Multiple Antibiotic Resistance as a Fecal Contamination Source Identification Method

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Abstract
A variety of methods have been developed over the last 30 years to determine the sources of fecal contamination of surface water. Identification of sources is a major step required in the process of prevention or remediation of such pollution. This paper provides a brief overview of the various methods available, then an in-depth discussion of a specific method: Multiple Antibiotic Resistance (MAR) testing. The MAR method relies on the idea that fecal bacteria from different sources will have differing levels of resistance to antibiotics and combinations of antibiotics, based on previous exposure of the sources to antibiotics. The method first involves developing a library of known sources for a watershed and then using the library to identify the sources of new samples. Several case studies are provided showing the effectiveness of the method in field conditions.

Key Words
Fecal contamination, source identification, multiple antibiotic resistance (MAR) analysis, discriminant analysis,

Introduction

Fecal contamination in surface waters is a growing problem worldwide. In order to tackle this problem at its sources, the sources need to be identified. The goal of this paper is to provide an overview of some of the methods for identifying fecal contamination sources, and a detailed explanation and evaluation of a particular method – multiple antibiotic resistance (MAR).

This introduction will include a very brief overview of the many potential methods available for source identification. These are separated into two broad categories – biological methods and chemical/physical methods. The focus is then narrowed to biological methods and, specifically, a method that uses the resistance of isolated fecal indicator bacteria to various antibiotics. A couple of variations in the testing and analysis methods will be described. Finally, some case studies will be discussed.

Overview of Source Identification Methods

The old standby of source identification has been the ratio of fecal coliform (FC) bacteria to fecal streptococci (FS) bacteria concentrations. A ratio of 4 or greater indicates human origins and a ratio of 0.7 or less indicates non-human origins (Sargeant, 1999). However, this method is no longer considered valid by many researchers (Sargeant, 1999; APHA, 1998; Hagedorn et al.,1999; Wiggins, 1996; and Maier et al., 2000). In (APHA, 1998) there were mentioned three major problems with this method: (1) Different FS bacteria can have significantly different survival rates in the environment; (2) The FC/FS ratio can be affected by wastewater disinfection; and (3) the ratio can be affected by the way in which the FS are counted (some methods have high false-positive rates). The FC/FS method is only valid for very recent contamination (Maier et al., 2000). “For these reasons, the FC/FS ratio cannot be recommended, and should not be used as a means of differentiating human and animal sources of pollution” (APHA, 1998). The reference does not suggest an alternative method.

The purpose of all of these methods is to determine if the source of contamination is from human or non-human animals. That means demonstrating differences in the indicators between human and non-human sources. For physical/chemical methods, an indicator is a substance or environment that is different; while microbiological methods look at differences in the morphology or behavior of microorganisms that come from the different sources. Note that the discussion of methods below is by no means exhaustive.

Table 1
This table shows some of the basic chemical and physical methods, along with pros and cons to their use. (Table adapted from Sargeant, 1999, and Meays et al., 2004)

Method	Description	Advantages	Disadvantages
Optical Brighteners	Found in laundry detergents; indicates human pollution	Simple, fast, low cost	Provides limited information. May not reflect recent pollution
Caffeine (and similar drugs)	Water samples tested for presence of caffeine; indicates human pollution	Indicates impact from human pollution	Expensive test, easily degraded by soil microbes, sensitivity issues
Coprostanol	Fecal sterol present in the feces of humans and other higher mammals. Primary sterol in domestic wastes and is unaffected by physical factors like temperature and salinity.	May work as an indicator of near-source bacteria pollution from sewage sources.	Expensive and complicated laboratory procedure. Not well studied.
Fluorescent Dye Tracing	Fluorescent dye and charcoal packets are used to determine if on-site septic systems are functioning properly. Dye is added to the waste stream ahead of the septic system and charcoal packets placed near the drainfield. Packets are collected and tested for presence of the dye.	Test is time intensive but thorough.	Requires landowner cooperation and intensive field sampling.
Land use (bracketing)	Information on land use is used to select monitoring sites that bracket potential sources. Monitoring sites are placed upstream and downstream of the potential source.	Identifies areas and possible sources of bacterial pollution. Inexpensive tests.	Time intensive and requires numerous samples. Identifies area where pollution is occurring but not specific source.

