Environmental Molecular Diagnostics: Current Capabilities and Future Trends

RITS 2013

Purpose of Presentation

- To identify the currently available major Environmental Molecular Diagnostic (EMD) tools
- To explain what these EMDs measure or detect and how they should be used
- To demonstrate how EMDs can be used to make site management decisions at contaminated sites
- To provide an overview of new EMDs that will be available in the future

Navy Resources on EMDs

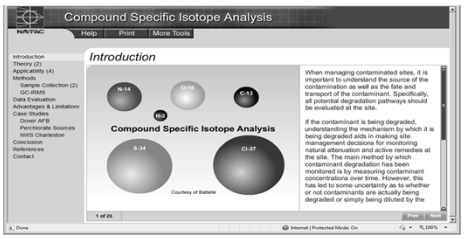
Past Related RITS Presentations

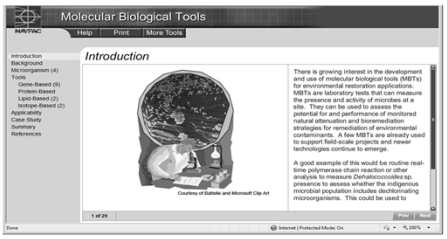
1. Applications of Molecular Biological Tools for Site Remediation
   Spring 2009
2. Compound Specific Isotope Analysis Today
   Fall 2008
3. Environmental Forensic Tools: Isotope Ratios
   Fall 2005

Navy Resources on EMDs (cont.)

T2 Tools

1. Compound Specific Isotope Analysis (CSIA) Web Tool
2. Molecular Biological Tools





Presentation Acronyms

CSIA	compound specific isotope analysis
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
EMDs	Environmental Molecular Diagnostics
mRNA	messenger ribonucleic acid
PLFA	phospholipid fatty acid
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
SIP	stable isotope probing
T-RFLP	terminal restriction fragment length polymorphism

Presentation Glossary

- **Gene Expression –**
  - **Gene expression is the most fundamental level at which the genotype gives rise to the phenotype. The genetic code stored in DNA is "interpreted" by gene expression, and the properties of the expression give rise to the organism's phenotype evidence that a gene is either being used to produce RNA or protein**
- **Functional Gene –**
  - **Gene in microorganisms' DNA that codes a protein or enzyme with a specific activity, *i.e.*, reductive dechlorination**
- **Metabolite –**
  - **A product of a compound undergoing biodegradation**

These are some terms that will be used during this presentation

Presentation Overview

► Introduction

- Quantitative Polymerase Chain Reaction (qPCR)
- Stable Isotope Probing (SIP)
- Microbial Fingerprinting
- Future Diagnostic Tools
- Special Issues for EMDs
- Summary

Definition

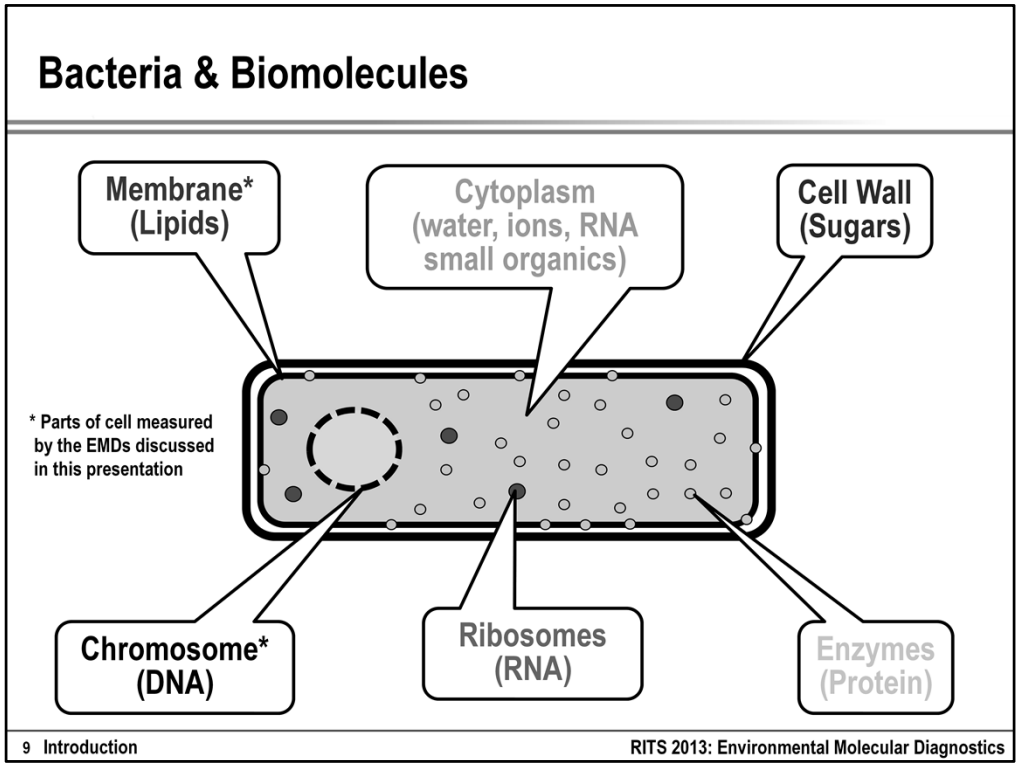
Environmental Molecular Diagnostics (EMDs)

A series of techniques that provide chemical and biological data that can help determine what natural processes are occurring at a site

- **Chemical-Based (*e.g.,* CSIA)**
  Changes in isotopic composition of contaminants – CSIA
- **Molecular Biology-Based (*e.g.,* qPCR)** Focus of this presentation
  Changes in levels of microorganisms, genes, lipids and enzymes

8 Introduction RITS 2013: Environmental Molecular Diagnostics

The key distinction is that this presentation will focus on molecular biology-based EMDs (*e.g.,* qPCR) rather than techniques such as CSIA that focus on COCs themselves.



This slide shows the major structural components of a bacterial cell.

The cell wall is made of polymerized sugar-based materials. The cell wall acts as a structural support for the cell.

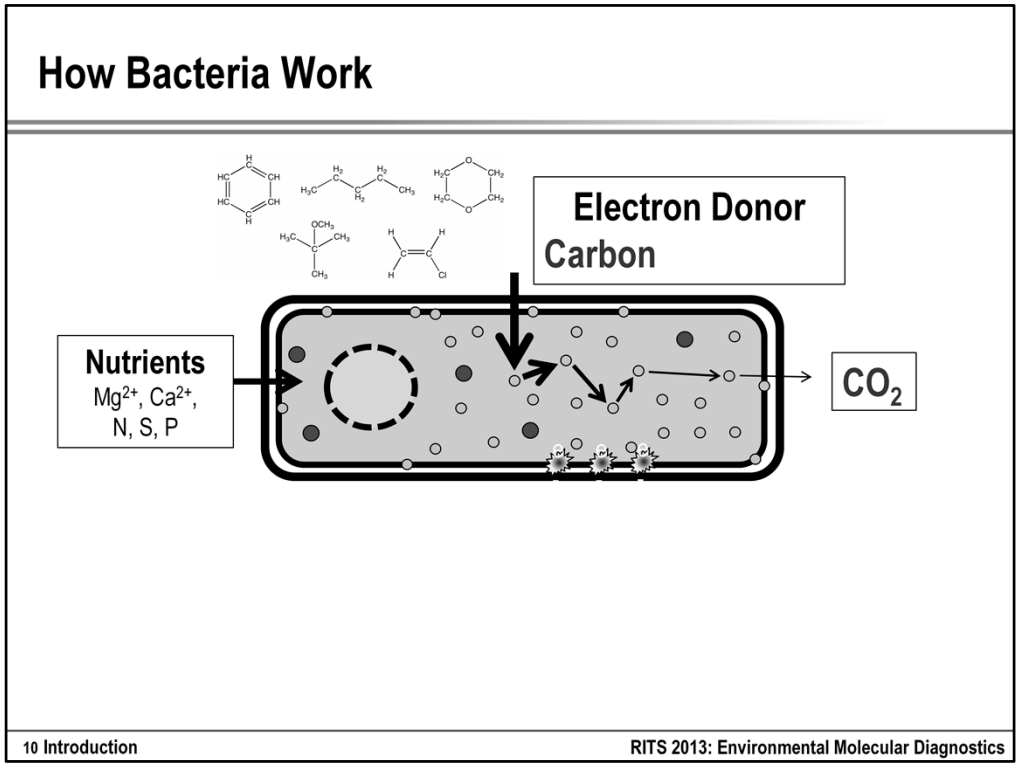
The cell membrane is made of phospholipids. This membrane acts as a selective barrier between the aqueous phases inside the cell and the outside medium.

The cytoplasm contains all of the other major structures of the cell. The cytoplasm is made up of water, ions, RNA and small organics.

