

## Addressing Microbial Pollution in Coastal Waters A Reference for Local Governments

### Appendix I: Technical Source Tracking Methods

#### Non-molecular Library-dependent Methods

Non-molecular, library-independent methods can provide basic information about microbial sources, generally indicating whether human or non-human sources contributed the majority of the contamination, however, these techniques do not deliver conclusive information. Non-molecular, library-dependent methods offer substantially more discriminating characteristics and because of library comparisons, can usually provide much more definitive information about the microbes within a sample<sup>1</sup>.

#### Antibiotic Resistance Methods

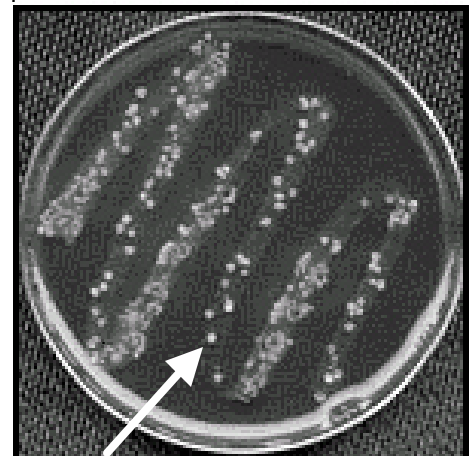
ARA (Antibiotic Resistance Analyses) and MAR (Multiple Antibiotic Resistance) are relatively new methods for determining fecal contamination sources. These methods have proven particularly useful for differentiating between human and non-human sources because bacteria strains from wildlife species are generally lacking in antibiotic resistance. Strains from humans and domestic animals usually have some varying degrees of resistance to the types and concentrations of antibiotics used and the development of antibiotic resistance profiles or “fingerprints” makes identification of source groups possible<sup>2</sup>. Most research to date has focused on *E. coli* from humans and fecal streptococci (*Enterococcus*) from animals to



Figure 2. Antibiotic resistance profile analysis of an *E. coli* isolate. The white disks on the plate are impregnated with different antibiotics and the clear zones of bacterial growth inhibition are measured to determine the resistance of each bacterial isolate. Photo courtesy of Dr. Joanna Mott, Texas A&M—Corpus Christi

determine antibiotic resistance to different types and strengths of antibiotics<sup>2</sup>. ARA and MAR methodology is similar and the terms are often used interchangeably. ARA exposes bacterial colonies to a single antibiotic used at different

concentrations. MAR exposes bacteria to multiple antibiotics at different concentrations<sup>3</sup>. Fecal bacteria colonies are isolated using the streak plate method (Figure 1) and then plated onto a series of agar (growth medium) plates, each containing disks of specific antibiotics and concentrations<sup>3</sup>. After incubation, colonies are examined for growth around the disks and a resistance pattern emerges that can be used in source differentiation (Figure 2)<sup>3</sup>. This is a library-dependent method and antibiotic resistance profiles from water samples are compared to known source profiles using statistical analyses<sup>3</sup>.



#### Colony Isolate

Figure 1. An example of the streak-plate method. A single bacterial colony isolate (single-species) is indicated. Photo courtesy of The Oklahoma Science Project:  
[www.okscienceproject.org](http://www.okscienceproject.org)

## Carbon Source Profiling (Nutritional Analysis)<sup>2</sup>

Carbon source profiling or nutritional analysis is based on differences in the nutritional requirements of bacterial groups. Particularly, analyses are based on carbon and nitrogen sources of nutrition. Nutritional analysis involves raising bacterial groups on different nutrient-based growth plates (i.e. carbon sources) and differentiating between bacterial species based on survival and growth rate on those nutrient sources.

### *Non-molecular Library-independent Methods*

#### **Fecal Bacteria Ratios**

This procedure is one of the earlier techniques developed in the source tracking field and is mainly based on the ratios of Fecal Coliform to Fecal Streptococci (FC:FS). In this method CFUs (Colony Forming Units) of fecal coliforms and fecal streptococci are counted on plates and a ratio between the two is determined. A ratio of >4 is considered human contamination and a ratio of <0.7 suggests non-human sources<sup>4</sup>. Die-off rates are monitored through time and the change in the FC:FS ratio is then used to further interpret possible sources<sup>3</sup>.

#### **Non-molecular Species-Specific (Host-Specific) Indicators<sup>3</sup>**

There are a few bacterial strains that are more specific to human and/or certain animal species and can therefore be used as indicators of the presence of microbial contamination from a particular host species. These methods usually measure raw numbers of bacteria or CFU (Colony Forming Units) counts.

