Tech NOTES 9

National Nonpoint Source Monitoring Program

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Through the National Nonpoint Source Monitoring Program **(NNPSMP)**, states monitor and evaluate a subset of watershed projects funded by the Clean Water Act Section 319 Nonpoint Source Control Program.

The program has two major objectives:

1. To scientifically evaluate the effectiveness of watershed technologies designed to control nonpoint source pollution
2. To improve our understanding of nonpoint source pollution

NNPSMP Tech Notes is a series of publications that shares this unique research and monitoring effort. It offers guidance on data collection, implementation of pollution control technologies, and monitoring design, as well as case studies that illustrate principles in action.

Monitoring for Microbial Pathogens and Indicators

Introduction

The U.S. Environmental Protection Agency's (EPA's) 2010 *National Water Quality Assessment* lists pathogens (including indicators) as the leading cause of impairment for rivers and streams, the number two cause of wetland impairment, and the third-ranked cause of impairments in the nation's bays and estuaries (USEPA 2012b). Pathogens have been the focus of more than 11,000 total maximum daily load (TMDL) determinations since 1995, by far the leading water quality impairment addressed by the TMDL process across the U.S. Microbial pathogens can cause serious illness in people and violations of water quality standards for bacteria can impact drinking water supplies, shut down shellfishing, and close beaches.

A 1993 outbreak of cryptosporidiosis in Milwaukee is the largest waterborne disease outbreak ever reported in the U.S. An estimated 400,000 people were reported ill. High tributary flows into Lake Michigan because of rain and snow runoff may have transported the parasites great distances into the lake from its watershed, and from there to the water plant intake. Although all applicable water quality standards were being met by the water treatment plant, the facility needed significant upgrades to reduce the risk of *Cryptosporidium* in treated water. (Rosen 2000)

Pathogenic bacteria and protozoa can come from many different animal sources in rural and suburban watersheds, including wildlife, pets, agricultural livestock, and humans. Urban development is also often associated with an increase in bacteria in stormwater runoff and receiving waters. Exposure to pathogens can occur during swimming or other recreational activities through ingestion, inhalation, or direct contact with contaminated water. Shellfish from pathogen-impaired estuarine waters may pose a health risk to consumers. Treated drinking water, where treatment includes disinfection and/or filtration, is normally free from pathogens, but chlorination alone may not remove all pathogens and treatment failures are possible. Untreated drinking water may be threatened by contaminated source water or by faulty well construction.

Threats to human health and the extent of pathogen-related water quality impairments drive the need to monitor for microbial pathogens and indicators in watershed programs.

Because pathogens and many associated indicators are living organisms, monitoring provides challenges that differ from the demands of typical physical and chemical monitoring in nonpoint source (NPS) projects. The generation of microorganisms from both domestic and wild animals, the transport of microbes through the environment, their survival or die-off in the environment, and sampling and analytical constraints all combine to require specific approaches to monitoring.

This Tech Note provides basic information about waterborne pathogens in watersheds and presents recommendations on how to conduct monitoring in NPS watershed projects using traditional fecal indicator bacteria (FIB) and microbial source tracking (MST) approaches. Unlike recent EPA guidance for beach monitoring that promotes techniques with shorter analytical timeframes to make rapid beach closure decisions to reduce public health risk, this Tech Note explores the broader use of FIB, pathogen, and MST approaches depending on specific project needs and budgetary constraints.

Purposes of Monitoring for Pathogens and Indicators

In NPS watershed projects, monitoring for microbial pathogens and indicators may be conducted for several purposes, comparable to objectives for monitoring other NPS pollutants:

- Documentation of water quality impairment;
- Regulatory compliance;
- Source identification;
- TMDL development; and
- Evaluation of treatment effectiveness (BMP or watershed level).

For the most part, monitoring of microorganisms for these purposes will follow the same design and operational principles as for other NPS pollutants. However, through the use of techniques of molecular biology, monitoring for microbial pathogens and indicators can contribute to pollutant source identification in ways not possible with most physical and chemical constituents commonly monitored in watershed projects (see later section on Microbial Source Tracking).

Because microbial pathogens and indicators are also involved in human health issues, monitoring may also be conducted for such special purposes as:

- Drinking water safety;
- Disease outbreak investigations;
- Regulation of shellfishing; and
- Recreation management (e.g., beach closure).

Recreational Water Quality Criteria

One key purpose of microbiological monitoring is to manage risk of illness in the use of recreational waters. In 2012, EPA released new Recreational Water Quality Criteria (RWQC) recommendations for protecting human health in waters designated for primary contact recreation (USEPA 2012a). These criteria (Table 1) rely on recent research that shows a link between illness and fecal contamination in recreational waters, based on the use of bacterial indicators (*E. coli* and enterococci).

Table 1. 2012 Recreational Water Quality Criteria (USEPA 2012a).

Criteria Elements	Recommendation 1 Estimated Illness Rate 36/1,000		Recommendation 2 Estimated Illness Rate 32/1,000	
Indicator	GM (cfu/100 mL)	STV (cfu/100 mL)	GM (cfu/100 mL)	STV (cfu/100 mL)
Enterococci (marine & fresh)	35	130	30	110
E. coli (fresh)	126	420	100	320

GM = geometric mean, STV = statistical threshold value, cfu = coliform forming unit

The RWQC consist of three components: magnitude, duration, and frequency. The magnitude of the bacterial indicators is described by both a geometric mean and a statistical threshold value for the bacteria samples. The statistical threshold value approximates the 90th percentile of the water quality distribution. The waterbody geometric mean should not be greater than the selected geometric mean magnitude, and no more than 10 percent of the samples should exceed the selected statistical threshold value (STV) magnitude in any 30-day interval.

These water quality criteria recommendations are intended as guidance in establishing new or revised water quality standards. Additional information on the 2012 RWQC can be found at EPA's ***Recreational Water Quality Criteria*** website.

Microbial Pathogens and Indicators

Organisms of Concern

A pathogen is any agent that causes disease in animals or plants. Microbial pathogens include bacteria, protozoans, and viruses. Many microorganisms are not themselves pathogenic, but are monitored because their detection is practical and inexpensive and their presence coincides with the presence of pathogens.

Bacteria

Bacteria are unicellular organisms that lack an organized nucleus and contain no chlorophyll. Bacteria may have various shapes: spherical (coccus), rod-shaped (bacillus), comma-shaped (vibrio), spiral (spirillum), or corkscrew-shaped (spirochete) and may

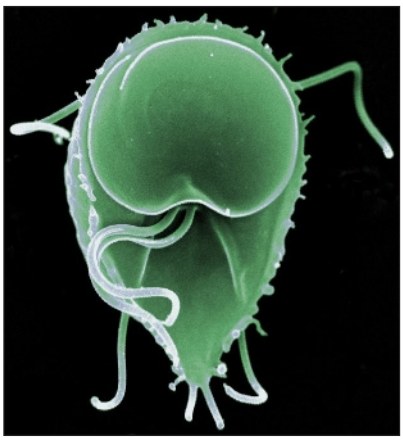
range from 0.5 to 5.0 µm in size. Some live in soil, plants, or water; others are parasites of humans, animals, and plants. Bacteria can be classified into three groups based on their need for oxygen. **Aerobic bacteria** thrive in the presence of oxygen and require oxygen for continued growth and existence. **Anaerobic bacteria** thrive in oxygen-free environments. **Facultative anaerobes** can survive in either environment, although they prefer the presence of oxygen.

Bacteria are ubiquitous in nature; many species perform functions essential or beneficial to human life, while others cause disease. Of concern in this Tech Note are the types of bacteria found in the feces of humans and other animals that are often found in waterbodies, including the coliform group, streptococcus, campylobacter, and others. It is important to understand that most fecal bacteria are not pathogenic or disease-causing.

E. coli

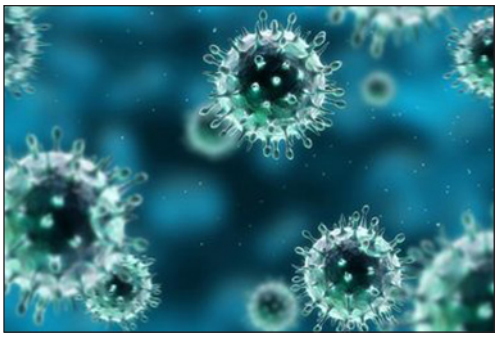
Important water-borne pathogenic bacteria include:

Escherichia coli O157:H7 is a potentially deadly bacteria strain that can cause bloody diarrhea and dehydration, especially in children. It is an unusually infectious organism with as few as 10 cells capable of causing illness. Although this organism is not pathogenic to cattle themselves, calf water troughs and moist mixed cattle rations have been cited as sources of *E. coli* O157:H7 on farms.



Giardia

Campylobacter (e.g., *Campylobacter jejuni*) is common in the environment and is shed in the feces of humans, livestock, and wildlife, including birds. *C. jejuni* can cause infection in humans. It is found in a variety of surface water, stream sediment, and sewage effluents. Cattle and poultry feces and effluent from poultry processing facilities have been shown to contain *C. jejuni* that, in some cases, are similar to strains found in humans.

