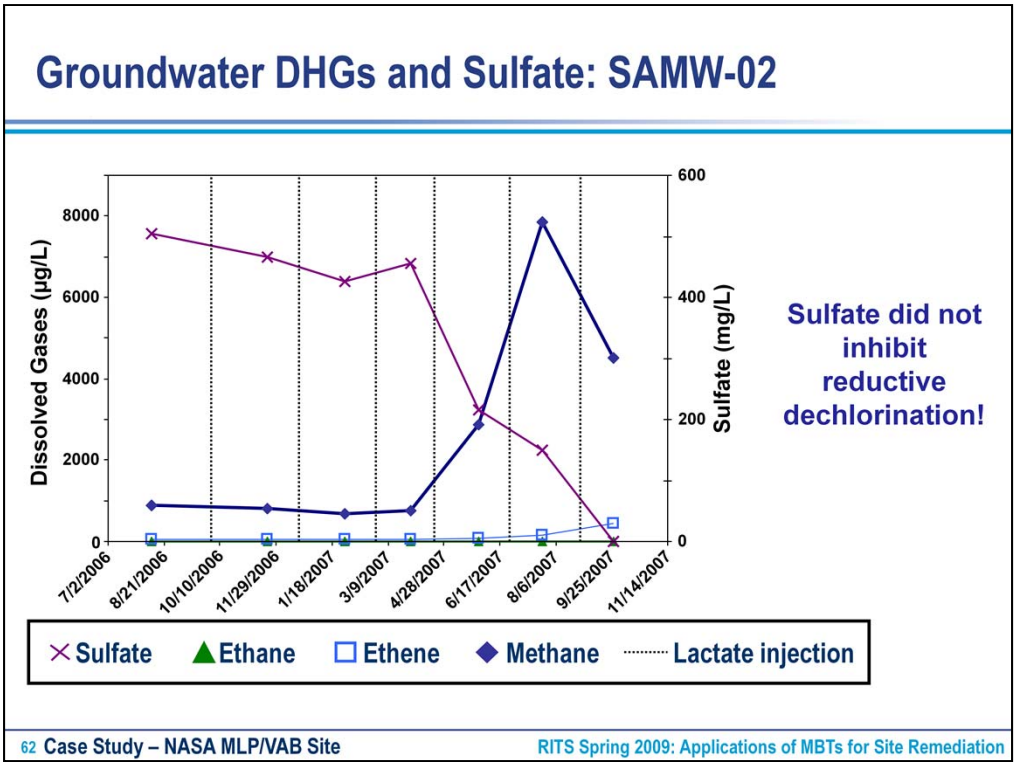


60 Case Studies RITS Spring 2009: Applications of MBTs for Site Remediation

- NASA Cape Canaveral Mobile Launch Platform



- Source area well
- Dhc data ~10^4 (not shown)
- Dhc and cDCE data indicated bioaug not needed
- Dhc increased to > 10^8



- Sulfate reduction kicked in, resulting in methane generation. No inhibition of RD.

Correlations: Spearman Test

Nonparametric (distribution-free) correlation

- **Covert data to ranks**
- **Perform Spearman calculation**
  - r_s up to 0.33 = weak relationship
  - $0.34 < r_s < 0.66$ = medium strength relationship
  - $r_s > 0.67$ = strong relationship

- Goal of data analysis is to identify correlations of Dhc data to other parameters using the Spearman test

Correlation of Dhc/*vcrA* to VOCs and Ethene

Spearman Test

TCE, DCE, VC, Ethene → *Dhc*

VC, Ethene → *vcrA*

- **Correlation results:**
  - **Dhc or *vcrA* to TCE, DCE or VC**
    - Weak correlation ($r_s < 0.33$) for all comparisons
  - **Dhc or *vcrA* to ethene**
    - *Dhc* to ethene = strong correlation ($r_s = 0.66$; $n = 10$; $p = 0.05$)
    - *vcrA* to ethene = strong correlation ($r_s = 0.67$; $n = 10$; $p = 0.05$)