Some of the more promising non-molecular species-specific indicators include the Fecal Streptococcus group (Streptococcal Population Profile method) and *Clostridium perfringens*. The Fecal Streptococcus group is composed of a variety of species that are generally found more in different types of animals than humans. Due to the low survival rate in the environment the discovery of Streptococcus species in a waterbody may indicate recent pollution. Because some Streptococcus species bacteria are more likely to come from certain hosts (both human and non-human) than others, profiles of different percentages of Streptococcus species can be constructed for different animal groups. At one time *S. faecalis* and *S. faecium* were thought to be more human-specific than other Streptococcus species<sup>7</sup>. Other species have been observed in human feces but less frequently than *S. faecalis* and *S. faecium*<sup>7</sup>. *S. bovis*, *S. equinus*, and *S. avium* are not exclusive to animals, although they usually occur at higher densities in animal feces<sup>7</sup>. *C. perfringens* is primarily associated with human wastes and is indicative of point source pollution.

#### **F+ coliphage serotyping**

Coliphages are viruses that predominantly infect *E. coli* bacteria. Coliphages are unable to reproduce outside their host and thus are indicators of recent fecal contamination<sup>6</sup>. They are commonly sorted into two groups: the somatic phage and the male-specific (F+ or F-specific) phage. The two different groups of coliphages are named for the two different strains of *E. coli* they infect, the somatic strain and the male-specific strain. Male specific strains (F+) may be host-specific and may distinguish between human and animal contamination. Within the F+ coliphages there are 4 main serological (antigenic) groups. Group I is associated with both human and non-human waste and therefore is not a definitive indicator of the pollution source<sup>8</sup>. Group II and III are associated with humans and group IV is associated with animals. This serotyping source tracking technique is based on identifying and categorizing coliphages depending on what antigen serums they react with<sup>2</sup>.

While it may be possible to distinguish between human and most animal wastes by serotyping F+ coliphage isolates, there is a problem with separation between human serotypes and serotypes associated with pigs, which can contain some groups II and III. In addition, some animal groups may not be detected by this method alone because some do not have F+ coliphage associated with their *E. coli*<sup>1</sup>.

## **Enterotoxin Biomarkers<sup>2</sup>**

The biomarker method utilizes several of the *E. coli* strains that secrete enterotoxins, biochemical substances poisonous to the host in large quantities. The enterotoxic *E. coli* strains are ideal for source tracking because their enterotoxins are described in both non-molecular (phenotypic) and a molecular (genotypic) ways, and are relatively easy to isolate from a suspected source. This is a relatively simple and low-cost technique that can differentiate human from animal waste. It is also highly sensitive and capable of finding one target *E. coli* out of 1,000,000 non-target *E. coli*.

## ***Molecular Library-dependent Methods<sup>1</sup>***

Genetic “fingerprinting” is a relatively new field, developed in the 1980’s, but is rapidly becoming a widely used research tool. Genetic fingerprinting is based on the premise that every organism has a genetic makeup that is highly individual, and as distinctive as a person’s fingerprint. The application of genetic fingerprinting to intestinal bacteria is highly effective because bacterial species can mutate (change genetic makeup) very rapidly on the order of days, weeks, and months. In addition, because the intestinal environment in each type of animal is slightly different, identical bacterial species mutate as they colonize different animals and rapidly develop slightly different genetic structure. The purpose of these molecular-based techniques, such as genetic fingerprinting, is to identify these slight differences in the genetic makeup of bacterial species isolated from different source animals. Once these slight differences in genetic structure (or “fingerprints”) are defined, the source of these bacteria, such as dogs, cats, humans, livestock, etc. can often be pinpointed much more accurately than with non-genetic methods.

Current techniques use sophisticated molecular-based tools that rely on DNA extracted from bacteria. Most molecular techniques use two central tools, Polymerase Chain Reaction (PCR) to copy DNA fragments to detectable levels, and Gel Electrophoresis to then sort the cut and copied DNA fragments by size for identification. For these approaches, the DNA must first be carefully extracted, purified, and quantified. Molecular techniques are all very technology-intensive procedures and expert personnel trained on expensive equipment must perform many meticulous tasks.