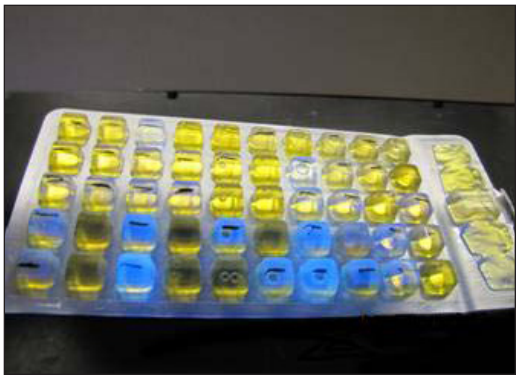


Enterovirus

Salmonella species cause diarrhea and systemic infections that can be fatal in particularly susceptible persons. An estimated 800,000 to 4 million human infections occur each year in the U.S. The majority of outbreaks are associated with foodborne illness, rather than water-borne exposure.

Other bacteria of generally secondary concern include *Yersinia*, *Shigella*, *Brucella*, and *Leptospirosis.*

Fecal Indicator Bacteria



Most pathogenic bacteria are present in the environment only sporadically, at very low levels, and are difficult and expensive to detect directly. For these reasons, we have traditionally monitored more common, easy-to-measure bacteria as indirect indicators of fecal contamination of water: fecal indicator bacteria. The presence of FIB provides evidence of the presence of fecal material and the potential presence of pathogenic organisms because FIB are believed to survive or die-out under similar physical, chemical, and nutrient conditions as true pathogens.

The choice of specific FIB for monitoring has evolved over the past 80 years.

The **Total Coliform** Group (comprising all aerobic and facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas formation within 48 hours at 35 °C) was once the standard indicator bacteria test. However, total coliforms have been found to not be useful for testing recreational or shellfishing waters because some species in the group are naturally present in soils or plant materials, so their presence does not reliably indicate fecal contamination. Total coliforms, however, continue to be useful for testing treated drinking water where contamination by soil or plant material would be a concern. Water-quality criteria for drinking water, based on total coliform density, are specified in the Safe Drinking Water Act, as amended in 1986 (USEPA 1986).

Fecal coliform bacteria are a sub-group of total coliform bacteria (that portion of the coliform group which will produce gas from lactose in a multiple tube procedure liquid medium within 24 hours in a water bath maintained at 44.5 °C) that are present in large quantities in the intestines and feces of people and animals. The presence of fecal coliform bacteria in a water sample is often believed to indicate recent fecal contamination. Water-quality criteria for shellfish growing areas based on fecal coliform have been developed by the U.S. Food and Drug Administration under the ***National Shellfish Sanitation Program***. The 2007 guide for the control of molluscan shellfish (U.S. Food and Drug Administration 2009) specifies criteria, based on total coliform and fecal coliform densities, to indicate the sanitary quality of water in shellfish-growing areas.

Fecal streptococci (a group of species of the genus *Streptococcus,* such as *S.faecalis, S.faecium, S.avium, S.bovis, S.equinus,* and *S.gallinarum)* were once used as an indicator of recent fecal contamination and to differentiate the source of fecal contamination based on the speciation of fecal streptococci. However, this approach was proven to be unreliable and the use of fecal streptococci generally has been discontinued for water-quality monitoring (Myers et al. 2007).

Escherichia coli (a subgroup of the fecal coliform group) and **enterococci** are the preferred bacterial indicators today for recreational waters because both are predictors of swimming-associated gastroenteritis. The presence of generic *E. coli* in water almost always indicates recent fecal contamination, and thus a risk that pathogens are present. In 1986, *E. coli* replaced total and fecal coliforms and fecal streptococci as the recommended indicator bacteria. Either *E. coli* or enterococci are recommended for monitoring fresh water, whereas enterococci are the preferred indicator bacteria for marine waters because of their salt tolerance (USEPA 2004). New recreational water quality criteria for *E. coli* and enterococci were promulgated in 2012 (Table 1).

Clostridium perfringens is another bacterium indicating contamination of water with treated or untreated sewage or other wastes. It is used as an alternative indicator of recent fecal contamination in tropical and subtropical waters because other indicator bacteria may regrow.

While FIB monitoring is widely practiced, the validity of the indicator concept has been increasingly questioned (e.g., Harwood et al. 2005). Recent epidemiological studies conducted as part of EPA's ***National Epidemiologic and Environmental Assessment of Recreational Water*** (NEEAR) have supported a strong link between increasing levels of exposure to FIB in recreational waters and increases in gastro-intestinal illness (e.g., Wade et al. 2010). However, the traditional assumption that the presence of FIB denotes **recent** fecal contamination because FIB die off quickly in the environment may no longer be completely valid, as research has documented survival and even regrowth of FIB in manure stocks, soils, biofilms, beach sand, and even stormwater catchbasins (e.g., Marino and Gannon 1991, Jamieson et al. 2002, Yamahara et al. 2009).

Furthermore, FIB are not believed to be reliable indicators for *Cryptosporidium* and *Giardia* in source waters. In comparison with FIB, oocysts and cysts are more resistant to disinfection and survive longer in the environment. Numerous studies have shown that absence of *E. coli* is not necessarily indicative of the absence of all pathogens, especially protozoa (e.g., Boyer and Kuczynska 2003). Outbreaks of cryptosporidiosis and giardiasis, for example, have occurred where water quality standards based on the absence of indicator organisms have been met (Craun et al. 1997).

Thus, while the approach to monitoring for pathogens using FIB has been traditional over the last 80 years, research over the last ten years has shown that relying exclusively on FIB might not be complete. So while use of FIB may be advised or required for determination of compliance with water quality standards, a full pathogen risk assessment might require selection of a different monitoring strategy that focuses on true pathogens.

Protozoa

Protozoa are complex single-celled eukaryotes (organisms whose cells have nuclei) that are mobile, consume food from external sources, and reproduce by fission. Pathogenic protozoans are found in the feces of humans and other warm-blooded animals. They are widely distributed in the aquatic environment and have been implicated in outbreaks of waterborne diseases (Lee et al. 2002, Rose et al. 1997). *Cryptosporidium* and *Giardia* species are the most common protozoan pathogens of concern in U.S. waters. Both organisms produce environmentally-resistant forms (oocysts for *Cryptosporidium* and cysts for *Giardia*) that permit their extended survival in natural and treated waters.

Cryptosporidium parvum is a protozoan parasite that infects many humans, agricultural livestock (cattle, sheep, goats, pigs, and horses), pets, and wildlife species, such as mice, voles, and raccoons (Fayer and Ungar 1986, Fayer 1997). Different species of *Cryptosporidium* are found in mammals, birds, and reptiles. Cryptosporidiosis is a cause of morbidity and mortality in animals and humans, resulting primarily in diarrhea; the most severe infections occur in immune-compromised individuals.

Production of oocysts is generally limited to livestock that are less than 30 days old. Infected humans can shed oocysts at any age. When the oocyst is ingested, sporozoites are released and parasitize the lining cells of the small intestine. Experimental studies in healthy humans determined that the infectious dose at which 50 percent of subjects acquired infection was 132 bovine-derived oocysts, although ingestion of as few as 30 oocysts has been shown to induce cryptosporidiosis (DuPont et al. 1995). The oocyst stage can remain infective for many months under cool, moist conditions where water temperatures in rivers, lakes, and ponds remain low but above freezing.

Studies have shown that *Cryptosporidium* sp. oocysts were present in 39–87 percent of surface water tested throughout the U.S. from 1988 to 1993 (Rose et al. 1991, LeChevallier et al. 1991, LeChevallier and Norton 1995). Groundwater is also impacted; Hancock et al. (1998) found that about 10–20 percent of U.S. groundwater samples tested positive for *Cryptosporidium*. Numerous reports of outbreaks of cryptosporidiosis related to drinking water in North America, the United Kingdom, and Japan indicate that water is a major vehicle for transmission of cryptosporidiosis.

Giardia is a genus of flagellated protozoa frequently found in rivers and lakes that infects the intestinal tract of mammals, such as humans, dogs, cats, bears, muskrats, and beaver, as well as some birds, reptiles, and amphibians. Giardiasis is typically characterized by diarrhea, abdominal cramps, bloating, and weight loss. Cysts are shed in feces intermittently, often in large numbers. The infectious dose is low; ingestion of 10 cysts has been reported to cause infection.

Even more widespread than *Cryptosporidium,* most surface water tested has been found to contain *Giardia* cysts (LeChevallier et al. 1991, Wallis et al. 1996). During 2009–2010, 46 U.S. states reported giardiasis cases (Yoder et al. 2012).