Correlation of Dhc/*vcrA* to Dechlorination Rates

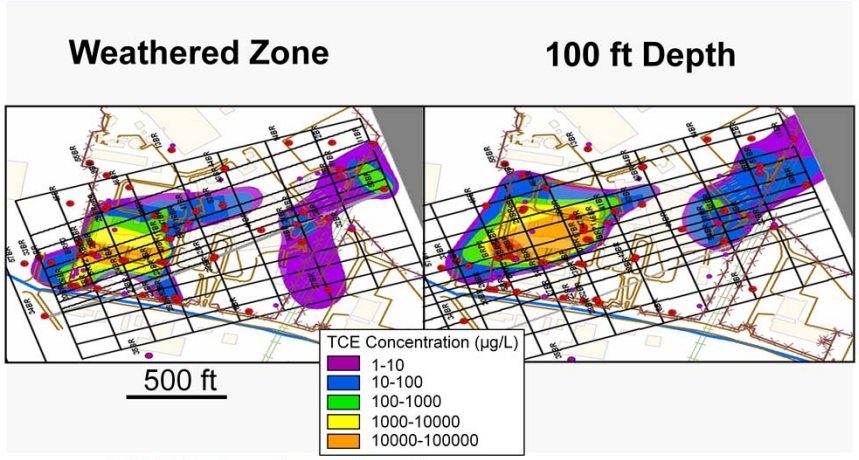
Spearman Test

TCE rate (yr^{-1})
DCE rate (yr^{-1})
VC rate (yr^{-1})
→ *Dhc*

DCE rate (yr^{-1})
VC rate (yr^{-1})
→ *vcrA*

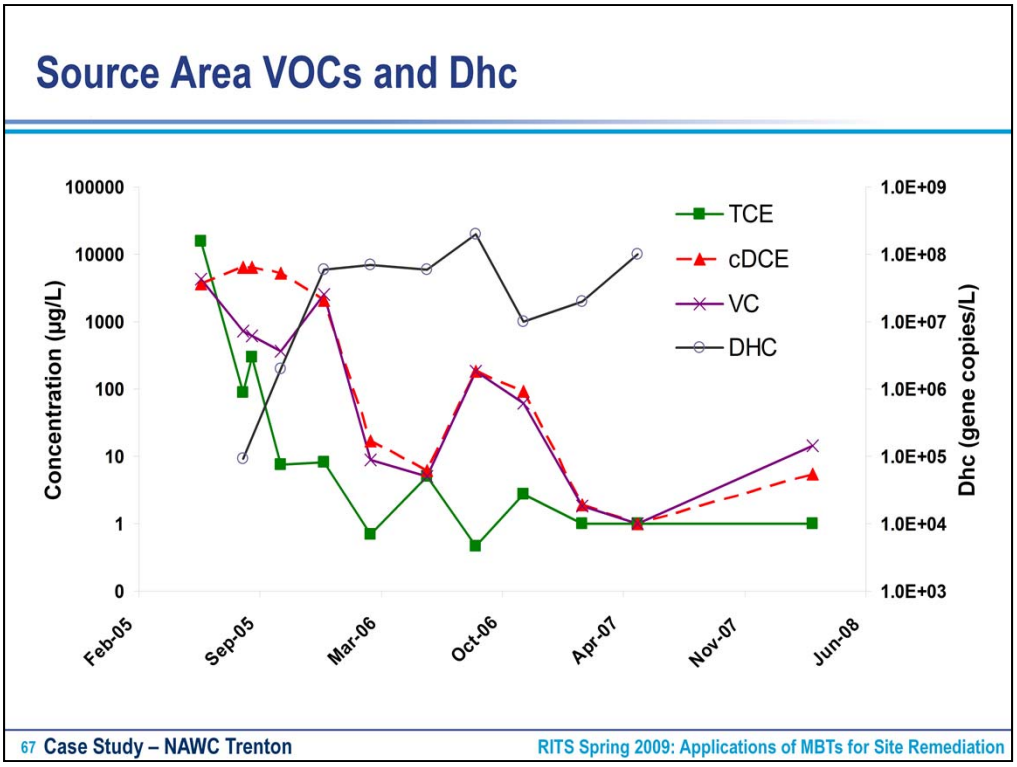
- **Correlation results:**
  - Strong correlations (rs = 1.00) for all comparisons to Dhc
  - Medium correlations (rs = 0.50) for all comparisons to *vcrA*
- **Limited validity to results (only three data points)**