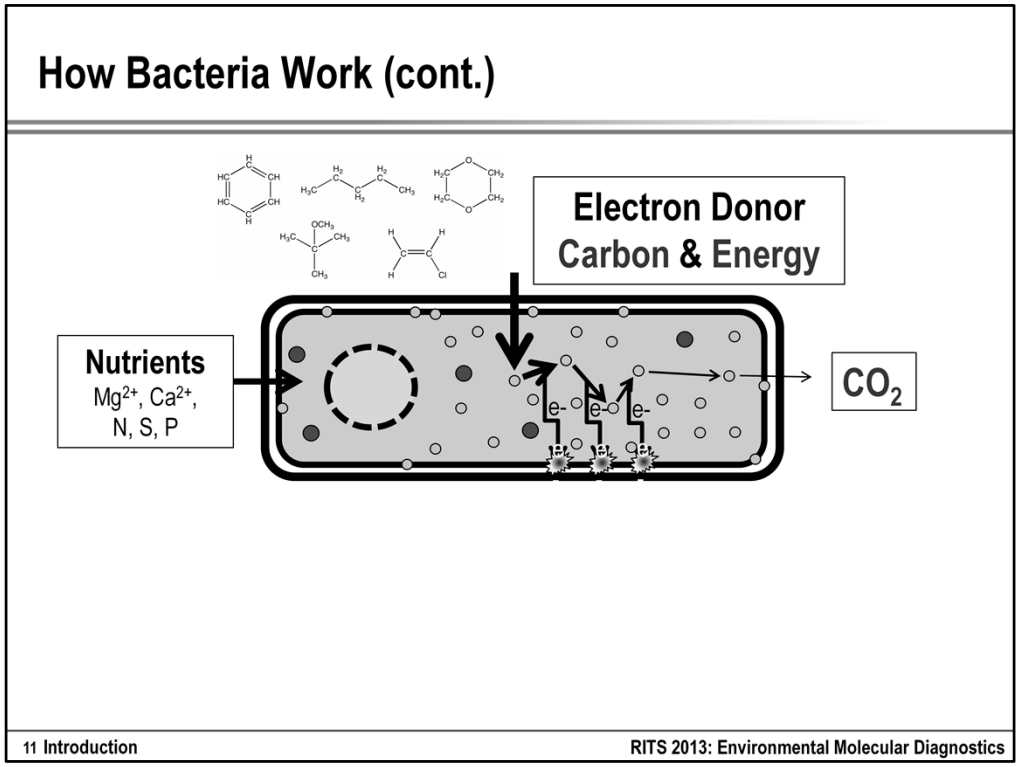
The chromosome is made up of DNA (deoxyribonucleic acid). This chromosome is the repository for all of the inheritable genetic material of the cell. The chromosome acts as the “hard drive” for the cell and the information in the chromosome dictates what the cell can, and cannot, do.

Ribosomes are made up of RNA (ribonucleic acid). This is similar to DNA but this RNA-based structure has a catalytic activity which is used in protein synthesis. Ribosomes are really protein making structures.

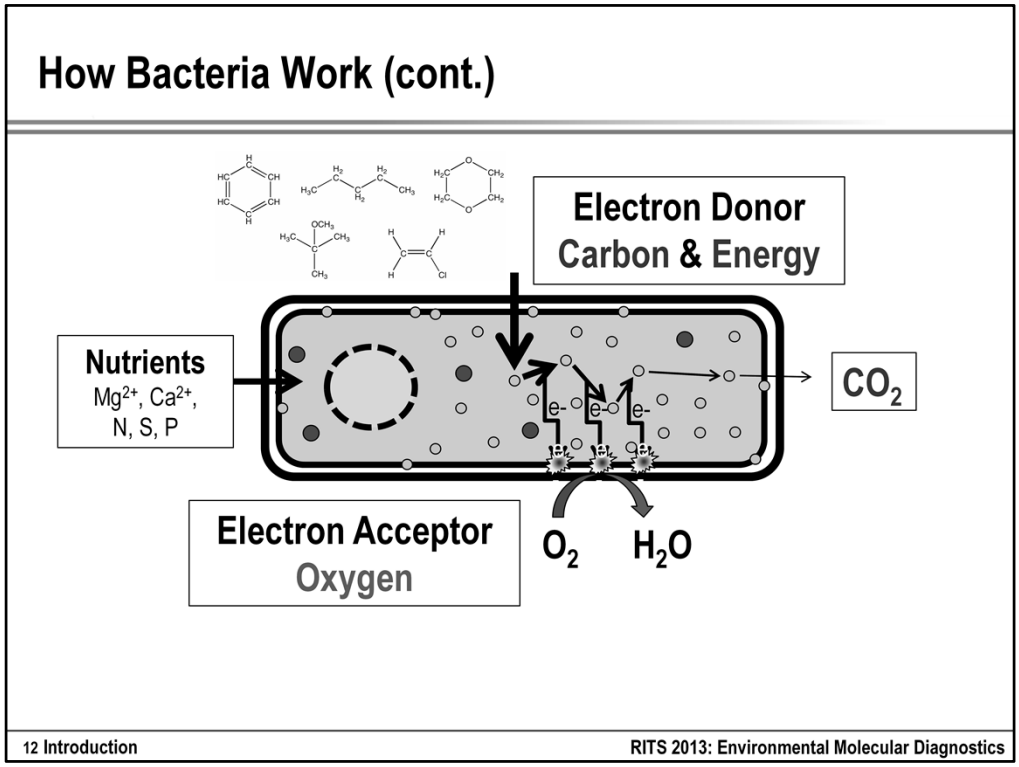
Enzymes are forms of proteins that catalyze reactions. Enzymes are found throughout the cytoplasm and they are responsible for all of the biochemical activities of the cell, including contaminant biodegradation.



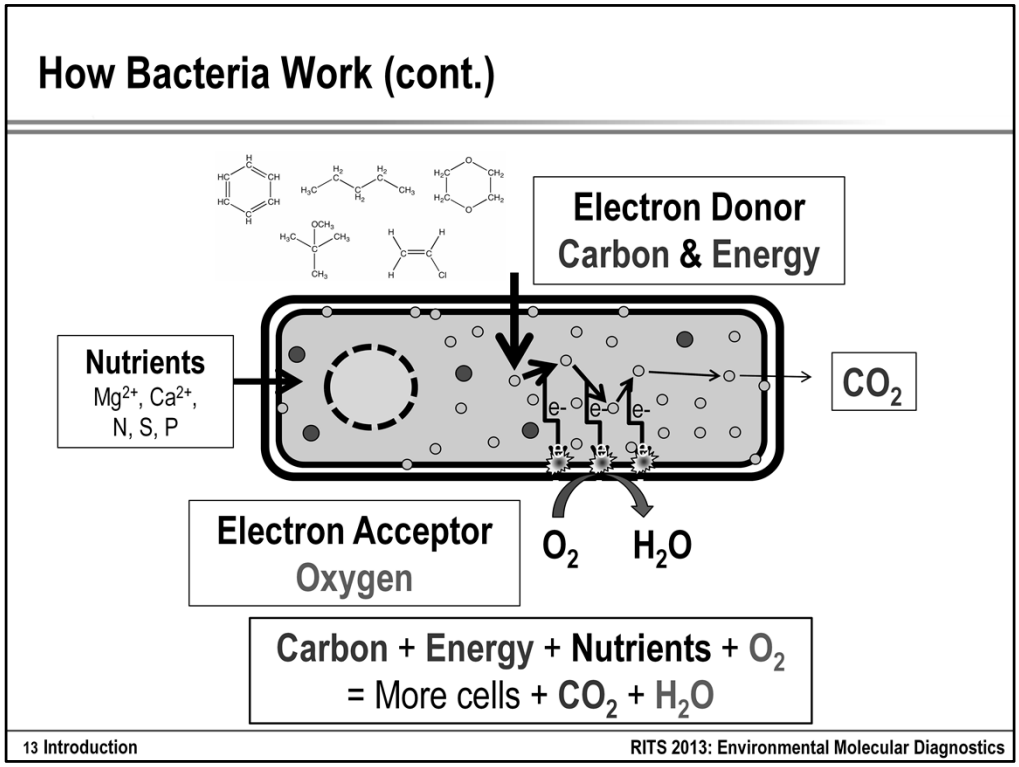
To grow bacteria, you need a carbon source. Carbon is needed as all of the major structures in a bacterium are carbon-based. In many bacteria the carbon source also acts as an energy source. This is known as the electron donor. The slide shows a variety of example COCs. The individual COCs are taken up by the cell and are biodegraded through sequential activities of enzymes until the COC has been fully oxidized to CO_2. CO_2 is then excreted as a terminal metabolite (product). To be able to grow bacteria, you also need nutrients such as metal ions. The electron donor and nutrients are all taken up by bacteria from the external medium.



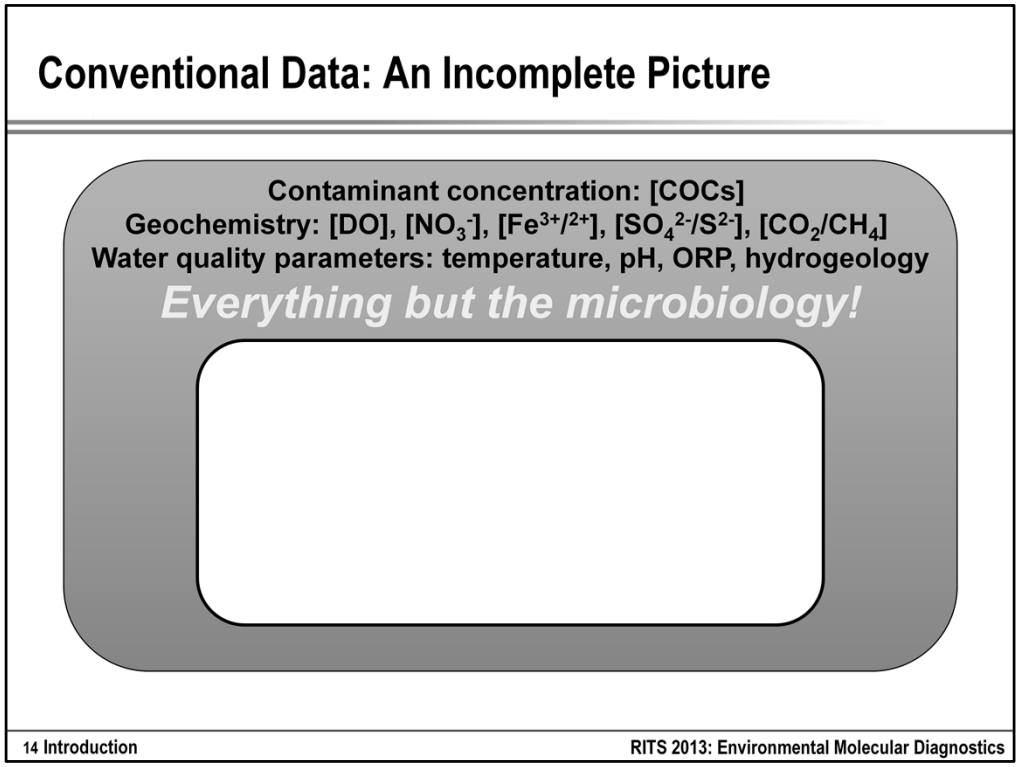
During the biodegradation of a COC by an enzyme pathway, electrons are removed from the COC. These electrons are passed to a series of proteins in the cell membrane. Reactions in the membrane allow the bacterium to generate biochemically useful forms of energy.