Giardia is primarily transmitted through ingestion of infected human or animal waste, either through exposure to fecally-contaminated water or food, through contact with an infected person, or occupational exposure to human waste. Drinking water is an important vehicle for *Giardia* transmission; *G. intestinalis* was the single most frequently identified pathogen in all drinking water outbreaks reported in the U.S. during 1971–2006 (Craun et al. 2010). Untreated drinking water was identified as a risk factor for sporadic giardiasis in studies in the U.S. (Chute et al. 1987). Untreated groundwater appeared to be particularly risky if it was acquired from poorly constructed or maintained wells that might have been subject to surface water contamination (Snel et al. 2009). Treated or untreated recreational water also has been implicated as a vehicle of giardiasis transmission; in studies of sporadic giardiasis, swallowing water while swimming and during other recreational contact with fresh water were both risk factors for contracting *Giardia* (Stuart et al. 2003, Snel et al. 2009). It is believed that most *Giardia* cysts in surface waters and contaminated water supplies are from wildlife and human waste sources, respectively; a link between livestock and human infection has not been conclusively documented (Yoder et al. 2012).

Viruses

Viruses are small infectious agents that can reproduce only inside the living cells of an organism. Most significant virus groups affecting water quality and human health grow and reproduce in the gastrointestinal tract of people and animals. Viruses potentially infective to humans present in animal waste include Hepatitus E virus, Reoviruses, Rotaviruses, caliciviruses (noroviruses), adenoviruses, enteroviruses, and retroviruses. Gastro-intestinal illness associated with swimming is often attributed to human enteric viruses.

More than 100 types of human pathogenic viruses may be present in fecal-contaminated waters, but only a small number of them can be detected by currently available methods (Bushon and Francy 2003). Coliphages (viruses that infect and replicate in coliform bacteria) are used as indicators of fecal contamination and of the microbiological quality of the water. Coliphages are not pathogenic to humans, but they have been suggested as potential indicators of enteric viruses because of their similar structure, transport, and persistence in the environment.

Runoff from spreading of municipal biosolids and manure may be a source of viruses to waterbodies. However, little evidence shows that viruses shed in the excrement of livestock have posed a major waterborne threat to human health in the U.S. (Rosen 2000). Septic tank effluent may be the most significant source of pathogenic viruses in the subsurface environment (CAST 1992).

Sources, Fate, and Transport of Waterborne Microorganisms

Numerous sources of pathogens exist in the environment, all associated with human and animal wastes. Complex pathways for their distribution are common.

Sources of pathogens and indicators

Agriculture is recognized as a major cause of water quality impairment based on indicator bacteria standards (USEPA 2012b) and may under some circumstances contribute microbial pathogens to water resources. Major agricultural sources of microorganisms include:

- **Animal feeding operations** are potential sources of pathogens and indicators including *E. coli* O157:H7, *Cryptosporidium*, and *Giardia*. Bare areas, such as open lots with heavy animal traffic, have the greatest potential for pathogen runoff into surface water. Direct deposit into streams where livestock have free access to waterways is also an obvious source. When not properly stored and managed, accumulations of manure and associated facility wastewater represent potent sources of microorganisms if a runoff event or discharge occurs.

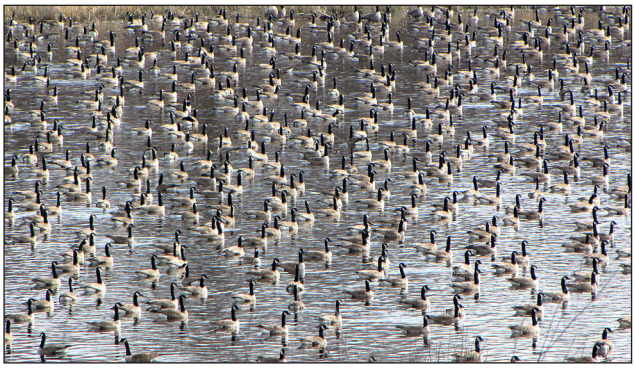
- **Land applied manure** can represent a major reservoir of microorganisms distributed across the landscape and available for loss to surface and ground water resources. While manure application can be managed to prevent much of the potential loss of pathogens and indicators (e.g., by managing application rates and forms, incorporation into the soil, and use of buffers and setbacks between fields and waterways), poorly managed manure applications (e.g., application at excessive rates, onto saturated or frozen soils, or without soil incorporation) can result in significant pathogen and indicator losses to surface and ground water. Manure applied to pasture land by grazing cattle—especially if cattle have direct access to waterways—can also yield high microbial loads to waterways.

Human wastes are obvious sources of pathogens of direct concern for human health. Potential sources of contamination include:

- **Treated wastewater** is generally disinfected before discharge by chlorination or other processes. However, some microorganisms such as *Cryptosporidium* are resistant to chlorine disinfection and may be discharged to receiving waters with wastewater effluent.
- **Septic system** effluent may be a significant source of pathogenic bacteria and viruses in the subsurface environment (CAST 1992). In the mid-1980s, overflow or seepage from septic tanks and cesspools was responsible for 43% of the reported outbreaks and 63% of the reported cases of illness caused by the use of untreated ground water in the U.S. (Craun 2010).
- **Urban runoff** from municipal combined sewer overflows, storm sewers, parking lot and impervious surfaces runoff, highway and road runoff, and permitted stormwater discharges may be a source of contamination.
- **Land application of biosolids** may represent a concentrated source of pathogens unless adequately treated and handled. Many pathogens can survive sewage treatment and some pathogens are adsorbed to particles that remain with the sludge during sedimentation processes. The class of biosolids directly affects the likely pathogen load. Class A biosolids are treated to reduce pathogens below detection levels, but Class B biosolids have received lesser treatment that may not completely eliminate pathogens. When farmers use Class B sludge, they are advised to avoid direct human contact or inhalation of dust or spray during and after application. The EPA should be consulted for a detailed review of regulations and technologies of pathogens in biosolids (USEPA 2003).

Pet wastes can be an important source of microbial contamination, especially in developed areas. Pets provide a potential reservoir for a number of pathogens including *Giardia* sp., *Cryptosporidium parvum,* and *Salmonella* sp. Dogs and cats release waste in yards and walking areas often adjacent to streams that are subject to direct runoff. Management of pet waste among owners is extremely variable, ranging from careful collection to complete neglect.

Wildlife, including mammals and birds, act as pathogen reservoirs. They are dispersed across forest land, idle land, pastureland, cropland, and the urban landscape. Their wastes most commonly enter surface water, although leaching to ground water can occur. Wildlife can contribute pathogenic microbes, such as *Salmonella* sp. and *E. coli* as well as large numbers of indicator organisms. The high density activities of these animals close to or in water provide little opportunity for terrestrial die-off of organisms during their lifecycle.



Transport of microorganisms

Wastes and associated microorganisms may be introduced into surface or ground water through direct discharges, livestock grazing, wildlife activity, and accidental spills. Introduction of wastes and associated microorganisms may also occur through transport from their source to waterways by overland or subsurface flow. Bacteria transport in field runoff is associated with both direct entrainment of organisms in overland flow and transport of sediments onto which microorganisms have been attached. Some reports have compared microbial detachment from the soil surface to soil erosion and sediment transport, while other reports have described microorganism release from land-applied manure as similar to the release of dissolved chemicals. As protozoa are generally in a size class between clay and silt, for example, their movement in runoff may be more comparable to particulate detachment and transport than to solution movement, and therefore should be considered as part of the particulate load. Regardless of the mode of transport, hydrology is an important driver of pathogen transport in surface runoff. Like most other pollutants, a strong positive association between flow and bacteria numbers is reported in most NPS situations.

Pathogen and indicator organism numbers reported in urban runoff may be due as much to hydrology as to the magnitude of sources. Stormwater systems—especially older systems where rapid collection and transport of stormwater was the design—are very efficient at moving available microorganisms—especially those deposited on impervious surfaces.

While soils can be effective filters for microorganisms, the existence of macropores, relatively large channels in soil resulting from worm-holes, voids left by decayed plant roots, etc., can allow pathogens and indicator organisms to bypass soil filtration. Significant movement of microorganisms through macropores into tile drainage and ground water has been documented from cropland receiving manure (especially high rates of liquid manure applied to reduced-tillage cropland) (Jamieson et al. 2002) and from grazing land. High bacteria counts have been observed in tile drainage from cropland receiving heavy manure application. In properly functioning septic system drain fields, bacterial movement is expected to be very slow, primarily due to formation of an organic mat at the soil boundary. However, in failed systems under saturated conditions or in systems installed in unsuitable soils, bacteria can move far more rapidly and reach ground waters.

Survival/die-off factors

One distinctive feature of microbial pathogens and indictors is that many species have limited survival in the environment, once removed from their hosts. The principal environmental factors that promote die-off of microorganisms (especially bacteria) include heat, sunlight (UV radiation), desiccation, and predation by native microorganisms. Other factors including aerobic conditions, freeze-thaw cycles, and low nutrient concentrations

have also been suggested as factors promoting microorganism die-off. High temperatures combined with low moisture appear to yield the highest die-off rates for bacteria. Decay in bacterial populations generally follows first-order kinetics:

$$N_t/N_0 = 10^{-kt}$$

where

N_t = number of bacteria at time t

N_0 = number of bacteria at time 0

t = time in days

k = first order or die-off rate constant

Reported values for k range from about 0.17–0.70/d for *E. coli* and 0.04–0.47/d for fecal coliform. This suggests, for example, that the time required for 90% of *E. coli* to die-off would be on the order of 1.5 to 6 days, depending on ambient conditions.