### **Rep-PCR (Polymerase Chain Reaction)<sup>11</sup>**

PCR gives researchers the means to copy a DNA fragment to create a readable fingerprint. PCR is a tool, not a tracking method in and of itself. The PCR “xeroxes” genes by rapidly copying a specific region of DNA into millions of exact replicate fragments. This process first involves cutting DNA strands into specific fragments (usually genes). Then the cut DNA fragments are chemically “unzipped” from the DNA’s double helix, leaving two separate DNA strands. As the two strands separate small DNA “primers” adhere to each DNA strand and then make exact copies of new DNA using each strand as a template. The process can be repeated 30 or more times because each newly copied DNA strand acts as a new template.

### **PFGE (Pulsed-Field Gel Electrophoresis)<sup>1</sup>**

Gel electrophoresis is one of the staple tools in MST. Gel electrophoresis makes it possible to determine very small genetic differences in similar portions of bacterial and viral DNA. Once PCR has been used to cut and copy DNA fragments, gel electrophoresis separates these DNA fragments on the basis of size<sup>3</sup>. The technique works by forcing the cut DNA fragments across a suspension gel with an electrical current. Individual fragment size determines how far the current can move each fragment through a gelatinous medium. After staining, the separated DNA fragments in each lane can be seen in a series of fluorescent bands spread from one end of the gel to the other. The number and position of bands formed on each lane of gel is the actual genetic “fingerprint” of that DNA sample.

## **Ribotyping<sup>2</sup>**

“Ribotyping” is a general term for any molecular technique that targets ribosomal RNA. RNA is very similar to DNA, except that it is single-stranded and carries out different functions within the cell than DNA. While DNA codes for instructions to the cell, it is RNA that actually carries out those instructions by creating proteins the cell uses for metabolism. Segments of RNA (16S and 23S regions) are often the target of multiple molecular techniques because these gene segments are considered to be very stable and do not mutate rapidly. Ribotyping involves cutting the bacterial DNA, followed by gel electrophoresis to separate the individual DNA fragments. Bacterial 16S and 23S RNA are then detected and bound to the DNA fragments isolated from the gel. This creates a fluorescent pattern of the fragments containing ribosomal RNA. A camera then creates an electronic image or “fingerprint” of the resulting RNA banding pattern. Fingerprints of possible sources are then compared to the banding patterns from the water samples using complex statistical analyses.

## **RAPD (Randomly Amplified Polymorphic DNA)<sup>1</sup>**

RAPD is another technique that copies selected portions of DNA using PCR. RAPD methodology uses DNA “primers” to identify unique sequences within the DNA of fecal bacteria. Performing RAPDs first involves DNA isolation, purification, and quantification of sequences of interest. Next, primers are added to the DNA sequences, then copied using PCR followed by gel electrophoresis to separate sequences by molecular weight. RAPD-based genetic fingerprints are based on gel banding patterns and compared to fingerprint libraries of known sources. This method is complex in that it requires screening over 1,200 commercially available DNA primers to find sets of sequences that occur in each fecal source of interest. Only after these sets of DNA sequences have been found can fecal bacteria be compared to **known sources**.

## *Molecular Library-independent Methods*

### **Bacteriophage Indicators<sup>3</sup>**

Bacteriophages are groups of viruses (phages) that infect bacteria. Any given virus strain may be able to grow inside several strains of bacteria and a wide variety are present in the natural environment wherever coliform bacteria are present. As previously discussed, a useful subgroup of bacteriophages are the F+ Coliphages, which are viruses that specifically infect male strains of *E. coli* bacteria. Another useful bacteriophage indicator is the *Bacteroides fragilis* phage; however, unlike the coliphage, *B. fragilis* is very host-specific and has been detected exclusively in human sewage. Some coliphages can persist for extended periods in the environment, but not much is known about the survival rates of *B. fragilis* phage. *B. fragilis* phage is very specific to human fecal contamination but more research is needed on host affinity and survival in freshwater and estuarine environments. In addition, *B. fragilis* is inappropriate for seawater projects because large sample volumes are necessary due to very low population densities.