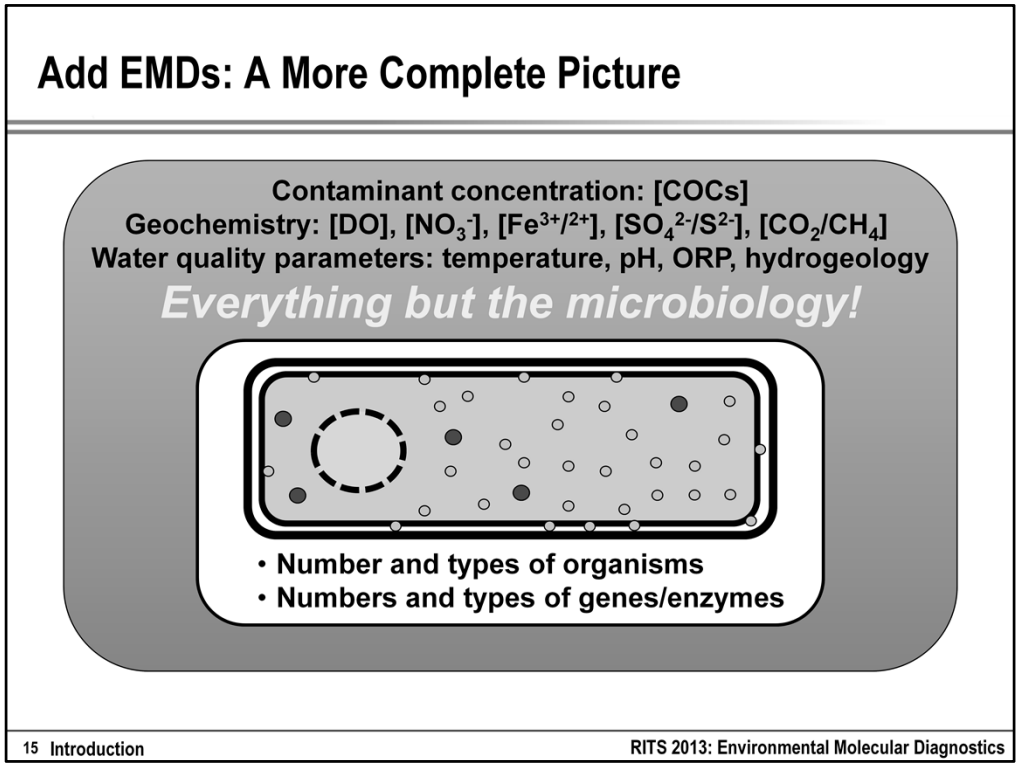
Once the energy has been extracted from the electrons, they are dumped on to a terminal electron acceptor. In the example shown here the electron acceptor is oxygen (O_2). This means this bacterium is an aerobic microorganism. There are also many examples of anaerobic microorganisms. The principal difference between an aerobe and an anaerobe is which electron acceptor they use. Aerobes use oxygen, different anaerobes can use compounds such as nitrate, sulfate and even COCs such as PCE and TCE as electron acceptors.



The net result of these reactions is that a bacterium oxidizes an electron donor and reduces an electron acceptor. The energy released during this process is used to convert carbon into biomolecules and new cells. The products of these reactions for an aerobic bacterium are more cells, CO_2 and water.



This slides shows many of the "conventional" data that are often collected during site characterization and monitoring. Many of these are examples of electron donors or electron acceptors used by microorganisms. These data also include important environmental factors that can influence microorganisms (*e.g.,* temperature). However, most conventional data do not include any measurements that directly determine the number and types of microorganisms at a site. We typically measure everything but the microbiology.

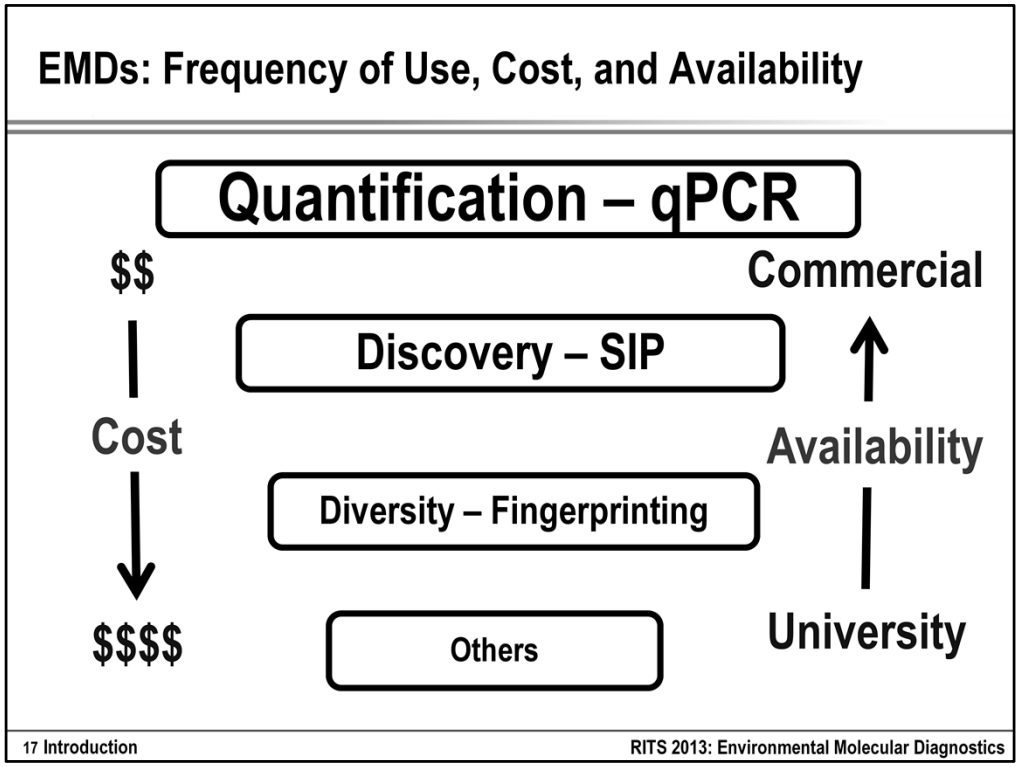


The role of molecular biology-based EMDs is to fill this data gap. EMDs can tell us what types of microorganisms are present and what genes and activities these microorganisms have. EMDs therefore provide a more complete and direct picture of the microorganisms at a site and their potential to biodegrade COCs. It should be noted that EMDs really supplement rather than replace more conventional data.

When do I need EMDs?

- When NOT TO USE them:
  - All conventional data indicate biodegradation is occurring
  - All conventional data indicate site closure is appropriate
- When TO USE them:
  - Conventional data is inconclusive about biodegradation
  - To determine if biostimulation or bioaugmentation is necessary

Key Point EMDs supplement, not replace, conventional data



There are a wide range of different EMDs that can do different things and provide different types of information. This slide summarizes the most frequently used EMDs and compares their relative cost and availability.

Quantitative PCR (qPCR) is the most widely used, least expensive and most available EMD. This technique can quantify the numbers of microorganisms at a site.

Stable Isotope probing (SIP) is not as frequently used as qPCR but is very useful as it enables RPMs to determine whether a COC can biodegrade at a site. This can be done without knowing which microorganisms are involved in this process. SIP is therefore a discovery tool. Although SIP is more expensive than qPCR, it is still commercially available.

Other techniques, including fingerprinting techniques, tend to be more specialized and less frequently used than either SIP or qPCR. They are also more expensive than qPCR or SIP and tend to be available through university labs and other specialized laboratories.

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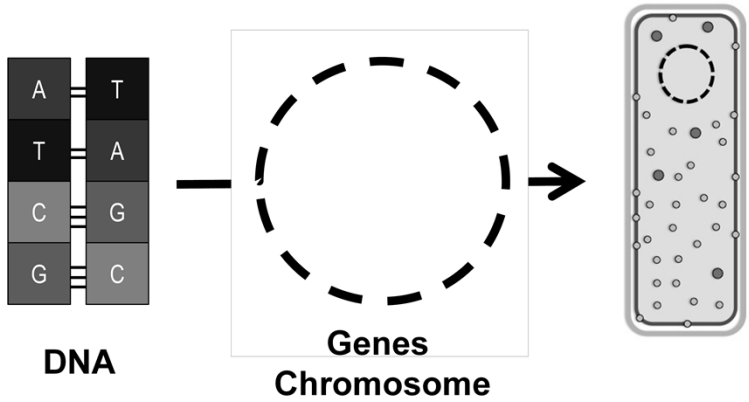
What the RPM Needs to Know About qPCR