Protozoa such as *Cryptosporidium* and *Giardia* produce environmental resistant forms (oocysts and cysts, respectively) that can tolerate temperature and moisture extremes and thus can survive considerably longer in the environment than can bacteria. Reported values for first-order decay coefficients (k) for *Cryptosporidium* are in the range of 0.006–0.008/d, but some oocysts may remain viable and infective for more than a year.

In agricultural settings, several important factors come into play regarding survival of microbial pathogens and indicators:

- **Waste storage:** Reductions of bacteria numbers of two to three orders of magnitude have been reported with passive manure storage for two to six months (Patni et al. 1985, Moore et al. 1988, Meals and Braun 2006). Thus, application of stored manure could be expected to introduce significantly fewer bacteria to agricultural land when applied to cropland than would fresh manure. Manure treatment (e.g., composting, digestion, chemical amendment) can further reduce bacteria loads in manure (Topp et al. 2008). Dynamics of protozoa and viruses in manure are considerably less well-documented.
- **Land application:** Microorganisms in surface-applied wastes may be exposed to high temperatures, desiccation, UV light, and other stresses, and experience significant die-off after application. Incorporating wastes into the soil by tillage may enhance survival of microorganisms because they are sheltered from lethal stresses, but incorporation also removes microorganisms from interaction with surface runoff and places fecal microorganisms in an environment where predation by soil organisms can further reduce their numbers. Liquid manure applied to hay land can be deposited on vegetation, where desiccation, high temperatures, and exposure to light may kill microorganisms. Soils can effectively remove

microorganisms from percolating water by adsorption, filtration, and predation. In livestock pastures, fecal bacteria appear to survive for long periods (e.g., ≥100 days) in cowpies, which remain a potential source for microorganisms in pasture runoff.

Once delivered to a surface water body, microorganisms are subject to additional environmental stressors. Pathogenic bacteria generally are not well suited to aquatic systems, as native bacterial flora outcompete them for nutrients. Many small protozoa feed on bacteria, including pathogens, and many invertebrates feed on both bacteria and protozoa in a waterbody. High temperatures and exposure to UV light will be lethal to fecal microorganisms. Once in a waterbody, microorganisms often become adsorbed to organic matter and soil particles. These settle out and accumulate at the bottom of rivers and lakes, although they may become a source of organisms if resuspended.

Monitoring Issues

Monitoring design

Overall watershed project monitoring design considerations for microbial pathogen and indicator monitoring do not differ fundamentally from those for other NPS pollutants (see Tech Note 2, ***Designing Water Quality Monitoring Programs for Watershed Projects***).

Monitoring must be designed to meet the main goals of the project. For NPS watershed projects, these goals may include documenting pre-implementation water quality conditions, measuring changes in water quality in response to implementation of management practices, or measurement of pollutant removal efficiencies of specific BMPs. When evaluating the effectiveness of watershed projects, the emphasis should be on testing a hypothesis rather than estimating parameters. The goal for the monitoring design would be to test the null hypothesis (e.g., that *E. coli* counts will not change between pre-implementation and post-implementation periods) and, if the null hypothesis is rejected, to conclude with some level of confidence that a change occurred. Monitoring the variables and the locations where a response is anticipated and monitoring close to the impaired or treated area will usually help collect the data necessary to test a hypothesis.

Key elements of designing a monitoring project include:

- Selecting a statistical design, e.g.,
  - — Upstream/downstream,
  - — Paired watersheds,
  - — Trend monitoring;
- Choosing sample type (e.g., grab vs. flow-proportional) ;
- Sample timing; and
- Sampling frequency.

When monitoring is used specifically for pathogen source assessment—especially when combined with MST—a synoptic survey can be a particularly useful design. Because of requirements for sterile sampling procedures, grab sampling is the most common approach for sampling FIB or pathogens. Timing of sample collection for FIB may be tied to known or suspected seasonal patterns or to compliance monitoring requirements for dry vs. wet-weather sampling.

The extreme variability that characterizes the occurrence of microbial pathogens and indicators in the environment demands special consideration with respect to monitoring frequency and timing. True pathogens (e.g., *Cryptosporidium*, *E. coli* O157:H7) are likely to occur only sporadically; monitoring for these organisms must be tied to likely sources such as livestock facilities as they occur in space (e.g., CAFOs) and time (e.g., calving schedules). Even for FIB, significant spatial, temporal, and hydrologic variability must be recognized. Figure 1 shows a plot of more than five years of weekly *E. coli* data from a Vermont agricultural watershed. Bacteria counts varied over five orders of magnitude and showed a strong seasonal pattern, driven by both weather and agricultural management. Sampling frequency would need to account for this extreme variability to achieve effective monitoring for change or trend (see Tech Note 7, ***Minimum Detectable Change Analysis***).

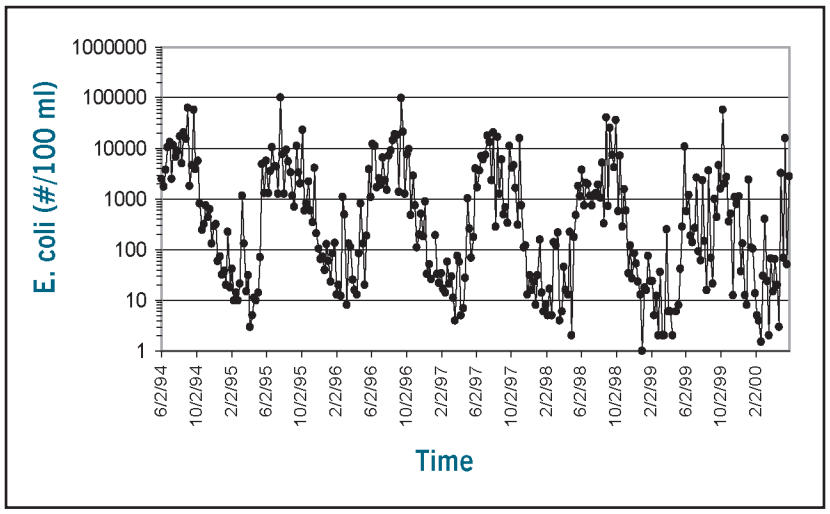


Figure 1. Five years of weekly *E. coli* data from a Vermont agricultural stream. (Meals 2001)

Even though all NPS pollutants generally show significant hydrologic variability and positive association with flow, this may be particularly important for pathogen monitoring, especially in urban stormwater systems where flows are very flashy. Moreover, water quality standards for FIB (e.g., for recreation and shellfishing) may require a certain level of monitoring effort in wet weather vs. dry weather or a certain number of samples over a specified time period to calculate a geometric mean. Beach sampling may require specific monitoring designs that account for temporal (storm event, tidal, seasonal, use-intensity) and spatial (depth, site features, shoreline location) variations (USEPA 2002).

Sample collection

Specific sample collection techniques should be selected based on the requirements of the organism(s) of interest. Some key principles for collecting microbiological samples include:

Sample Collection Guidance

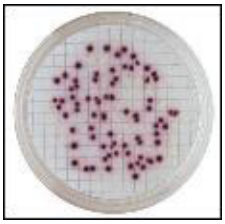
- U.S. Geological Survey. 2008. *Techniques of Water-Resources Investigations, Chapter A7, Biological Indicators*
- The Ocean Conservancy and USEPA. 2006. *Volunteer Estuary Monitoring: A Methods Manual*
- USEPA. 2010. *National Coastal Condition Assessment Field Operations Manual*

- Sterile technique must be followed and documented when collecting and processing samples for fecal indicator bacteria. This includes sterilizing sampling equipment and supplies with heat, chemical, or other treatment prior to sampling and decontaminating sampling equipment between site visits.
- Procedures for collecting representative samples from a waterbody are generally the same as for physical and chemical constituents except that sample containers are not to be field rinsed with native water, but should be autoclaved or otherwise sterilized before use.
- Higher sample volumes may be required for microbial pathogens than for other constituents. Analysis for protozoa, for example, generally requires 10 L or more of sample. This requirement may be challenging for sampling technology.
- Sterile conditions must be maintained during storage, transport, and analysis of microbiological samples.
- Holding times (at 1–4 °C) between sample collection and analysis are generally shorter than those for chemical constituents:
  - From 6 hours for FIB in nonpotable water to 30 hours for FIB samples collected from drinking water sources (Myers et al. 2007, USEPA 2013)
  - ≤ 96 hours for samples collected for protozoa analysis (Bushon and Francy 2003, USEPA 2013)
  - ≤ 48 hours for coliphage and enteric virus analysis (Bushon 2003)

Analytical methods

FIB are routinely cultured and enumerated by two general methods (Myers et al. 2007):

- Membrane-filtration (MF) and liquid broth; and
- Enzyme substrate tests (e.g., USEPA-approved IDEXX Colilert® and Enterolert®)



Membrane filter culture methods typically report results as bacteria density (#/100 mL or colony forming units (cfu)/100 mL). Enzyme substrate tests using multiple-well plates report results as most probable number (MPN)/100mL. Most state agencies and commercial water-testing laboratories are able to conduct routine analysis for FIB at a relatively low cost.