Case Study – NAWC Trenton



- TCE in fractured rock
- DCE stall, Dhc at ~10^3
- EVO and bacterial culture injection

- NAWC Trenton



- Note Dhc from 10^3 to 10^8

Correlation of Dhc/*vcrA* to VOCs and Ethene

- Dhc to TCE = Strong correlation (r_s = -0.84; n = 14; p = 0.01)
- Dhc to cDCE = Strong correlation (r_s = -0.73; n = 9; p = 0.05)
- Dhc to VC = Strong correlation (r_s = -0.78; n = 9; p = 0.05)
- Dhc to Ethene = Strong correlation (r_s = 0.85; n = 9; p = 0.01)

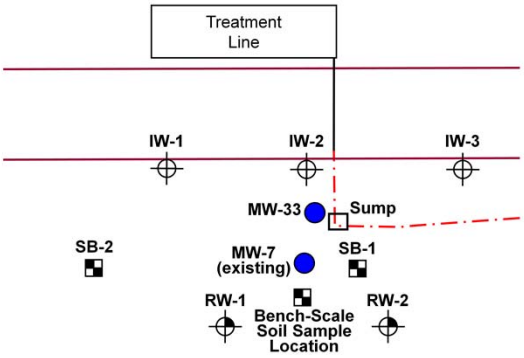
Correlation of Dhc/*vcrA* to Dechlorination Rates

$$\frac{VC + ethene}{DCE} \rightarrow Dhc$$

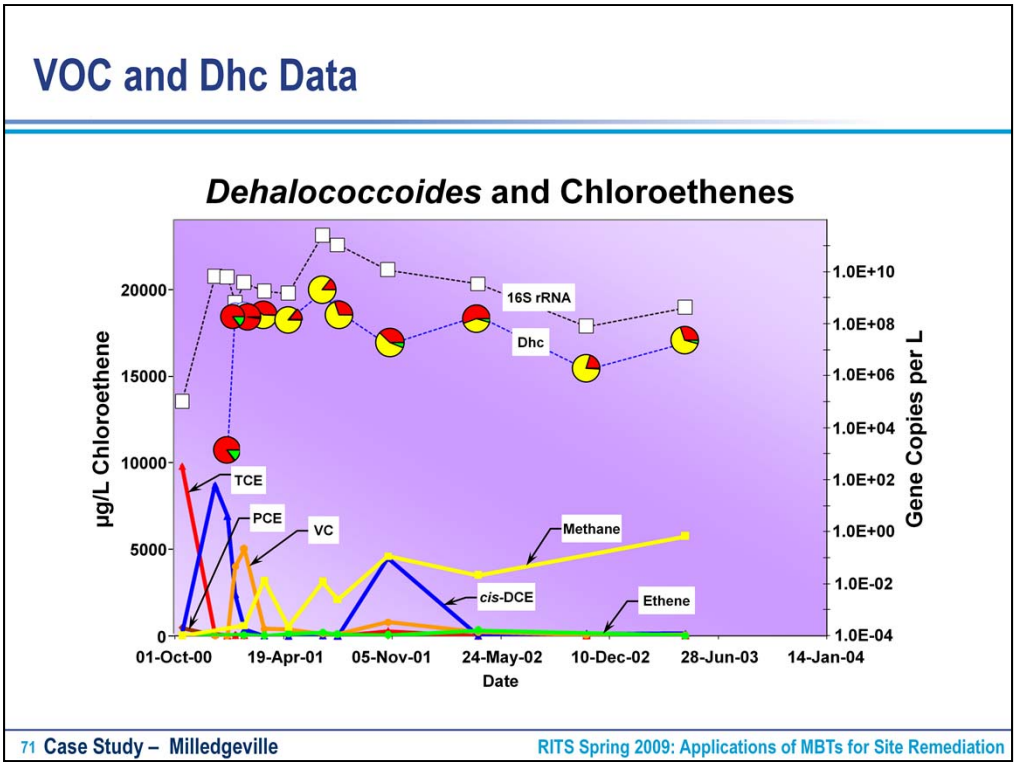
- Medium correlation (r_s = 0.54; n = 14; p = 0.05)
- TCE, cDCE, or VC dechlorination rates to Dhc
  - Medium correlations (r_s = 0.40) for all correlations
  - Limited validity to results (only four data points)