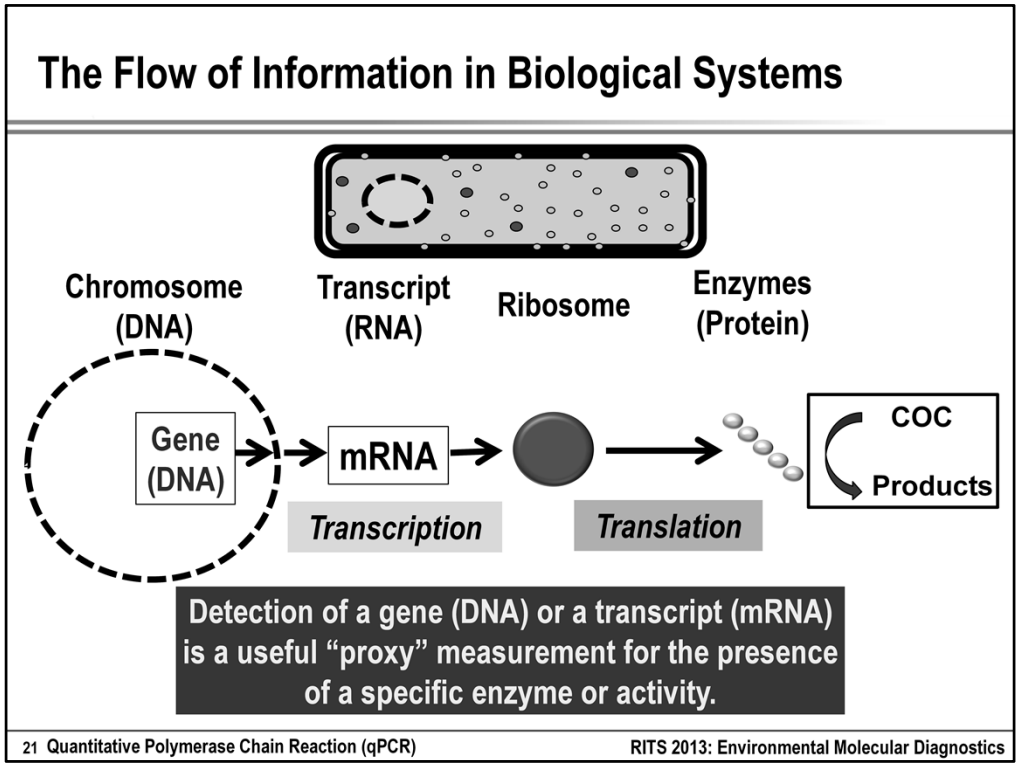
QUANTIFICATION

- qPCR tells you how many of a specific organism are present at a site
  - Are sufficient BTEX-degraders present to use MNA?
  - Did biostimulation increase number of PCE-degraders?
  - Why do I have a cis-stall.... Am I missing key organisms?
- Available for many important organisms, but not for all organisms (inquire at commercial laboratory)

DNA Structure

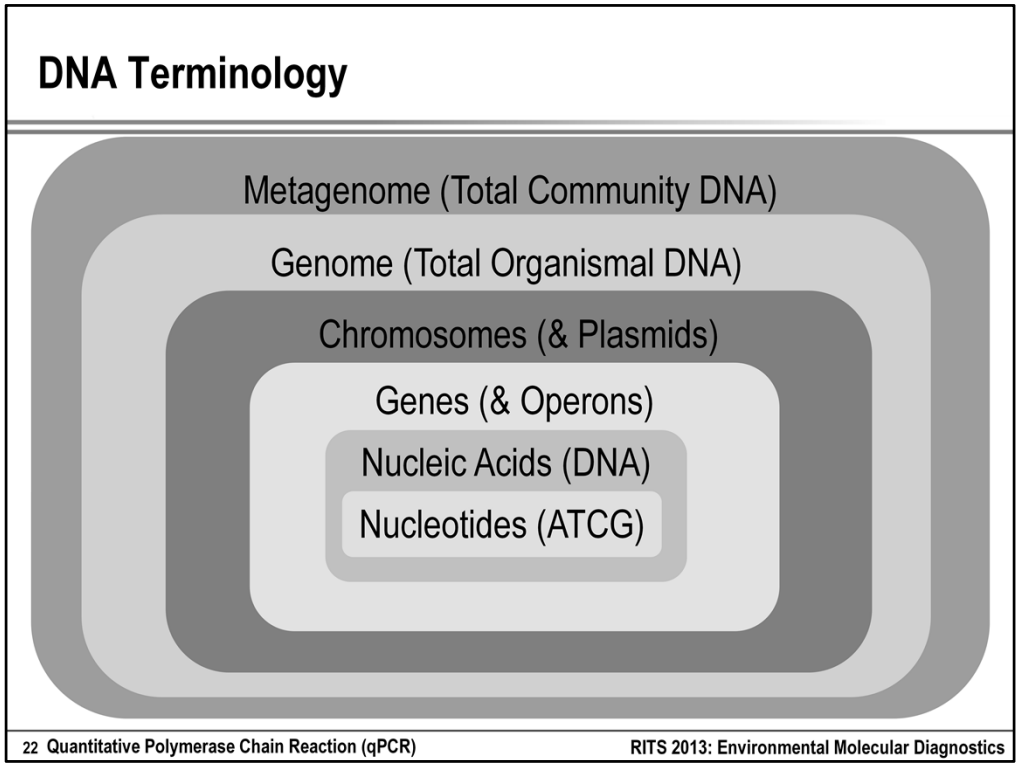


- Made up of 4 bases (A,T,C, and G)
- 2 complimentary strands
- Information lies in sequence of bases
- Information is organized in genes

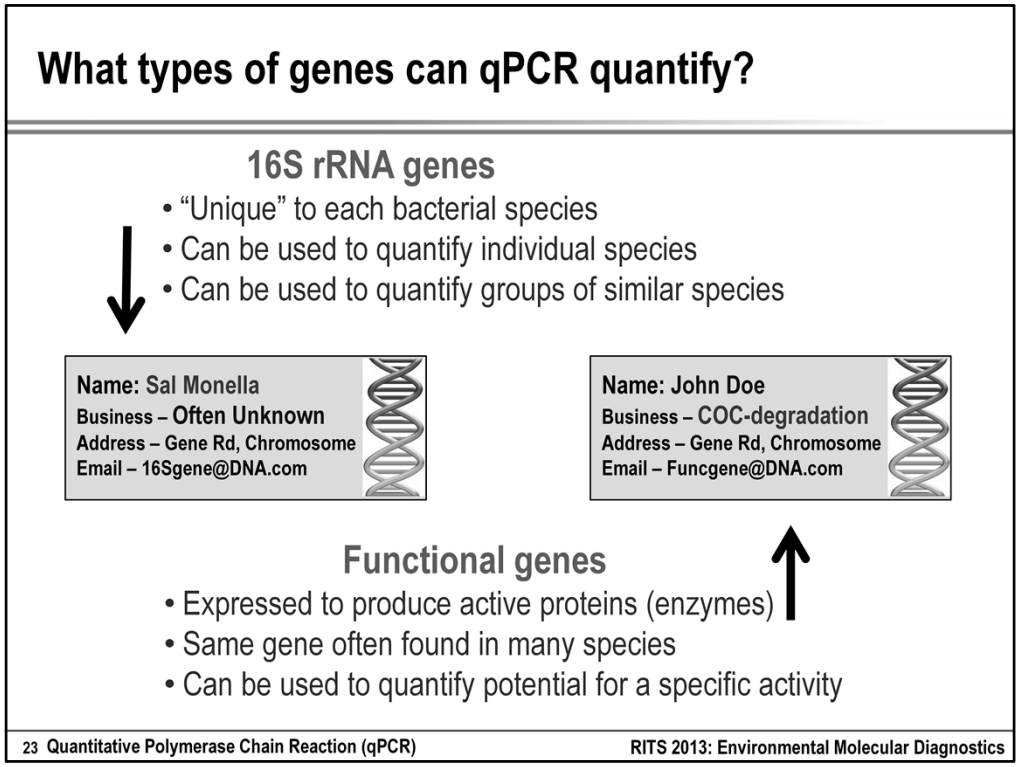


This slide summarizes the flow of information that occurs in a typical bacterial cell. The information in DNA is made up of long sequences of bases that are organized into genes. Genes are rather like programs used on a computer. A specific program enables a computer to do a specific thing. Likewise, a gene enables a bacterium to do a specific task. These specific tasks or reactions such as COC degradation are catalyzed by enzymes. The information in genes effectively dictates which enzymes are made by a cell. The information needed to make proteins cannot be read directly from DNA but needs to pass through an intermediate step or form made of RNA. The information in RNA is then used to direct protein synthesis by ribosomes. There are therefore two major information changes in bacteria. A gene (made of DNA) is read (transcribed) and a temporary form of this information is generated in the form of RNA. The ribosomes then read (translate) the mRNA and make the correct protein.

Because of this direct flow of information from DNA to RNA to protein, a central assumption of many EMDs is that the detection of a gene (DNA) is a useful proxy measurement of the ability of a microorganism to catalyze a specific reaction.