Because they cannot be cultured, the presence of protozoan pathogens in water must be verified by identification of the pathogens themselves. *Cryptosporidium* and *Giardia* are

generally analyzed in water using USEPA Method 1623 (USEPA 2005b). Method 1623 must be performed in a certified laboratory by a qualified analyst, and involves the following steps:

1. **Filtration** — *Cryptosporidium* oocysts and *Giardia* cysts from the water sample are concentrated on a filter, eluted from the filter with an elution buffer, and reconcentrated by centrifugation.
2. **Immunomagnetic separation (IMS)** — The oocysts and cysts are magnetized by attachment of magnetic beads conjugated to antibodies and then separated from extraneous materials in the sample with a magnet.
3. **Immunofluorescence assay (FA)** — Fluorescently labeled antibodies and vital dye are used to make the final microscopic identification of the oocysts and cysts. The organisms are identified by microscopy when the size, shape, color, and morphology agree with specified criteria.

Laboratory analysis for protozoa requires a specialized microbiology laboratory and involves considerably higher costs than those for FIB. It should be noted that most laboratory analysis for *Cryptosporidium* and *Giardia* focus on drinking water analysis, and samples of other matrices (e.g., manure, soil, highly turbid runoff) may present major challenges to many labs.

Analysis for viruses in environmental water samples focuses on coliphages analyzed by one of two techniques (USEPA 2005b):

- USEPA Method 1602, a single-agar layer (SAL) plaque assay method recommended for surface water samples; and
- USEPA Method 1601, a two-step enrichment method that determines presence/absence of coliphages, and is recommended for ground water samples.

Laboratory analysis for viruses also requires a specialized microbiology laboratory and involves high costs compared to FIB analysis.

Other more advanced techniques of molecular biology are also used to analyze samples for waterborne pathogens and indicators. These are discussed under Microbial Source Tracking.

Microbial Source Tracking

Unlike monitoring for most chemical pollutants, microorganism monitoring can provide insight into pollution sources through the tools of molecular biology. The term "Microbial Source Tracking" — also referred to as "genetic fingerprinting" — refers to procedures that use host-specific (i.e., found only in one host species or group) or host-associated (i.e., largely confined to one host species or group) microbial indicators to establish the origin of fecal pollution in water. MST is based on the principles that some microorganisms have an exclusive or preferential association with a particular host, and that these

host-associated microorganisms are shed in feces and can be detected in water bodies (Hagedorn et al. 2011, USEPA 2005a).

MST methods are distinguished by whether they use genotypic vs. phenotypic analysis and whether they use cultivated target organisms or conduct direct analysis of environmental samples. There is no widespread consensus among researchers or regulatory agencies regarding the best methods for MST; each approach has its own advantages and limitations. MST approaches fall into two general groups:

Library dependent methods (LDMs)

LDMs require the development of databases of **genotypic** (genetic makeup) or **phenotypic** (observable physical or biochemical characteristics) fingerprints for bacterial strains isolated from suspected fecal sources. Fingerprints of isolates from contaminated water are compared with these libraries for classification. The LDM approach is based on the hypotheses that certain characteristics of fecal bacteria are associated with specific animals or groups of animals, that these characteristics in environmental strains are similar to those found in host groups, and that the relative proportion of the identifying characteristic remains constant in the environment over time.

The majority of LDMs use characteristics of fecal indicator bacteria, most commonly *E. coli*, which can link source tracking results to the routinely monitored bacteria used in water quality standards. In order for the source of environmental isolates to be correctly identified, it is essential that the library be large and diverse enough and also contain a set of profiles representative of all the potential animal sources in a particular watershed; at the same time, the library must be geographically constrained enough to be applicable to the study area. One advantage of LDMs is that the work can be tailored specifically to a watershed based on the sources actually present.

Some common LDMs for MST include:

Phenotypic Typing Methods

- **Antibiotic Resistance Analysis** (ARA) relies on bacterial resistance to antimicrobials to distinguish sources of fecal bacteria. The theory behind this method is that normal gut flora from different animal hosts are exposed to antibiotics in varying degrees and will develop resistance to antibiotics over time due to selective pressure. Patterns of resistance can be determined for isolates from different animal groups, which can then be used to identify sources of fecal pollution. ARA is generally much less expensive and technically demanding than genotypic library-dependent methods. These techniques can distinguish multiple sources, including human and domestic animals.

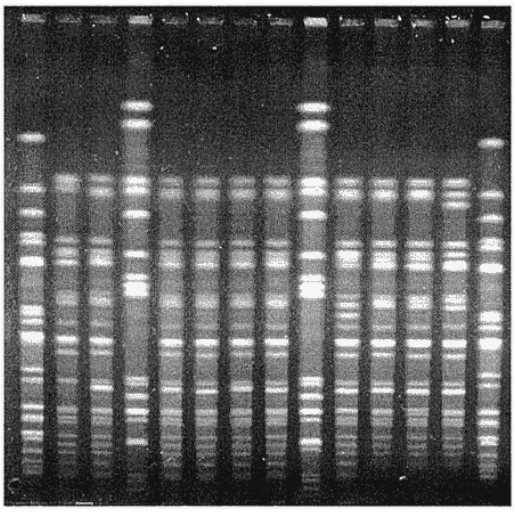
- **Biochemical Fingerprinting** (e.g., carbon source utilization) is based on measuring the ability of bacteria to metabolize specific carbon and nitrogen substrates. Compared to molecular methods, this method is rapid, requires

less technical expertise, and uses commercially available supplies. However, its application in field studies has not yet been widespread.

Genotypic Methods

- **Pulsed-Field Gel Electrophoresis (PFGE)** is considered to be one of the best methods for biochemical and molecular typing among molecular MST methods and is widely used to identify bacteria implicated in disease outbreaks. PFGE uses the entire DNA genome for identification. The method is highly sensitive and accurate, but both costly and technically demanding and requires an extensive library.

- **Ribotping** has been one of the most widely used LDM applications. Ribotyping is based on the detection of genetic differences in the genomic sequences of ribosomal RNA. Although highly accurate, ribotyping is technically demanding, expensive, and labor intensive.



PFGE typing of *Shigella*

Many of the library-dependent techniques use typical FIB species, thus offering direct comparison with commonly monitored indicators. However, LDMs have some serious disadvantages that increasingly discourage their use; these disadvantages focus on the large and uncertain size of the library required for source identification (Sargeant et al. 2011). LDMs are based on the belief that specific bacterial strains are associated with specific animal species. However, recent studies suggest that subspecies of *E. coli* change significantly with respect to geography, time, and habitat. Thus, a library would need to contain a very large number of isolates to account for this variability. Furthermore, most *E. coli* and *Enterococcus* strains have been found to occur in many host species (Stoeckel and Harwood 2007). Very large libraries tend to contain large proportions of these cosmopolitan strains, reducing the specificity of source identification.

Library independent methods (LIMs)

LIMs do not depend on the isolation of targeted source identifiers because identification is based on detection of specific DNA or other genetic markers in isolates from contaminated water. This technique can be applied to both bacteria and viruses. LIMs are based on the selective detection of source-specific bacterial populations (through direct culture/enumeration or analysis of nucleic acids). Methods looking at genetic material often use polymerase chain reaction (PCR), a biochemical technology that amplifies a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence to facilitate identification. Specific PCR applications may be qualitative (i.e., presence/absence) or quantitative

(qPCR) (i.e., measuring the magnitude of the source) in the detection of a host-associated target organism or gene.

Some common LIMs include:

- **Cultivation of different species/groups.** For example, *Rhodococcus coprophilus* has been proposed as a specific indicator for farm animal fecal contamination. Its natural habitat is herbivore waste on which the bacterium proliferates and can be found in high numbers.

- **Combining cultivation with genetic marker detection.** Application of PCR techniques can detect bacterial genes for toxin production that are specific to source type. For example, analysis of the 16S rRNA gene can detect the presence of bacteria specific to individual source groups. The group *Bacteroidales* is the most widely used taxon targeted for source identification for livestock (pigs, cattle, sheep, horses, and chicken) and domestic pets (dogs and cats), while *Bacteroides* is considered the predominant genus of human fecal bacteria.

- **PCR analysis of viral markers.** Because enteric viruses exhibit a high degree of host specificity, viruses are increasingly used as species-specific water quality indicators.

The principal advantage of LIM methods is that they do not require development of a library database; this saves time and resources. Furthermore, the techniques that do not require culture of microorganisms can be quite rapid (i.e., completed and reported in the same day in which sampling occurred) (Sargeant et al. 2011). Several drawbacks of LIM techniques should be mentioned. One of the major limitations of library-independent techniques is the lack of techniques for host species beyond humans and a few domestic animal species. Second, non-cultivation methods like PCR measure microbial genetic material and do not distinguish between living and dead organisms. Thus, such assessments may overestimate infectious microorganism levels. Second, no single genetic marker can be 100% specific and sensitive at the same time and each assay has its own bias. Probabilistic statistical models must be used to evaluate genetic marker data.