Table 2
This table (adapted from Sargeant, 1999, and Meays et al., 2004) describes several of the available biological methods.

Method	Description	Advantages	Disadvantages
Species specific indicators	There are a number of bacterial strains that are more specific to certain animal species. (Sargeant, 1999) Describes five specific strains and the related animal sources.	Can provide very good selectivity. Some are useable in saline waters.	None provide quantification of the source. Lab costs and testing complexity can be high.
Multiple Antibiotic Resistance Testing	Bacteria are tested for their ability to grow in contact with a variety of antibiotics. Newer methods also vary the antibiotic concentrations.	Relatively high fraction of correct classifications. Can discriminate isolates from multiple sources. (Meays et al., 2004) claims testing is rapid.	Requires a reference database. Geographically specific. Can be prone to false positives. Difficulty with mixed samples. (Sargeant, 1999) claims this is time intensive.
Bacteriophages/Coliphages	Coliphage survival characteristics and inability to reproduce outside their host make them good fecal indicators. *Bacterioids fragilis* phage specifically indicates human fecal contamination	Can be very specific.	Unknown use in saline waters. Laboratory methods are difficult and costly. Small percentages of fecal samples contain coliphages.(Wiggins, 1996) Large water sample volumes may be necessary.
DNA ribotyping (genetic fingerprinting)	Involves isolating pure cultures from both the receiving waters and the suspected sources. The DNA of the two sources are compared.	Excellent specificity.	Laboratory analysis is expensive. Quantification is not possible. Samples must be obtained from all possible sources.

Chemical/Physical Methods

Chemical methods do not detect fecal bacteria. Instead these methods are designed to detect chemical compounds that are associated with humans, such as caffeine or laundry detergent optical whiteners (Stiles, 2003, and Sargeant, 1999). Sources can also be determined from physical aspects of the surface water course. For example, dye tracer studies can be performed to verify point sources. And sampling that brackets a potential source can narrow the possible source locations (Sargeant, 1999). Some examples of chemical and physical methods are shown in Table 1.

Biological Methods

Biological methods look at how different microbial organisms look or behave when they come from human or non-human sources. This generally means looking at the presence/absence or abundances of different indicator organisms. Table 2 lists several of the methods available.

Multiple Antibiotic Resistance (MAR) Testing

Overview

Along with the use of antibiotics in humans and livestock there has been an increase in the resistance of bacteria to those antibiotics. The introduction of new antibiotics leads to bacteria evolving eventually, to become resistant to those as well. The premise behind the use of antibiotic resistance methods is that fecal bacteria originating from wildlife species generally should be lacking in antibiotic resistance, while strains from humans and domestic animals will exhibit various amounts of resistance (Sargeant, 1999). So if a researcher can find differences in antibiotic resistance of bacteria in a sample, she can make predictions about the source of those bacteria.

Antibiotics are used in humans and other animals to treat bacterial infections. But they are also used in commercial livestock and poultry feed to increase growth (usually at lower dosages than if used to treat infections). This low dose, frequent use of antibiotics is thought to significantly increase the evolution of resistance in bacteria (Sayah et al., 2005).

Several analysis methods have been used to study antibiotic resistance patterns. The one being focused on in this paper is called discriminant function analysis. “Discriminant function analysis is used to determine which variables discriminate between two or more naturally occurring groups” (StatSoft, 2003). Discriminant function analysis is similar to analysis of variance (ANOVA) testing. In single variable ANOVA, two groups of data are compared with a statistical test to determine if the means of the two groups are significantly different. Discriminant function analysis looks at the effect of a large number of variables, (resistances to antibiotics in this case) and determines which of those variables can statistically separate sources into different categories.

A simplified example will help to illustrate the process [this example is adapted from (StatSoft, 2003)]. Samples of fecal bacteria from several known sources are tested for resistance to three antibiotics: A, B, and C. The human sources are very resistant to A and B, but not resistant to C. Samples from cattle are resistant to B but to neither of the others. Now this same testing can be done with unknown samples from the same watershed to determine if the bacteria present are from human or cattle sources. Evaluating patterns of resistance (e.g. A and B but not C) provides a more robust test than the results of single antibiotic tests (Wiggins, 1996).