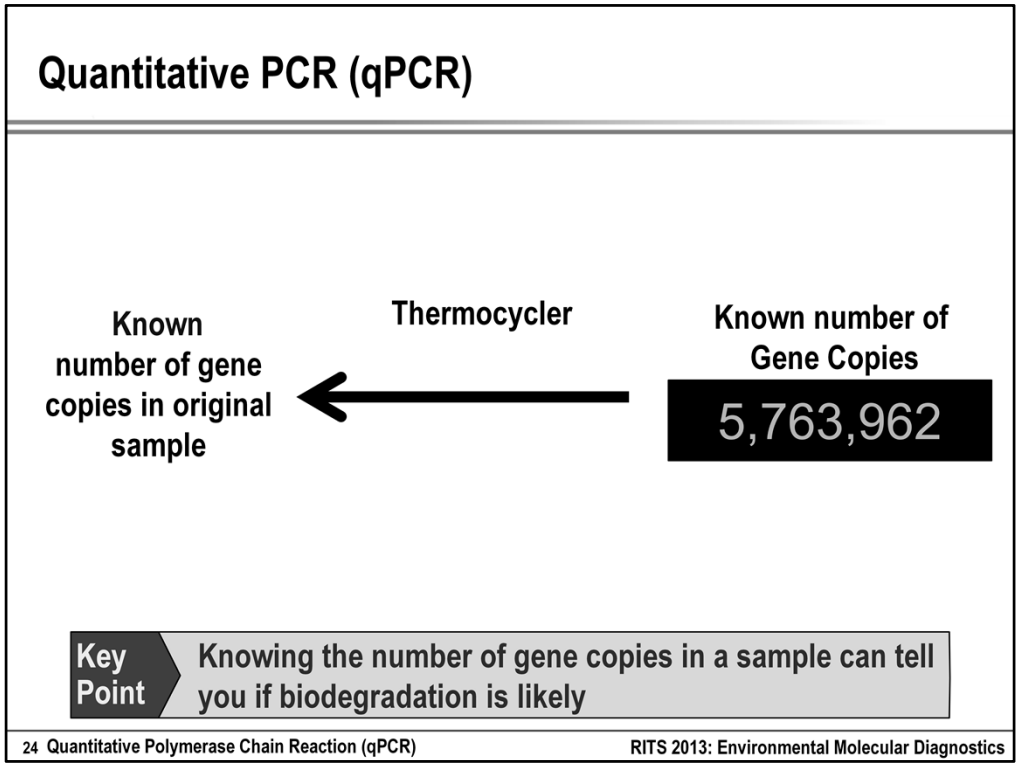
This slide summarizes some of the common terms used to describe the DNA-based information in cells and microbial communities. At the center of the image are nucleotides. These are the four bases that encode information in DNA. As we expand outwards we see that strings of nucleotides are organized into functional units called genes. Genes are present in chromosomes and so forth.



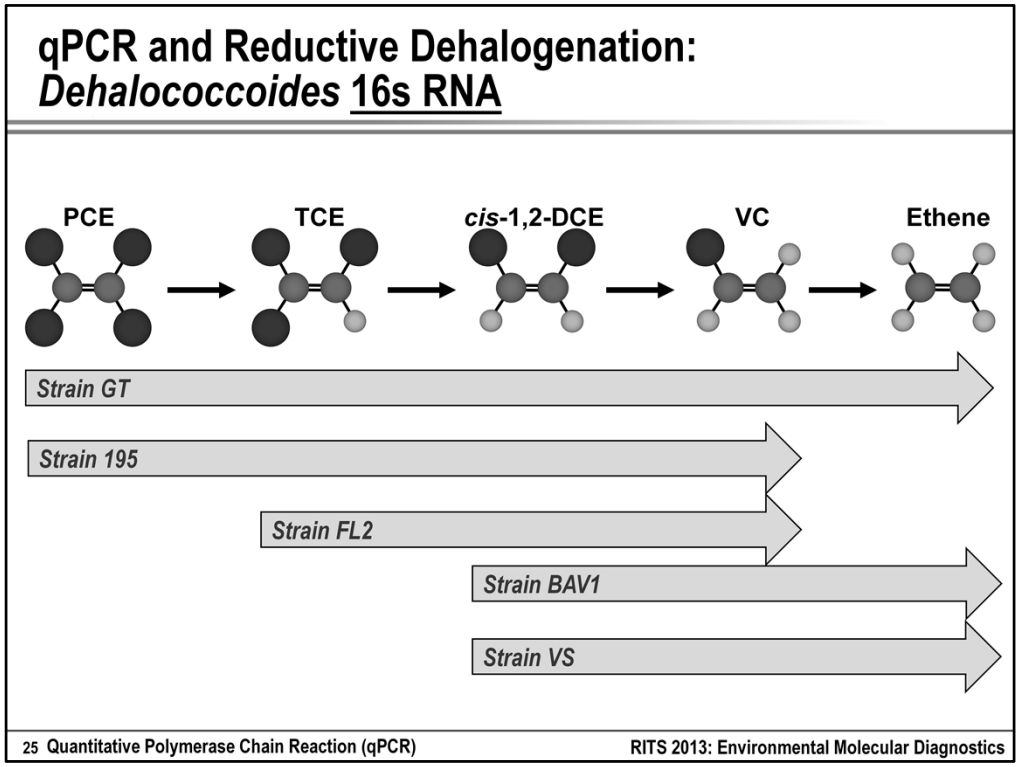
The are really two different types of genes that are useful for RPMs.

16S rRNA genes: these are the business cards of microorganisms. These genes can tell you which organisms are present in a sample. In many cases the specific activities of these organisms revealed from 16s rRNA genes are unknown.

Functional genes: These are genes that encode specific proteins or enzymes that catalyze specific reactions. Determining the numbers of functional genes can be useful because it can help explain whether a particular reaction or biodegradation process is likely to occur in a specific environment.

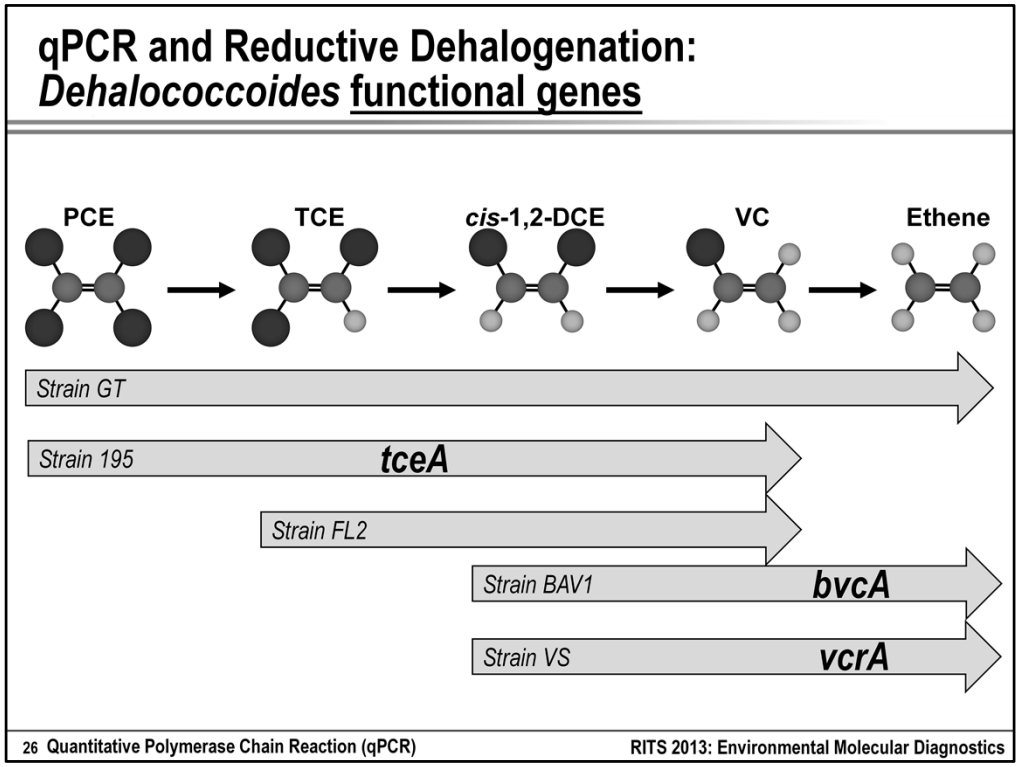


The polymerase chain reaction is effectively just a way to amplify (copy) DNA. A small amount of a specific gene in an environmental sample can be copied using PCR to make billions of new identical copies. qPCR is a quantitative version of PCR that counts the number of copies made during a PCR reaction. If the final number of copies made is known, this information can be used to determine how many copies of the original gene were present in a sample.



This slides shows how qPCR can be applied to investigate the potential for reductive dehalogenation of PCE. In this case the qPCR analysis focuses on 16S rRNA genes. The slide shows the pathway of PCE reduction and shows the various strains of *Dehalococcoides* bacteria that can reduce various compounds in this pathway. Using a qPCR analysis that focuses on 16s rRNA genes we can determine how many of each different strain of *Dehalococcoides* is present in a sample and therefore which reaction is likely (and unlikely) to occur.

For example, if we only detected high levels of strain VS we would expect that *cis*-1,2-DCE could be reduced to ethene but PCE and TCE reduction to *cis*-1,2-DCE would be unlikely.



This slide shows the same organisms and pathway but with functional genes detected rather than 16S rRNA genes. In this example we would expect rapid conversion of VC to ethene if we detected high levels of either *bvcA* or *vcrA*. Similarly, if we detected low levels of *tceA* we would not expect rapid reduction of TCE to *cis*-1,2-DCE.

Information we can get from qPCR

- Quantity of target determines potential for biodegradation
- Typically represented as cells/mL or cells/gram

Quantity (cells/mL)	Practical Interpretation
~10^6	MNA is probably appropriate treatment
10^1 to < 10^4	Biostimulation would be appropriate
< 10^1	Bioaugmentation would be needed

Key Point Knowing the quantity of the genes or organisms of interest can help determine treatment options

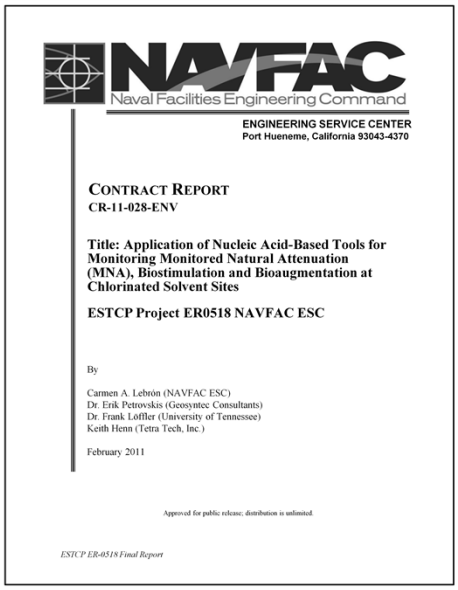
Best Practices for qPCR – Sampling Method

- In line filtration using Sterivex™ filters
  - Groundwater sample (≤10L) is obtained using low flow (500 mL/min) peristaltic pump and 0.22 μm sterile filter
  - Low flow purge followed by surging to increase particulates in groundwater sample
  - Samples water and particles, decreases shipping and laboratory processing costs
  - Filter is stored and shipped at 4°C (not frozen)

Key Point Keep method consistent throughout project – sampling (not qPCR) is the biggest source of variability

Best Practices for qPCR – Sampling Location/Timing

- **Locations:**
  - **Source areas, downgradient, stratification**
- **Correlate sampling location with potential activity:**
  - **Presence of daughter products (TCE, *cis*-1,2-DCE)**
  - **Appropriate redox conditions**
- **Frequency:**
  - **Baseline and 1-2 months after injections**
  - **Quarterly in first 12-18 months**



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CONTRACT REPORT
CR-11-028-ENV

Title: Application of Nucleic Acid-Based Tools for Monitoring Monitored Natural Attenuation (MNA), Biostimulation and Bioaugmentation at Chlorinated Solvent Sites

ESTCP Project ER0518 NAVFAC ESC

By

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February 2011

Approved for public release; distribution is unlimited.