Applications of MST

MST can be used to identify sources of FIB impairments (e.g., human, livestock, wildlife) as part of watershed planning and prioritization at the program level. In pathogen TMDL development, MST has been used during source assessment to supplement the identification and characterization of FIB sources developed through a watershed inventory (Hagedorn et al. 2011). Where multiple sites have been monitored within a watershed over time, MST can be used to identify spatial and temporal trends that may link with specific sources or source-specific characteristics that influence bacterial fate and transport. Because MST data are not yet sufficiently quantitative to provide accurate and defensible estimates of the relative loadings of fecal contamination from potential

upland sources, the most appropriate use of MST data in TMDLs is to supplement source assessment data collected in more traditional ways, assist in load partitioning, and corroborate water quality model results (Hagedorn et al. 2011).

Finally, application of molecular-based MST methods has raised the prospect of direct monitoring for pathogens in surface waters, rather than using indicator organisms. Experimental application of PCR-based technology has successfully detected the presence of *Salmonella* spp., *Campylobacter* spp., different pathogenic strains of *E. coli*, protozoan parasites, and enteroviruses (Hagedorn et al. 2011). MST is, however, an evolving science and until such time as routine direct measurements of pathogen presence is possible, MST should be viewed as one of a suite of methods for microbiological assessment, including watershed characterization, sanitary surveys, and synoptic sampling.

MST can be an effective tool for water-quality management if used judiciously and with a clear understanding of the benefits and limitations of the specific method(s) employed. MST technology is a rapidly evolving field and watershed project managers should consult sources of technical expertise in the field before selecting an approach for a specific application. Those wishing to apply MST to TMDL development or other watershed projects should consult ***Microbial Source Tracking Guide Document*** (USEPA 2005a) and ***Using Microbial Source Tracking to Support TMDL Development and Implementation*** (USEPA 2011).

Balancing the cost of MST versus traditional monitoring

Microbial source tracking can be expensive and time-consuming in comparison to traditional FIB monitoring. And sometimes MST results that conflict with pre-conceived notions of pollution sources are not trusted by the public. Hartel (2011) recommends a common-sense approach to pathogen source identification that combines **local knowledge** and **existing data** with a general survey of the waterway to identify sources of fecal bacteria in contaminated waterways without any MST testing at all. If sources of fecal contamination are still unclear, then targeted sampling in a synoptic survey can identify hot-spots of microbial contamination. Volunteer monitoring efforts can be highly useful in harnessing local observations of possible pathogen sources like animal concentrations, wildlife scat, and failed treatment systems.

Additional Resources

Boehm A.B., L.C. Van De Werfhorst, J.F. Griffith, P.A. Holden, J.A. Jay, O.C. Shanks, D. Wang and S.B. Weisberg. 2013. Performance of forty-one microbial source tracking methods: A twenty-seven lab evaluation study. *Water Res*. 2013 Jul 5. doi:pii: S0043-1354(13)00549-6. 10.1016/j.watres.2012.12.046. [Epub ahead of print]

Hagedorn, C., A.R. Blanch, and V.J. Harwood, eds. 2011. *Microbial Source Tracking: Methods, Applications, and Case Studies*. Springer, NY, NY. 644 p. DOI 10.1007/978-1-4419-9386-1. ***http://www.springer.com/life+sciences/microbiology/book/978-1-4419-9385-4*** (Accessed 5-27-2013).

Rosen, B.H. 2000. Waterborne Pathogens in Agricultural Watersheds. Watershed Science Institute, Natural Resources Conservation Service, US Department of Agriculture. *ftp://ftp-fc.sc.egov.usda.gov/WSI/pdffiles/Pathogens_in_Agricultural_Watersheds.pdf* (Accessed 3-4-2013).

Sargeant, D., W.R. Kammin, and S. Collyard. 2011. Review and Critique of Current Microbial Source Tracking (MST) Techniques. Environmental Assessment Program, Washington State Department of Ecology, Olympia, WA. *https://fortress.wa.gov/ecy/publications/publications/1103038.pdf* (Accessed 8-20-2013).

Stoeckel, Donald M. and V J. Harwood. 2007. Performance, Design, and Analysis in Microbial Source Tracking Studies. *Appl. Environ. Microbiol.* 73: (8): 2405-2415.

USEPA. 2002. National Beach Guidance and Required Performance Criteria for Grants. EPA-823-B-02-004. Office of Water, Washington, DC. *http://water.epa.gov/grants_funding/beachgrants/upload/2008_11_24_beaches_grants_guidance_all.pdf* (Accessed 3-7-2013).

USEPA. 2005a. Microbial Source Tracking Guide Document. Office of Research and Development, Washington, DC. EPA-600/R-05/064. 131 p. *http://cfpub.epa.gov/si/si_public_record_Report.cfm?dirEntryID=133523* (Accessed 3-7-2013).

USGS. 2008. Biological Indicators. Chapter A7, *National Field Manual for the Collection of Water-Quality Data*. F.D. Wilde, ed. U.S. Geological Survey Techniques of Water Resource Investigations, Book 9. *http://pubs.water.usgs.gov/twri9A/* (Accessed 3-7-2013).

USEPA. 2011. Using Microbial Source Tracking to Support TMDL Development and Implementation. Prepared for U.S. Environmental Protection Agency, Region 10, Watersheds Unit by Tetra Tech, Inc. and Herrera Environmental Consultants, Seattle, WA. *http://www.epa.gov/region10/pdf/tmdl/mst_for_tmdls_guide_04_22_11.pdf* (Accessed 8-20-2013).

FIB Monitoring Case Study
Vermont Agricultural Watersheds NNPSMP Project

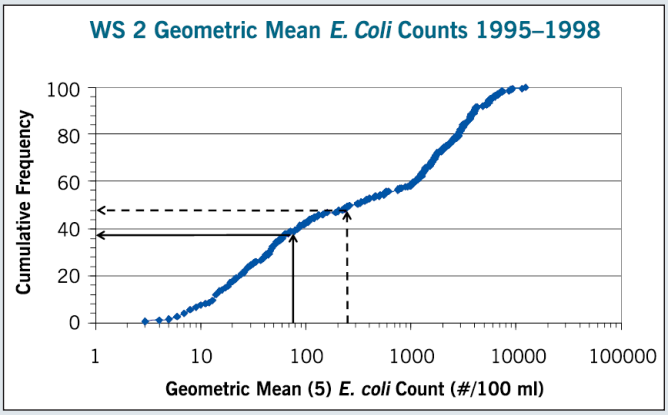
Setting:
The study was conducted in three small (690–1422 ha) agricultural watersheds in the Missisquoi River watershed within the Lake Champlain Basin in northern Vermont. The designated uses of many of the streams in the region are impaired for recreation by agricultural NPS pollution, particularly phosphorus, indicator bacteria, and organic matter. The study watersheds were selected to be representative of dairy agricultural watersheds within Vermont's Champlain Valley.

Objective:
The overall goal of the project was to evaluate the effectiveness of grazing management, livestock exclusion from streams, and streambank protection as tools for control of agricultural NPS pollution in small agricultural watersheds in the Lake Champlain Basin. Project objectives included:

- Implement practical, low-tech practices to protect streams, streambanks, and riparian zones from livestock grazing;
- Determine changes in concentrations and loads of NPS pollutants—sediment, nutrients, and fecal indicator bacteria—at watershed outlets in response to treatment.

Monitoring Methods:
The study was conducted from 1994–2000 using a paired watershed design that included one control watershed and two treatment watersheds. Streamflow was continuously monitored and automated flow-proportional composite samples for total suspended solids, total phosphorus, and total Kjeldahl nitrogen were collected weekly at fixed stations located at watershed outlets. Grab samples for fecal coliform and *E. coli* indicator bacteria were collected at the monitoring stations twice weekly, and transported on ice to the laboratory within 3 hours of collection. Fecal coliform and *E. coli* bacteria were cultured and enumerated by the APHA 9222-D and USEPA 1103.1 membrane filtration methods, respectively.

Results:
Pre-treatment FIB data were used to document water quality impairment in the streams, which were added to the Vermont 303(d) list. Following treatment, paired-watershed analysis of weekly mean FIB counts showed that mean weekly fecal coliform counts declined by 38 to 46%, and mean weekly *E. coli* counts declined by 29 to 40% in the two treatment watersheds. This response occurred within the first year following treatment, suggesting that much of the change could be attributed to immediate prevention of manure deposition in the stream. Frequency distribution of *E. coli* data from the seven-year database was used in an analysis of the impact of potential changes in Vermont water quality standards for *E. coli* by the Vermont Department of Environmental Conservation. This analysis showed that proposed changes in standards would not result in significant reductions of documented impairments.