Case Study – Milledgeville

- TCE source (18,000 ppb)
- Bioaugmentation pilot testing
  - 3 injection wells and 2 recovery wells oriented perpendicular to the prevailing direction of groundwater flow (southwest)
  - Soluble electron donor (lactate) and dechlorinating culture distributed by recirc



- Industrial site
- Pilot test in source area



- Note Dhc from 10^4 increase to 10^9

Correlation of Dhc or RDases to VOCs

- *Dhc* or *RDases* to VOCs
  - No correlations to *Dhc*
  - Strong correlation of *bvcA* to cDCE (rs = 0, n = 10, p = 0.02)
  - Strong correlation of *vcrA* to cDCE (rs = -0.80, n = 10, p = 0.01)
  - Strong correlation of *tceA* to VC (rs = 0.76, n = 10, p = 0.02)
  - No other correlations identified

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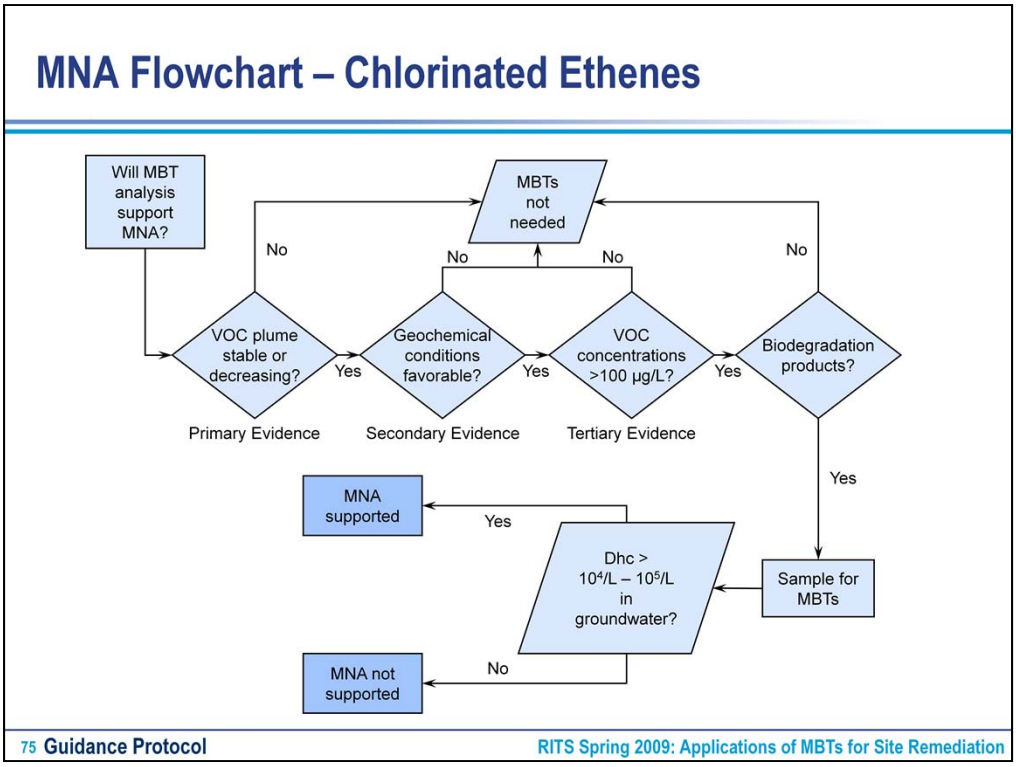
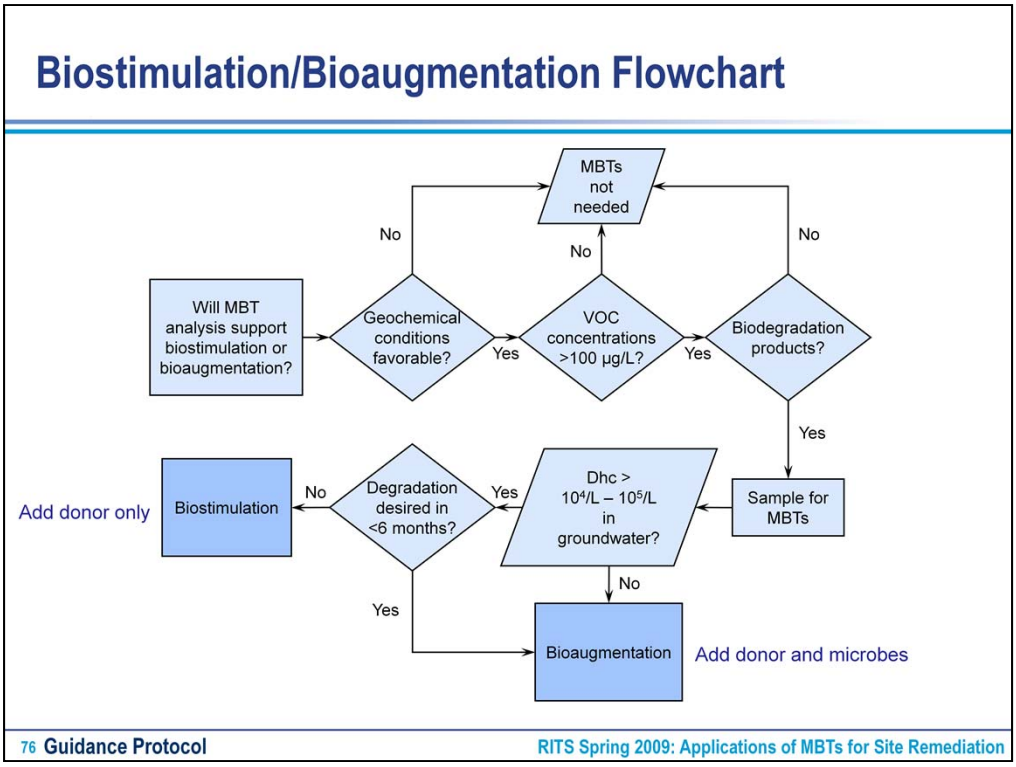
Guidance Protocol – Table of Contents