ESTCP ER-0518 Final Report

qPCR Strength and Limitations

- **Strengths:**
  - **Inexpensive:**
    - $300/sample for one gene, $75/additional gene per sample
  - **Commercially available through several vendors**
  - **Applicable to all media (soil, water, *etc.*)**
- **Limitations:**
  - **Utility of qPCR is limited to known biodegradation processes**
    - Anaerobic reductive dehaolgenation (PCE, TCE, DCE, VC)
    - Aerobic petroleum hydrocarbon degradation

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- Introduction
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What the RPM Needs to Know About SIP

DISCOVERY

- Does not require prior knowledge of microorganisms involved
- Tracks environmental fate of forms of COC with enhanced (10-100%) levels of ^{13}C (or ^{15}N) (not natural abundance levels)
- Can be applied to both well characterized and novel contaminants
- Analyzes changes in isotopic composition of biomolecules and metabolic products (*e.g.,* CO_2) made by microorganisms, not COCs
- Typically limited to compounds used as carbon (and energy) sources (*e.g.,* PAHs, BTEX, MTBE)
- Some applications to COCs used as N-sources (*e.g.*, RDX, TNT)

Basics of Isotopes

- Elements are defined by the number of protons (*e.g.,* H = 1 proton)
- Isotopes of an element have different number of neutrons (*e.g.,* H, D, T)
- Stable and radioactive isotopes can both be used to analyze fate of COCs in the environment

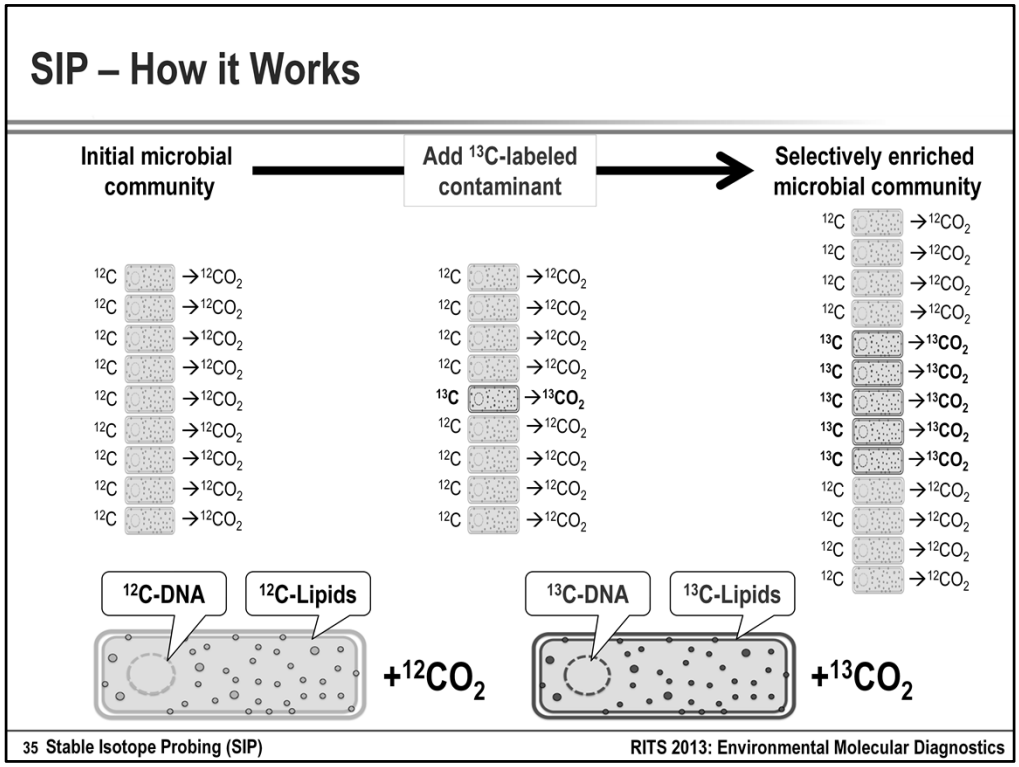
Why focus on Carbon (C) isotopes?

15 different C isotopes (^{8}C to ^{22}C) but only 3 occur naturally

Stable	Stable	Radioactive
^{12}C	^{13}C	^{14}C
12.00000	13.00335	14.00000
98.89%	1.11%	trace

Many COCs are organic (C-containing)

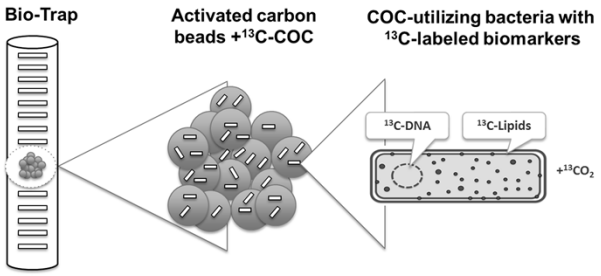
Carbon = 50% dry weight of microorganisms



This slide shows how stable isotope probing (SIP) works at the most basic level. On the left hand side of the slide there is a community of bacteria that is growing on normal ^{12}C-containing materials in the sample. In the middle of the slide a ^{13}C-containing COC is introduced. Only one of the several different types of bacteria in this sample are able to biodegrade this COC. As a result of this activity the numbers of the ^{13}C-COC-degrading bacteria increases. These organisms all generate $^{13}CO_2$ from the ^{13}C-COC and all of the biomolecules (DNA, lipids, proteins etc.) in these new cells also become ^{13}C-labeled. The various different SIP approaches can detect this ^{13}C-incorporation into these biomolecules. This information can be used to assess whether or not biodegradation of the ^{13}C-COC actually occurred and the types of microorganisms responsible for this activity.

In Situ Application of SIP

- ^{13}C-labeled COC added to Bio-trap, *in situ* incubation for 30-60 days



- Subsequent analyses of beads and surface-attached microorganisms
  - Depletion of ^{13}C-labeled COC (Was COC consumed?)
  - ^{13}C enrichment of phospholipids (Did biodegradation occur?)
  - Accumulation of $^{13}CO_2$ (DIC) (Supporting evidence)
  - Separation and molecular analysis of ^{13}C-labeled DNA/RNA (Which microorganisms were responsible for biodegradation?)

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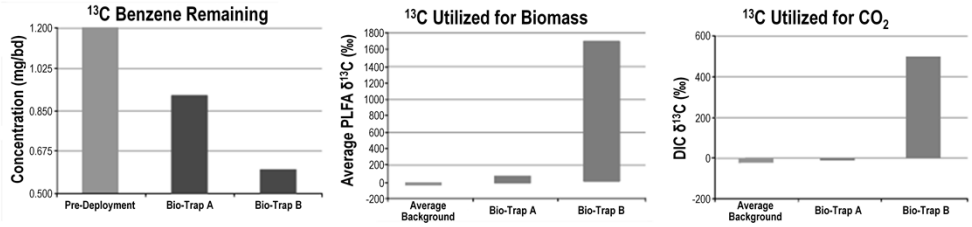
Bio-traps are a common way of conducting SIP studies. These traps contain activated carbon pellets that have been supplemented with a ^{13}C-labeled COC. These traps are introduced into monitoring wells. Native microorganisms in the ground water can then grow on the ^{13}C-COC and become attached to the activated carbon. The Bio-trap can then be retrieved from the well and the levels of ^{13}C-incorporation into the attached bacteria can be analyzed. The remainder of the slide describes the types of information that these subsequent analyses can provide.

SIP – Data Format

Data typically presented as

- ^{13}C contaminant remaining on beads (mg/bd), can calculate % loss
- ^{13}C in biomass (cells/bd), can calculate % incorporated (biomass from PLFAs)
- ^{13}C present in CO_2 (DIC) from complete biodegradation of COC (‰ ^{13}C)



- **Bio-trap A shows very limited biodegradation**
- **Bio-trap B shows clear evidence for biodegradation**

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This slide shows representative data obtained from a typical Bio-trap-based SIP study. It compares the results obtained at two different sites and summarizes the conclusions that can be drawn from the data obtained.

SIP Strengths and Limitations

- **Strengths:**
  - Can be conducted under field conditions (Bio-Traps)
  - Provides unequivocal evidence for biodegradation
  - Can be applied to both well-characterized and novel COCs
- **Limitations:**
  - Requires ^{13}C-labeled COCs that are often expensive
  - Not appropriate for COCs used as electron acceptors (TCE, perchlorate)
  - Molecular analysis can be time consuming (DNA-SIP)

Key Point **SIP is most useful when potential for environmental conditions or native microorganisms to support biodegradation is unknown**

38 Stable Isotope Probing (SIP) RITS 2013: Environmental Molecular Diagnostics

These include chlorinated ethenes or perchlorate. Atoms from these compounds are typically not incorporated into biomolecules by organisms that reduce these compounds as electron acceptors.