Source:
Meals, D.W. 2001. *Lake Champlain Basin agricultural watersheds section 319 national monitoring program project, final project report: May, 1994–September, 2000*. Vermont Dept. of Environmental Conservation, Waterbury, VT, 227 p.

MST Case Study
Lake Granbury, TX

Setting:

Lake Granbury, a 48-km long lake, provides drinking water for 150,000 residents. Because monitoring showed consistently high *E. coli* levels located in shallow coves with high-density housing that rely on septic systems, most of the bacteria were believed to come from human sources. Most septic systems were installed prior to current regulations in soils not well-suited for leach fields. The shoreline is densely populated and it was not uncommon to run lateral lines in the lake bed and to use 55-gallon drums as septic tanks.

Objective:

Although the primary sources of fecal pollution seemed obvious, bacterial source tracking studies were undertaken to identify the likely human and animal sources of fecal pollution in Lake Granbury. The aims of the studies included aiding the development of watershed protection plans and providing scientific evidence for informed water treatment infrastructure decision-making. The main goal was to help protect surface water resources and reduce public health risks.

MST Methods:

Several MST tools were employed to identify the likely sources of fecal pollution to Lake Granbury, including: *E. coli repetitive-sequence* polymerase chain reaction (ERIC-PCR) and RiboPrinting using a state-wide library of genetic fingerprints, library-independent *Bacteriodales* PCR, and *Methanobrevibacter smithii* and human polyomavirus PCR for the detection of human fecal pollution.

Lake Granbury water samples were collected monthly for 6 months from selected sites for *E. coli* detection using USEPA Method 1603, *Bacteroidales* analysis, and detection of human polyomavirus and *Methanobrevibacter smithii*. Known source fecal samples collected from wildlife, domestic septage/sewage, pets, and livestock from the study area were used to evaluate the distribution of *Bacteroidales* host-specific markers in the watershed. A total of 94 different human and animal fecal samples were analyzed for the presence of *Bacteroidales* markers: 28 samples from livestock, 36 samples from wildlife, 16 samples from domestic human sewage, and 14 samples from pets. A total of 80 *E. coli* isolates were obtained from 59 of the Lake Granbury human and animal fecal samples: 21 isolates from 17 human sewage samples, 8 isolates from 7 livestock samples, 48 isolates from 33 wildlife samples, and 3 isolates from 2 pet samples.

E. coli isolates from water and source samples were DNA-fingerprinted using ERIC-PCR. For source samples, ERIC-PCR was used to identify unique *E. coli* isolates from each sample. Following ERIC-PCR analysis, *E. coli* water isolates and selected source isolates were RiboPrinted using the automated DuPont Qualicon RiboPrinter, which uses standardized reagents and a robotic workstation, providing a high level of reproducibility.

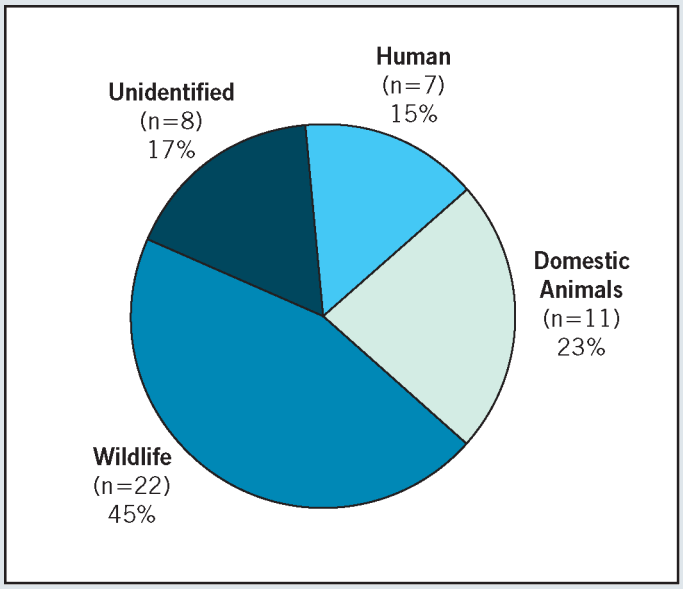
Genetic fingerprints of *E. coli* from ambient water samples were compared to fingerprints of known source *E. coli* isolates in the Texas *E. coli* MST library. The Texas library consists of 1,190 *E. coli* isolates from 1,063 different human and animal fecal source samples. Host sources were divided into three groups: (1) human, (2) domestic animals (including livestock and pets), and (3) wildlife (including deer and feral hogs). Composition and rates of correct classification for the Texas *E. coli* MST library (ver. 1–10) used in this study were in the 80–90% range.

MST Case Study: **Lake Granbury, TX** *(continued)*

Library-independent methods (PCR and qPCR) were used to analyze genetic markers of *Bacteroides* and *Prevotella* spp., fecal bacteria that are associated with humans, ruminants (including cattle and deer), and pigs.

Results:

E. coli and *Bacteroidales* MST results suggested that Lake Granbury was impacted primarily by animal-derived (wildlife) fecal pollution These findings were surprising because it was assumed that the site was highly impacted by human fecal pollution from leaking septic systems. 45% of the *E. coli* isolates were identified as originating from wildlife sources, while only 15% were identified originating from human sources. Further, none of the water samples were positive for the *Bacteroidales* human marker, while all were positive for the ruminant marker.



E. coli source identification for the Lake Granbury Port Ridglea East site. The number of water isolates identified in each source category is included in parentheses. The *E. coli* long-term geometric mean at this site is high (120 MPN/100 mL).

Intensive follow-up sampling (base flow) again confirmed the presence of animal fecal pollution and the absence of human source pollution, despite some of the samples having *E. coli* levels up to 2,400 cfu/100 mL. The *Bacteroidales* ruminant marker was detected in 17 of the 20 (85%) follow-up samples, the hog marker was detected in five (25%) of the samples (presumably from feral hogs in the watershed), while all samples tested negative for the human marker. In addition, only one of the follow-up water samples tested positive for human polyomavirus, and none tested positive for human *M. smithii*.

Source:

Hagedorn, C., A.R. Blanch, and V.J. Harwood, eds. 2011. *Microbial Source Tracking: Methods, Applications, and Case Studies*. Springer, NY, NY. 644 p. DOI 10.1007/978-1-4419-9386-1.
http://www.springer.com/life+sciences/microbiology/book/978-1-4419-9385-4

References

Boyer, D.G., and E. Kuczynska. 2003. Storm and seasonal distributions of fecal coliforms and *Cryptosporidium* in a spring. *J. Am. Water Resour. Assoc.* 39(6):1449-1456.

Bushon, R.N. 2003. Fecal indicator viruses: U.S. Geological Survey Techniques of Water-Resources Investigations, book 9, chap. A7 (3d ed.), section 7.2. ***http://pubs.water.usgs.gov/twri9A/*** (Accessed 3-4-2013).

Bushon, R.N., and D.S. Francy. 2003. Protozoan pathogens: U.S. Geological Survey Techniques of Water-Resources Investigations, book 9, chap. A7 (3d ed.), section 7.3. ***http://pubs.water.usgs.gov/twri9A/*** (Accessed 3-7-2013).

Chute, C.G., R.P. Smith, and J.A. Baron. 1987. Risk factors for endemic giardiasis. *Am. J. Public Health* 77:585–7.

Council for Agricultural Science and Technology (CAST). 1992. Water quality: agriculture's role, task force report 120. Ames, IA.

Craun, G.F., P.S. Berger, and R. L. Calderon. 1997. Coliform bacteria and waterborne disease outbreaks. *J. Am. Water Works Assoc.* 89(3):96-104.

Craun, G.F., J.M. Brunkard, J.S. Yoder, et al. 2010. Causes of outbreaks associated with drinking water in the United States from 1971 to 2006. *Clin. Microbiol. Rev 23*:507–28.

DuPont, H.L., C.L. Chappell, and C.R. Sterling. 1995. The infectivity of *Cryptosporidium parvum* in healthy volunteers. *N. Engl. J. Med.* 332:855-859.

Fayer, R., and B.L.P. Ungar. 1986. *Cryptosporidium* sp. and cryptosporidiosis. *Microbiol. Rev.* 50:458-483.

Fayer, R. 1997. *Cryptosporidium* and *Cryptosporidiosis.* CRC Press, New York, 251 p.

Hagedorn, C., A.R. Blanch, and V.J. Harwood, eds. 2011. *Microbial Source Tracking: Methods, Applications, and Case Studies*. Springer, NY, NY. 644 p. DOI 10.1007/978-1-4419-9386-1. ***http://www.springer.com/life+sciences/microbiology/book/978-1-4419-9385-4*** (Accessed 5-27-2013).

Hancock, C.M., J.B. Rose, and M. Callahan. 1998. Crypto and *Giardia* in US groundwater. *J. Am. Water Works Assoc.* 90, 58–61.

Hartel P.G. 2011. Minimizing Microbial Source Tracking at All Costs. Ch. 16 in Hagedorn, C., A.R. Blanch, and V.J. Harwood, eds. 2011. *Microbial Source Tracking: Methods, Applications, and Case Studies*. Springer, NY, NY. 644 p.