In the case of MAR, researchers have investigated many variables, such as resistance to particular antibiotics, resistance patterns for a grouping of antibiotics and resistance patterns for various concentrations of antibiotics. They then used knowledge of the actual sources of samples to determine which of the variables (characteristics) allowed for the best discrimination between types (StatSoft, 2003).

Test Method

The first step is to set up a sample database, called a library. This is made up of samples of fecal bacteria from as many sources in the area of study as possible. Samples are taken from humans, livestock, pets, septic systems, and sewage systems. Samples are also taken from surface waters with and without known human or domestic animal inputs.

Then, using standard laboratory techniques, the fecal bacteria of interest are isolated and cultured. Note that (Wiggins, 1996; Wiggins et al., 1999; and Wiggins, et al., 2003) which are referenced many times in this paper, chose FS instead of FC for testing antibiotic resistance. This was a break from previous researchers who generally used FC. The reason given for this was "because FS survive well in the environment and are found in all potential pollution sources (e.g. composted poultry litter (Noblet et al., 2004)), in contrast to FC" (Wiggins et al., 1999). Both (Hagedorn et al., 1999 and Harwood et al., 2000) also recommend FS instead of FC as FS may make a better indicator organisms because they survive better in marine environments and through wastewater treatment.

These bacteria isolates are divided into multiple replicate samples placed on plates with growth medium and one concentration of one of several different antibiotics. The samples are incubated and examined. The isolated FS is considered resistant to that particular concentration and type of antibiotic if growth is observed (Wiggins, 1996).

Data Analysis Methods

Discriminant function analysis is very similar to analysis of variance (ANOVA). The basic idea is to determine whether groups differ with regard to the mean of a variable and then to use that variable to predict group membership. In the case of MAR, the variables are resistance to concentrations of antibiotic or groups of antibiotics and the predicted group memberships are the sources of the bacteria (StatSoft, 2003).

(StatSoft, 2003) Describes the steps involved in the analysis using an example. "Suppose we measure height in a random sample of 50 males and 50 females. Females are, on average, not as tall as males, and this difference will be reflected in the difference in means (for the variable *Height*). Therefore, variable height allows us to discriminate between males and females with a better than chance probability: if a person is tall, then he is likely to be a male, if a person is short, then she is likely to be a female."

This same approach is used for discriminant analysis of the antibiotic resistance data. Resistance patterns are tested against known sources. When the test is successful (i.e. when the pattern of resistance is associated with just one source) then the pattern is said to have correctly classified that source – in just the same manner as did the heights for classifying men and women in the example above (StatSoft, 2003).

On an aside, this type of analysis seems to be made for a neural network approach. (Brion and Lingireddy, 1999) describes a source identification using neural networks. In this case, the measurements were coliform and streptococcal counts, as well as bacteriophages and levels of coprostanol. The network was set up to determine if water samples were from an urban or rural setting (all livestock sources). The model worked very well, even with noisy and limited data.

Several strategies were used by researchers to improve successful classification rates. Pooling of groups was occasionally very helpful. For example, if it's not necessary to determine if pollution is from turkeys or chickens or from horses or cows; the groups can be combined to poultry and livestock respectively. For that matter, if it's only important to classify between human and non-human, pooling into those groups can improve classification (Wiggins, 1996, and Wiggins, et al.,

1999). The resulting classification in (Wiggins, 1996) improved to 95%. If prior knowledge of sources is available, such as there are human and poultry influences but no cattle in the area; then the classification rate can be improved further (Wiggins, 1996).

Challenges

The following concerns have been expressed by several of the references. In the opinion of the author of this paper, the proof is in the actual experiments. Although the fraction of samples that are classified incorrectly could be from any of these potential problems; the fact that the method has been successful in a large number of cases (see case studies later) indicates that it is likely a valid one. Which is not to say anything about the practicality of the method.

The assumption that all livestock will have been exposed to some level of antibiotics is not necessarily true. Also, wildlife often live in close proximity to livestock and consume their feed, resulting in them being exposed to the same antibiotics. This method is not useful for differentiating wildlife sources (Meas et al., 2004).

If one of the sources making up the library is from primary sewage influent, it could be contaminated with other sources by overland flow (especially if the sewer system has combined stormwater flow) (Wiggins et al., 1999).