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What RPMs Needs to Know About Fingerprinting

DIVERSITY

- Fingerprinting techniques summarize microbial diversity in a sample using analyses of biomolecules (*e.g.,* phospholipids, DNA)
- They can demonstrate large changes in microbial community structure (size and numbers) and identify dominant microbes (*e.g.,* did biostimulation impact numbers and types of microorganisms at a site?)
- These techniques require no prior knowledge about which microorganisms are of interest

Key Point Fingerprinting techniques are useful for tracking large changes in microbial community structure

It's a diverse microbial world out there!

- ~20,000 strains/species of prokaryotes (bacteria) in 1 gram topsoil
- << 1% of all prokaryotes can be cultivated in the lab
- Total number of microbial species >1 million

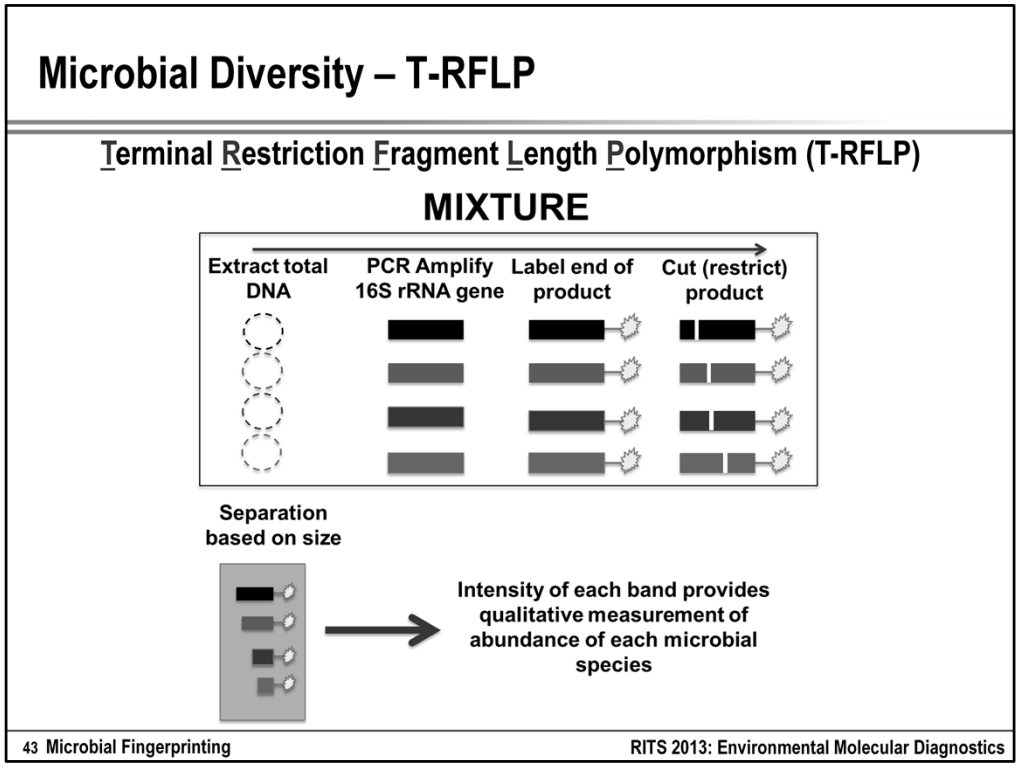
Microbial Diversity – PLFA Analysis

Quantitative
Each peak = individual PLFA

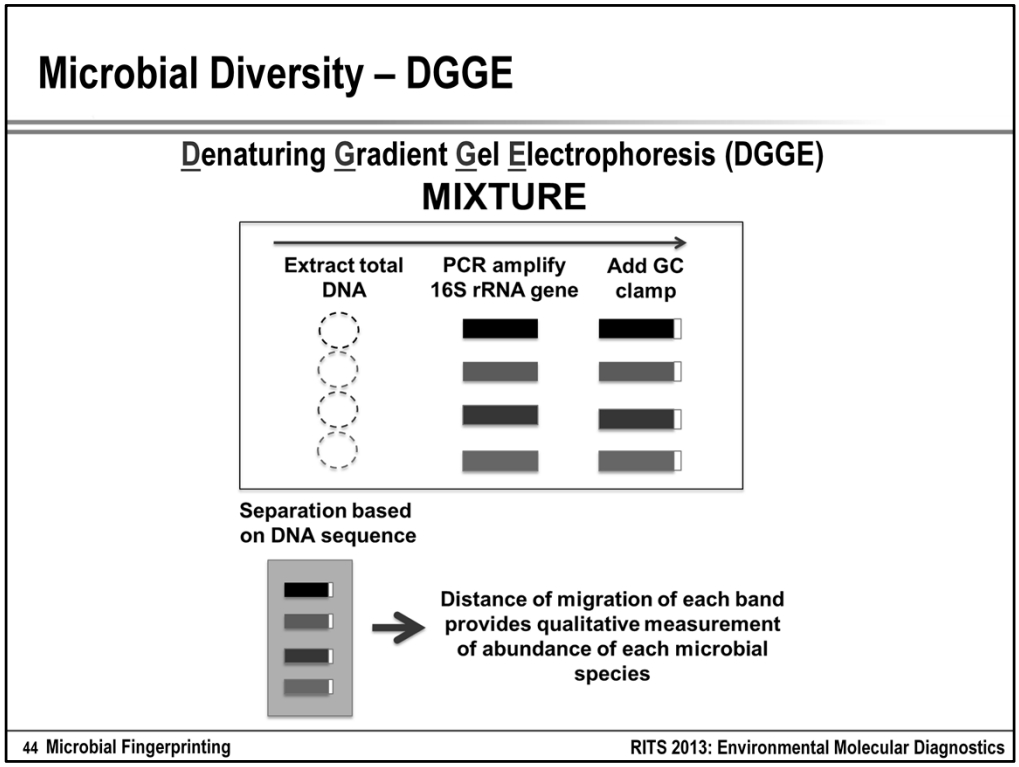
- Can quantify total amount of microorganisms
- Can demonstrate large changes in community structure
- Can identify functional groups of microorganisms

42 Microbial Fingerprinting RITS 2013: Environmental Molecular Diagnostics

PLFA analysis involves the extraction of microbial phospholipids from a sample using a solvent. The fatty acids are chemically released from the phospholipids. After derivitization, the fatty acids are analyzed by GC. The types, numbers and amounts of each fatty acid can be quantified. Some PLFAs are distinctive of particular types of microorganism but generally this approach lacks the ability to identify particular species of bacteria.



T-RFLP analysis can provide a quantitative estimate of microbial diversity as each unique fragment generated by cutting the DNA is, to a first approximation, derived from the same microorganism.



DGGE analysis provides a qualitative estimate of microbial diversity as each band detected is typically derived from the same microorganism. DGGE is not quantitative as it involves a PCR amplification of specific genes as part of the procedure.

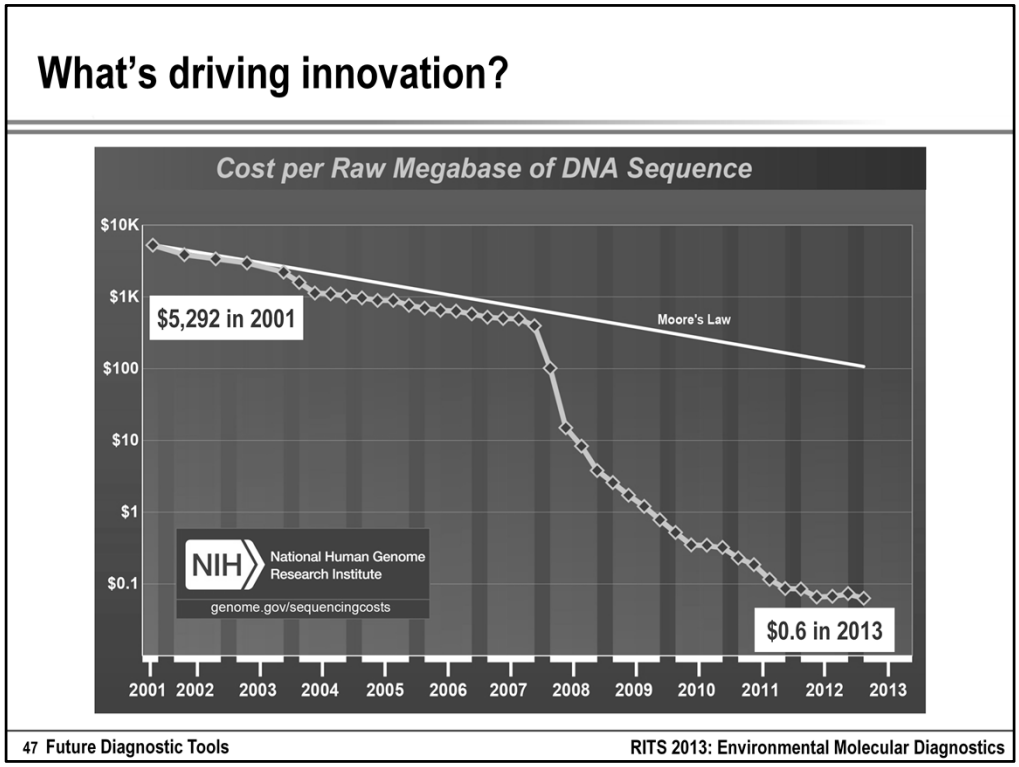
Microbial Fingerprinting Strengths and Limitations

- **Strengths**
  - All three techniques can demonstrate changes in community structure
  - Both PLFA and DGGE are commercially available
  - Relatively simple and rapid analyses
- **Limitations**
  - PLFA analysis cannot identify specific microorganisms
  - DGGE can be used for species identification, not quantification
  - Important microbial processes may be performed by a small portion of the total community (<1%) not detected in a DGGE profile

Key Point Emerging EMDs such as pyrosequencing are replacing these older fingerprinting methods

Presentation Overview

- Introduction
- Quantitative Polymerase Chain Reaction (qPCR)
- Stable Isotope Probing (SIP)
- Microbial Fingerprinting
- ► Future Diagnostic Tools
- Special Issues for EMDs
- Summary



This slide shows how dramatically the costs of DNA sequencing have fallen in the last few years.