1.0 Executive Summary
2.0 Introduction
3.0 Background
4.0 Molecular Biological Tools
4.1 qPCR Analysis for Dechlorinating Bacteria
5.0 Application of MBTs in Bioremediation Evaluation
5.1 MBT Use in Supporting Monitored Natural Attenuation
5.2 MBT Use in Supporting Biostimulation and Bioaugmentation
6.0 Field Sampling Methods
6.1 Collection of Sample Volume and Sample Filtration
6.2 Sampling Locations and Frequency
7.0 References

- Guidance protocol drafted for ESTCP project.



- Flowchart in protocol
- Walk through box by box



- Flowchart in protocol
- Walk through box by box

Reminder – Dhc Rules of Thumb

Dhc 16S rRNA gene copies per L	Interpretation
10^3 or lower	Suboptimal *Dhc* to sustain dechlorination rates
$10^4 – 10^6$	May sustain appreciable dechlorination rates
10^7 or greater	Often associated with high rates of dechlorination and ethene production

- Reminder that our target Dhc gene copy number is 10^7

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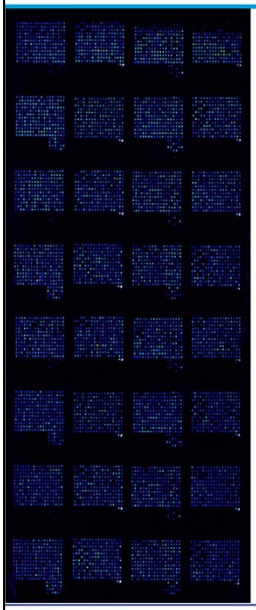
- Now, I'll finish with the summary

Summary

- MBTs are available to monitor a variety of biodegradation processes
- SOPs are available for MBT sampling. Field filtration is reliable.
- Biomarker genes (*bvcA*, *vcrA*) are indicators of field dechlorination activity
- Rules of thumb and draft guidance are available
- Understand limitations of the data