The library may be limited to local use. Few sources looked at possible use in multiple watersheds. However, (Wiggins et al., 2003) discussed the size required for the library for it to be useable in multi-watershed studies. This may be another area in which a neural network approach might be useful.

Case Studies

There are a large number of case studies listed below – the first two will be discussed in some detail. For the sake of brevity, just a small overview of each of the remaining projects is given.

(Wiggins, 1996) Discriminant Analysis of Antibiotic Resistance Patterns in Fecal Streptococci, a Method to Differentiate Human and Animal Sources of Fecal Pollution in Natural Waters.

This study by Wiggins was the first major one to use a modification of MAR testing, called antibiotic resistance analysis (ARA). ARA uses discriminant function analysis, described earlier, to categorize the isolates by source. The researcher also used FS instead of FC as described earlier.

The researchers isolated some 1,435 bacteria from 17 samples of water with different known contamination inputs. Using the patterns of antibiotic resistance he was able to classify the sources into categories quite effectively. The measure of success in this effort was named the average rate of correct classification (ARCC). Overall, researchers achieved 74% ARCC across all of the categories (beef, chicken, dairy, human, turkey and wild). The results for some specific groups were higher; for example 92 % of human isolates were correctly classified. When the researchers pooled groups, the ARCC improved dramatically. As an example, comparing human to wild animal groups, the ARCC improved to 98%.

Using the library, researchers then examined unknown samples from two polluted surface waters (streams running through heavy agriculturally impacted land and houses with septic systems). The results were 68% to 72% classified as cattle sources. When the analysis was performed in a pooled fashion with classification as human or animal, the results showed 95% and 96% animal sources.

(Wiggins et al., 1999) Use of Antibiotic Resistance Analysis to Identify Nonpoint Sources of Fecal Pollution.

This study was a follow up to that in (Wiggins, 1996). The research was developed in the same manner as the first study but with a much larger data pool, both for samples tested and antibiotics tested. Samples were obtained over a four year period from sources similar to the previous research project.

Another aspect of the analysis that the researchers explored was isolate level testing compared to sample level testing. The former involves classifying every isolate into one of the four categories (human, cattle, poultry, and wild). While the latter is concerned with classifications of the sample sources – each sample of water having provided multiple isolates. Classification results from isolates were not as good as they had been in the 1996 project, despite the much larger amount of data. However, the sample level testing resulted in ARCC values at or near 100%. The researcher noted an important assumption underlying sample level analysis. That is the assumption that all of the isolates in that sample are from the same source. One would need additional information about the environment to justify this assumption.

(Parveen et al., 1997) Association of Multiple-Antibiotic-Resistance Profiles with Point and Non-Point Sources of Escherichia coli in Apalachicola Bay

This research project used *E. coli* as the indicator organism and tested with just one concentration of antibiotic in each case. It was a slightly different approach in that researchers were mainly attempting to classify sources as point and non-point sources. The results were that point source bacteria were resistant to a wider variety of antibiotics than non-point source bacteria.

(Carroll et al., 2005) Sourcing Faecal Pollution from Onsite Wastewater Treatment Systems in Surface Waters Using Antibiotic Resistance Analysis.

The main goal of this project was to distinguish between human and non-human sources. As such, researchers had the benefit of pooling sources from the start. The research indicated that a much smaller library provided adequate rough classification of sources. But a larger library was recommended for a more reliable classification. ARCC rates of 93.8% between human and non-human sources were reported.

The point/non-point results were less clear – some bacteria counts were higher or lower with different water flow levels than expected. Researchers speculated that this was due to poorly performing septic systems providing a continuous supply of contamination.

This research used some innovative data analysis techniques. As a test of the predictive capabilities of the model, classification was done with some of the data removed. Then the removed data was reentered and used to test the classification. Also, the researchers used a very good graphical representation of the Discriminant analysis. A plot of the data in two dimensions showed clumping of the various sources, which nicely described both the classification of sources and the overlapping regions where classification was not as clear.