Moore's law is the observation that the number of transistors on a silicon chip doubles approximately every 18 months. This increases the speed of computers and decreases the cost over time for the same amount of computing power.

DNA sequencing involves the use of automated systems to determine the sequence of bases (A,T,C and G) in a DNA molecule. The cost of DNA sequencing followed a similar trend to Moore's law from 2001 until about 2008. After this the cost of DNA sequencing plummeted. The cost of sequencing the same 1 Mb (1 million base pairs) piece of DNA has now dropped 10,000-fold in 10 years.

DNA sequencing is key to knowing which organisms are present in a sample (16S rRNA genes) and what they are capable of (functional genes). The ability to determine the sequence of large amounts of DNA very quickly has important implications for new and emerging EMDs.

What are these emerging techniques and what will they be able to tell me?

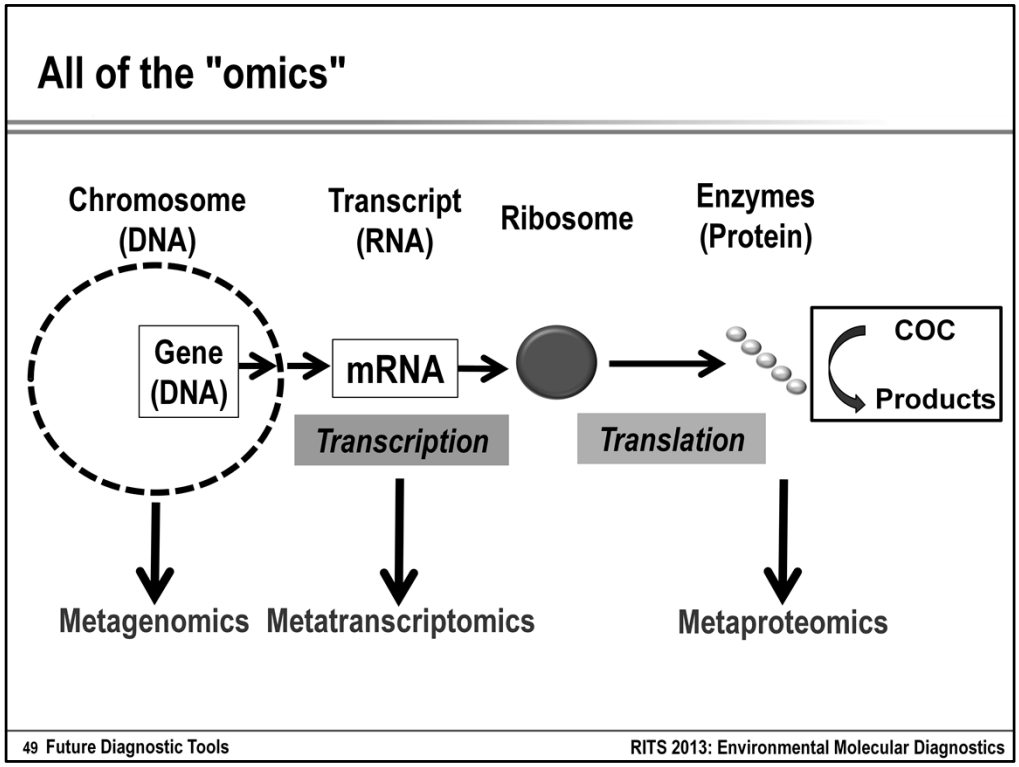
MULTIPLEXING

(Doing the same things many times simultaneously)

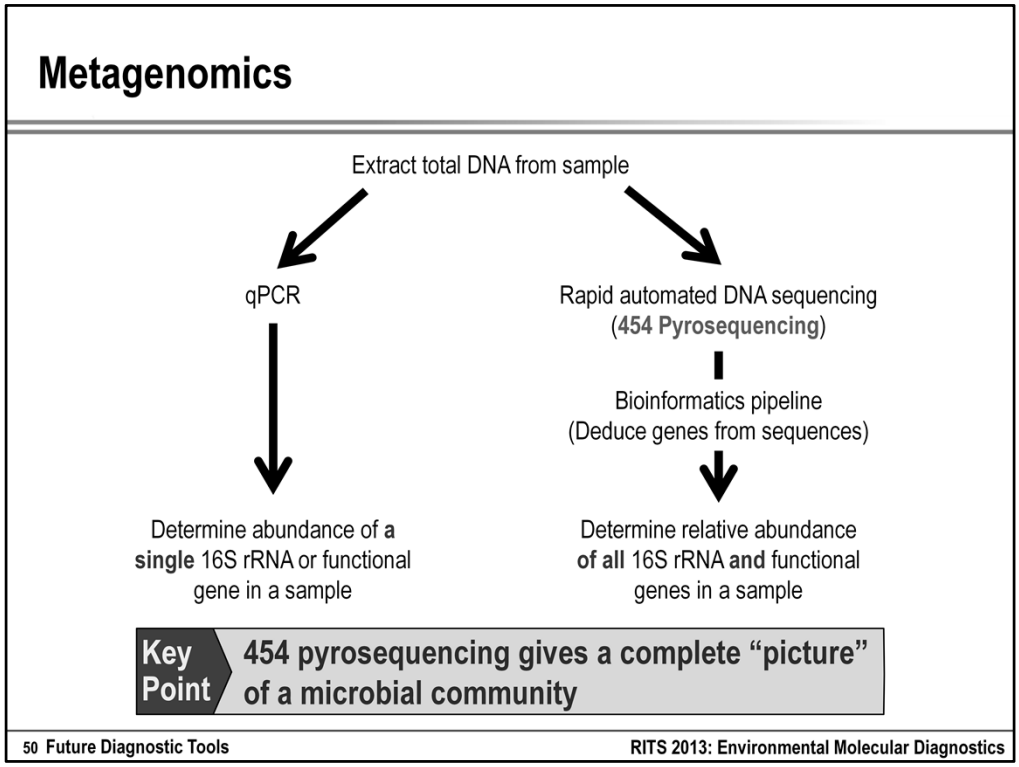
- **Metagenomics – (DNA) analysis of the genomes of all microorganisms in a sample**
  - **Rapid and commercially available: *e.g.,* 454 pyrosequencing**
- **Metatranscriptomics – (RNA) analysis of the transcriptional activities of all microorganisms in a sample**
  - **Slower, commercially unavailable**
- **Metaproteomics – (Proteins) analysis of the all of the microbial proteins in a sample**
  - **Specialized, expensive, and challenging**

48 Future Diagnostic Tools RITS 2013: Environmental Molecular Diagnostics

The new EMDs on the horizon all tend to exploit the rapid drop in the cost of DNA sequencing. The slide summarizes what these techniques are called, what they do and how available they are at the current time.



This slide shows the key “meta” analyses in terms of the flow of information in biological systems



This slide shows that the use of metagenomics (*e.g.,* 454 pyrosequencing) has a fairly simple pipeline. Unlike qPCR which can only determine the abundance of a single 16S rRNA or functional gene at a time, a metagenomics approach can determine all of these genes in multiple samples simultaneously. This approach has the potential to transform the amount and quality of data that an RPM can expect about the composition and activity of a microbial community at a contaminated site.

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Special Issues for EMDs

- **Think in terms of microbiology:**
  - **Sterility-microorganisms are everywhere**
    - Use inexpensive sterile disposable plastic collection vessels
    - Filters are inexpensive
  - **Delicate living organisms**
    - Temperature (freezing breaks apart cells, elevated temperatures promote growth)
    - Preservatives (pH extremes can kill or break apart cells)
    - Time (microorganisms can grow/die quickly in new conditions)

QA/QC

- Field and equipment blanks, or matrix and trip spikes, should be added to sampling programs
- Multiple extractions on the same sample increases yield
- Positive and negative controls should be run with all samples
- Long Term Preservation – DNA (-80°C), RNA (-80°C), Whole cells (4°C or -20°C with a preservative *e.g.,* DMSO)

Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples" (EPA 815-B-04-001, 2004).

Method A: Enterococci in Water by TaqMan® Quantitative Polymerase Chain Reaction (qPCR) Assay" (EPA-821-R-10-004)

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Summary

- EMDs can be used in site management decision making
- qPCR determines the number of copies of a specific gene in a sample
- SIP can be used to demonstrate active biodegradation and to identify unknown microorganisms
- Standard protocols are not currently available but are being actively developed as EMDs become more widely used
- Emerging EMDs will enable multiple measurements to be made simultaneously and will provide more data at a lower cost than some current methods

References

- ITRC: Environmental Molecular Diagnostics Fact Sheets (EMD-1) Nov-11
- ITRC Environmental Molecular Diagnostics Technical Regulatory Document 2013
  (Coming soon)
- EPA (U.S. Environmental Protection Agency). 2004. Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples. EPA/815/B-04/001. Office of Water.
- 2008. An Overview of Current Approaches and Methodologies to Improve Accuracy, Data Quality and Standardization of Environmental Microbial Quantitative PCR Methods. SERDP ER-1561.
- Application of Nucleic Acid-Based Tools for Monitored Natural Attenuation (MNA), Biostimulation and Bioaugmentation at Chlorinated Solvent Sites. ESTCP Project ER0518 NAVFAC ESC.