- Info on slide

Emerging Technology MBT: Microarrays

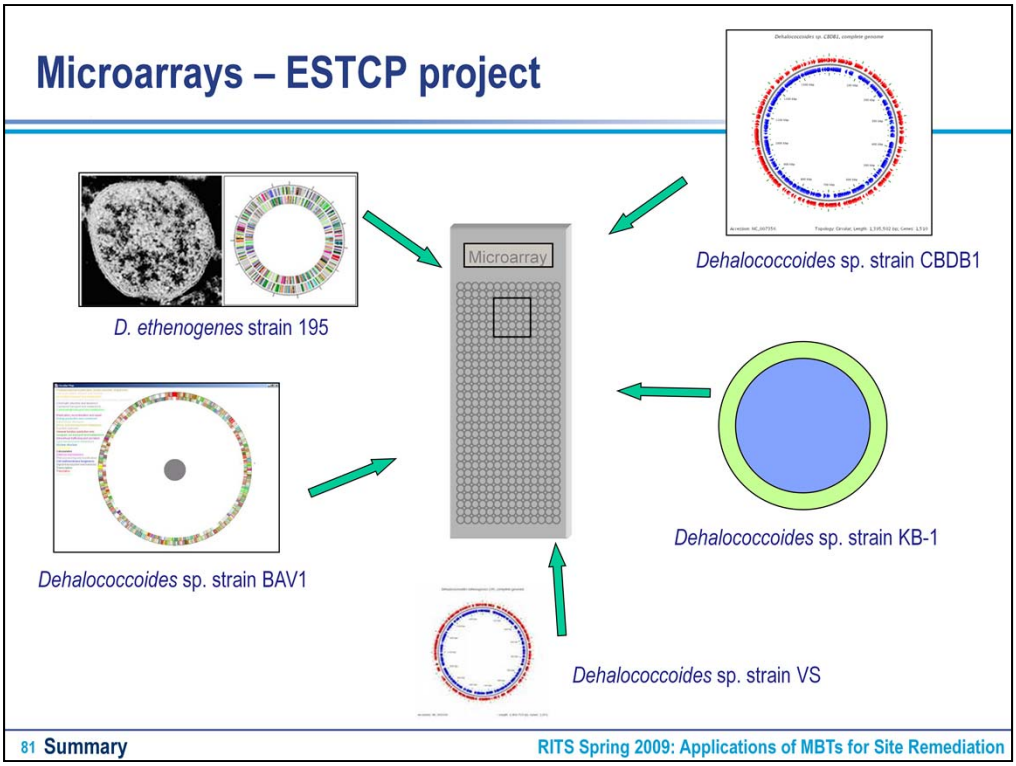


- Rapidly screens sample (DNA or RNA) for thousands of gene concentrations
- Allows varying levels of discrimination/resolution (e.g., strain, species, phylum and domain)
- Functional Gene Arrays (FGA) contain 20,000-50,000 genes involved in all known geochemical processes
- No known process specific (i.e., reductive dechlorination, perchlorate, UXO) arrays
- High initial design cost $15-50k per array
- Low usage cost $100-500 per array
- Lower overall cost:data-acquisition ratio than current MBTs

Monitor health and activity of whole community

80 Summary RITS Spring 2009: Applications of MBTs for Site Remediation

- Technology is emerging that can be used to screen tens of thousands of genes, rather than 3 or 4 as we do now



- A new microarray covering the known diversity of Dehalococcoides species and strains is in the design stage. This array will be used to test and validate genome:genome comparison based hypotheses. It will ultimately also be used in order to track and identify the nature of dechlorination taking place at contaminated sites based on expression of rdh genes specific to known strains/contaminants. Useful tool for screening a site to determine presence/effectivity of dechlorination.

Documents

- ESTCP. 2006. Interim Guidance Protocol for Application of Nucleic Acid-Based Tools for Monitoring Monitored Natural Attenuation (MNA), Biostimulation, and Bioaugmentation at Chlorinated Solvent Sites. ER-0518
- SERDP. 2008. An Overview of Current Approaches and Methodologies to Improve Accuracy, Data Quality and Standardization of Environmental Microbial Quantitative PCR Methods. ER-1561

- We have two documents available that provide detailed info that I presented today.