(Moore et al., 2005) Evaluation of Antibiotic Resistance Analysis and Ribotyping for Identification of Faecal Pollution Sources in an Urban Watershed

This research compared the results of antibiotic resistance classification to the classification that was accomplished using ribotyping techniques. The results showed ARCC rates of 44% for *E. coli* and 48% for FS using antibiotic resistance analysis and 69% for *E. coli* using rybotyping. The researchers concluded that neither method was accurate enough to be useful in the field.

(Hagedorn et al., 1999) Determining Sources of Fecal Pollution in a Rural Virginia Watershed with Antibiotic Resistance Patterns in Fecal Streptococci

Researchers on this project used FS isolates. They set up a library with isolates from human, cattle, deer, poultry and waterfowl. The library was used to classify a 892 isolates from an independent study with ARCC results of 88%. Pooling to human and non-human increased the ARCC to more than 95%.

As a further test, samples from three polluted stream sites were classified as having a cattle source. Part of a watershed improvement program involved fencing the pasture areas upstream of the sampling sites to block cattle. This resulted in a drastic improvement in water quality. The FC were reduced by 94% overall and the number of isolates classified as from cattle sources decreased to less than 45 %.

(Harwood et al., 2000) Classification of Antibiotic Resistance Patterns of Indicator Bacteria by Discriminant Analysis: Use in Predicting the Source of Fecal Contamination in Subtropical Waters

Researchers in this project evaluated antibiotic resistance from both FC and FS, finding similar results from each (ARCC of 63.9% and 62.3% respectively). The library was used for a field test on surface waters with a known input of human contamination via malfunctioning septic systems. The isolates were well classified with an overall ARCC of 89% and 74.5% respectively for FC and FS. After repair of the offending systems, the classifications as human dropped to under 8% for both. Additional testing was performed with samples from surface water with little human impact. The rates of animal classification for these areas were close to 77%.

This research also explored the data analysis from a different point of view. In cases where the classification was lower than expected, (Harwood et al., 2000) recommend looking at the "expected frequency of misclassification." In the example given by the researchers, the ARCC for human sources was 54.2% which is not as high as some of the others. But a portion (~10%) of the isolates known to be from sources other than human were misclassified as human. So it is plausible that as much as 10% of the field samples were incorrectly classified as human. This can be used as a standard of comparison.

(Choi et al., 2003) Application of Enterococci Antibiotic Resistance Patterns for Contamination Source Identification at Huntingto Beach, California

This was a field test that was set up for a specific problem of high FC levels in seawater outside of Huntington Beach, California. The sources selected for the library were birds, sewage, urban runoff, coastal marsh sediments, and seawater. It is unclear what the rational was for the latter three sources. While doing so will provide indications if the contamination is from one of those areas, it won't necessarily provide information about the animal source. The field testing determined that the sources of pollution were bird feces, run-off from the salt marsh and effluent from a sewage outfall at different times. A point of interest in this research was that a significant percentage of the wild bird population had fecal bacteria that were resistant to several antibiotics.

(Wiggins et al., 2003) Use of Antibiotic Resistance Analysis for Representativeness Testing of Multiwatershed Libraries.

Researchers in this project wanted to accomplish two main goals: (1) Determine how big the library of resistance patterns needed to be and (2) If libraries from different watersheds can be combined to be used over broad areas. The combined library contained data from six different watersheds in Virginia. When all of the libraries were included (6,587 isolates), the combined library was effective in classifying samples from the entire region. An interesting note from this research is that the combined library was still effective at classifying sources after a year.

(Sayah et al., 2005) Antibiotic Resistance Analysis of Fecal Coliforms to Determine Fecal Pollution Sources in a Mixed-Use Watershed

This research looked at patterns of resistance of isolates from a large number of animals, both wild and domestic (it would be interesting to watch the Michigan Department of Natural Resources obtain the cloacal swab samples from wild geese). One helpful attribute of this study was that researchers had assistance from farmers in the study. The farmers whose animals were sampled were able to provide antibiotic usage data. Results showed that the isolates with the highest levels of resistance came from swine. Also, there was consistency in resistance patterns within species on the farms and even between species for select antibiotics such as tetracycline.

Conclusion

The majority of case studies evaluated in this paper support the accuracy of this method for determining the sources of contamination of surface waters. If potential sources are pooled, or if local environmental conditions are known a priori, the classification becomes very good.

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