### **Virus (Human Pathogen) Indicators<sup>3</sup>**

Certain intestinal viruses may indicate the presence of human fecal contamination and the overall viral quality of water. Virus indicators are usually detected using molecular-based methods such as PCR. One potential indicator virus, Adenovirus, is a DNA-based virus belonging to the family Adenoviridae. Adenoviruses are frequently found in urban rivers and polluted coastal waters and their infection rate in humans occurs year-round. Poliovirus, Hepatitis A virus, and a variety of enteroviruses (viruses found in intestines) have also been researched for value as viral indicators. Adenoviruses have been consistently found in greater numbers than enteroviruses in raw sewage around the world and are more stable than either Poliovirus or Hepatitis A virus in tap water and seawater. Although Adenovirus can be detected in most samples of fecal contamination, enterovirus and Hepatitis A viruses can often be detected where Adenovirus is not. Therefore, more than one human virus should be monitored for to better reflect contamination sources and viral quality of the water.

### **Molecular Species-Specific (Host-Specific) Indicators<sup>3</sup>**

Some of the more promising molecular species-specific indicator bacteria include: *Bifidobacteria* sp., *Bacteroides fragilis*, and *Rhodococcus coprophilus*. Both *Bifidobacterium* and *Bacteroides* groups are highly associated with the human intestinal tract. These groups have a low survival rate and are indicative of recent human pollution. *Rhodococcus* sp. is a strong indicator of domestic farm animal contamination, but is sometimes associated with dog and bird feces.

### **Bacterial Endemism and Co-Speciation<sup>2</sup>**

Endemic organisms are those that live in only one very specific habitat on Earth. Endemism is fairly commonplace in plants and animals and usually consists of organisms that are threatened with extinction because of their narrow environmental tolerance and habitat range. Little evidence exists for free-living endemic bacteria. In fact most bacteria, such as *E. coli*, have been identified as cosmopolitan (having a world-wide distribution). However, some commensal intestinal bacteria may be specific to certain hosts only, thus being close to having an endemic distribution. The basic assumption for source tracking applications is that if a bacterium is endemic in only one host, the bacteria should have evolved (mutated) independently from other related organisms.

### **tRFLP (Terminal Restriction Fragment Length Polymorphism)**

tRFLP is a tracking method that can determine the diversity of a whole bacterial community by examining differences in a specific gene. Basically, if two organisms' structure of the same gene differs, the length of DNA fragments produced will differ when these genes are cut. In tRFLP a single "target" gene is amplified in the entire bacterial sample (whole community) using a fluorescent-tagged primer and PCR. All copied genes are then examined for different fragment lengths and then abundance of the different fragments is determined. In general, each population of the community contributes a terminal fragment of a distinct size<sup>2</sup>. This method is considered library-independent because it does not require the isolation of environmental strains, but does depend on the extent to which the DNA sequences from the environmental strains are represented in the available molecular databases<sup>8</sup>.

## ***Chemical Methods***

### **Whitening Agents & Optical Brighteners**

Whitening agents and optical brighteners are chemicals used in laundry detergents. They are often a large component of grey water discharge and have been used as indicators of sewage fallout with varying degrees of success<sup>9</sup>. Optical brighteners emit a blue color when exposed to UV light and are measured by their fluorescent intensity. Problems with this method are mainly due to high variability of natural background fluorescence in the environment<sup>3</sup>.

### **Caffeine<sup>3</sup>**

Caffeine is generally found in highest concentrations in highly urbanized areas; however, urbanization levels have not yet been matched to known caffeine concentrations in the environment. Caffeine levels must be present in very high levels (<200:1) to be detected by current methods. In addition, some plants contain substantial levels of natural caffeine that is indistinguishable from caffeine associated with human waste.

### **Coprostanol<sup>10</sup>**

Coprostanol is a byproduct of the breakdown of cholesterol and is present in human and some other mammal species. Human secretion of coprostanol can be inconsistent but in general it is a good indicator of human fecal pollution. It is present in high quantities in human sewage, and is not altered by changes in temperature or salinity; however the stability of coprostanol in the environment can be variable. This method is most suitable for monitoring point source sewage discharge from nearby areas. Other fecal sterols such as epicoprostanol, cholestanol, and epicholestanol are also being studied for research applications. Recent work has shown that a ratio of coprostanol:cholestanol greater than 1.0 indicated human sources while ratios less than 1.0 could be either mixed or non-human sources.

## FAME (Sterols/Fatty Acid Methyl Ester Analysis)<sup>1</sup>

Sterols are biochemical components of fatty acids that make up the cell walls and membranes of bacteria. The fatty acid analysis method is proposed to differentiate between the types and amount of sterols found both in human and animal *E. coli*.

## References

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- <sup>11</sup> Access Excellence at the National Health Museum: <http://www.accessexcellence.org/>