Harwood, V.J., A.D. Levine, T.M. Scott, V. Chivukula, J. Lukasik, S.R. Farrah, and J.B. Rose. 2005. Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. *Appl. Environ. Microbiol.* 71(6):3163-3170.

Jamieson, R.C., R.J. Gordon, K.E. Sharples, G.W. Stratton, and A. Madani. 2002. Movement and persistence of fecal bacteria in agricultural soils and agricultural drainage water: A review. *Can. Biosyst. Eng.* 44:1.1–1.9.

LeChevallier, M.W., and W.D. Norton. 1995. *Giardia* and *Cryptosporidium* in raw and finished water. *J. Am. Water Works Assoc.* 87:54-68.

LeChevallier, M.W., W.D. Norton, and R.G. Lee. 1991. Occurrence of *Giardia* and *Cryptosporidium* sp. in surface water supplies. *Appl. Environ. Microbiol.* 57:2610-16.

Lee, S.H., D.A. Levy, G.F. Craun, M.J. Beach, and R.L. Calderon. 2002. Surveillance for waterborne-disease outbreaks—United States, 1999–2000: Morbidity and Mortality Weekly Report—Surveillance Summaries, November 22, 2002, 51 (SS08), p. 1–28.

Marino, R.P., and J.J. Gannon. 1991. Survival of fecal coliforms and fecal streptococci in storm drain sediment. Water Res. 25(9):1089-1098.

Meals, D.W. 2001. *Lake Champlain Basin agricultural watersheds section 319 national monitoring program project, final project report: May, 1994-September, 2000.* Vermont Dept. of Environmental Conservation, Waterbury, VT, 227 p.

Meals, D.W., and D.C. Braun. 2006. Demonstration of methods to reduce *E. coli* runoff from dairy manure application sites. *J. Environ. Qual.* 35:1088– 1100.

Moore, J.A., J. Smyth, S. Baker, and J.R. Miller. 1988. Evaluating Coliform concentrations in runoff from various animal waste management systems. Special Report 817, Agricultural Experiment Stations, Oregon State University, Corvallis, OR.

Myers, D.N., D.M. Stoeckel, R.N. Bushon, D.S. Francy, and A.M.G. Brady. 2007. Fecal indicator bacteria: U.S. Geological Survey Techniques of Water-Resources Investigations, book 9, chap. A7, section 7.1 (version 2.0). ***http://water.usgs.gov/owq/FieldManual/Chapter7/index.html*** (Accessed 3-4-2013).

Patni, N.K., H.R. Toxopeus, and P.Y. Jui. 1985. Bacterial quality of runoff from manured and unmanured cropland. *Trans. ASAE* 28:1871–1877.

Rose, J.B., C.P. Gerba, and W. Jakubowski. 1991. Survey of potable water supplies for *Cryptosporidium* and *Giardia. Environ. Sci. Technol.* 25:1393-1400.

Rose, J.B., J.T. Lisle, and M. LeChevallier. 1997. Waterborne Cryptosporidiosis—Incidence, outbreaks, and treatment strategies, in Fayer, R. ed., *Cryptosporidium* and cryptosporidiosis: Boca Raton, Fla., CRC Press, pp. 93–109.

Rosen, B.H. 2000. Waterborne Pathogens in Agricultural Watersheds. Watershed Science Institute, Natural Resources Conservation Service, US Department of Agriculture. ***ftp://ftp-fc.sc.egov.usda.gov/WSI/pdffiles/Pathogens_in_Agricultural_Watersheds.pdf*** (Accessed 3-4-2013).

Sargeant, D., W.R. Kammin, and S. Collyard. 2011. Review and Critique of Current Microbial Source Tracking (MST) Techniques. Environmental Assessment Program, Washington State Department of Ecology, Olympia, WA. ***https://fortress.wa.gov/ecy/publications/publications/1103038.pdf*** (Accessed 8-20-2013).

Snel, S.J., M.G. Baker, V. Kamalesh, N. French, and J. Learmonth. 2009. A tale of two parasites: the comparative epidemiology of cryptosporidiosis and giardiasis. *Epidemiol. Infect.* 137:1641–50.

Stoeckel, Donald M. and V. J. Harwood. 2007. Performance, Design, and Analysis in Microbial Source Tracking Studies. *Appl. Environ. Microbiol.* 73: (8): 2405-2415.

Stuart, J.M., H.J. Orr, and F.G. Warburton. 2003. Risk factors for sporadic giardiasis: a case-control study in southwestern England. *Emerg. Infect. Dis.* 9:229–33.

Topp, E., A. Scott, D.R. Lapen, E. Lyautey, and P. Duriez. 2008. Livestock waste treatment systems for reducing environmental exposure to hazardous enteric pathogens: some considerations. *Bioresource Technol.* 100:5395–5398.

USEPA. 1986. Ambient water quality criteria for bacteria—1986: Washington, D.C., Office of Water Regulations and Standards Division, EPA 440/5–84–002, p. 15.

USEPA. 2002. National Beach Guidance and Required Performance Criteria for Grants. EPA-823-B-02-004. Office of Water, Washington, DC. ***http://water.epa.gov/grants_funding/beachgrants/upload/2008_11_24_beaches_grants_guidance_all.pdf*** (Accessed 3-7-2013).

USEPA. 2003. Environmental regulations and technology: Control of pathogens and vector attraction in sewage sludge. Report No. EPA/625/R-92/013, Revised July 2003, Washington, DC. ***http://www.epa.gov/region8/water/biosolids/pdf/625R92013ALL.pdf*** (Accessed 4-5-2013).

USEPA. 2004. Water Quality Standards for Coastal and Great Lakes Recreation Waters. 40 CFR Part 131 [OW-2004-0010; FRL-7837-5] RIN 2040-AE63. ***http://www.epa.gov/fedrgstr/EPA-WATER/2004/November/Day-16/w25303.htm*** (Accessed 3-4-2013).

USEPA. 2005a. Microbial Source Tracking Guide Document. Office of Research and Development, Washington, DC EPA-600/R-05/064. 131 p. ***http://cfpub.epa.gov/si/si_public_record_Report.cfm?dirEntryID=133523*** (Accessed 3-7-2013).

USEPA. 2005b. Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA. EPA 815-R-05-002. December 2005.

USEPA, 2011. Using Microbial Source Tracking to Support TMDL Development and Implementation. Prepared for U.S. Environmental Protection Agency, Region 10, Watersheds Unit by Tetra Tech, Inc. and Herrera Environmental Consultants, Seattle, WA. ***http://www.epa.gov/region10/pdf/tmdl/mst_for_tmdls_guide_04_22_11.pdf*** (Accessed 8-20-2013).

USEPA. 2012a. Recreational Water Quality Criteria. Office of Water 820-F-12-058. ***http://water.epa.gov/scitech/swguidance/standards/criteria/health/recreation/index.cfm*** (Accessed 7-26-2013).

USEPA. 2012b. Water Quality Assessment and Total Maximum Daily Loads Information, 2010. ***http://www.epa.gov/waters/ir/*** (Accessed 3-5-2013).

USEPA. 2013. Microbiological Methods/Online Publications. ***http://www.epa.gov/nerlcwww/online.html#protos*** (Accessed 5-27-2013).

U.S. Food and Drug Administration. 2009. Guide for the Control of Molluscan Shellfish. National Shellfish Sanitation Program. 2007 Revision. Interstate Shellfish Sanitation Conference and U.S. Food and Drug Administration. ***http://www.fda.gov/downloads/Food/FoodSafety/Product-SpecificInformation/Seafood/FederalStatePrograms/NationalShellfishSanitationProgram/UCM241512.pdf*** (Accessed 3-4-2013).

Wade, T.J., E. Sams, K.P. Brenner, R. Haugland, E. Chern, M. Beach, L. Wymer, CC. Rankin, D. Love, Q. Li, R. Noble, and A.P. Dufour. 2010. Rapidly measured indicators or recreational water quality and swimming-associated illness at marine beaches: A prospective cohort study. *Environ. Health* 9: 66.

Wallis, P.M., S.L. Erlandsen, J.L. Isaac-Renton, M.E. Olson, W.J. Robertson, and H. van Keulen. 1996. Prevalence of *Giardia* cysts and *Cryptosporidium* oocysts and characterization of *Giardia* sp. isolated from drinking water in Canada. *Appl. Environ. Micro.* 62:2789-2797.

Yamahara, K.M., S.P. Walters, and A.B. Boehm. 2009. Growth of Enterococci in unaltered, unseeded beach sands subjected to tidal wetting. *Appl. Environ. Microbiol.* 75(6):1517-1524.

Yoder, J.S., J.W. Gargano, R.M. Wallace, and M.J. Beach. 2012. Giardiasis Surveillance — United States, 2009–2010. Morbidity and Mortality Weekly Report (*MMWR*). Centers for Disease Control, September 7, 2012 / 61(SS05);13-23. ***http://www.cdc.gov/mmwr/preview/mmwrhtml/ss6105a2.htm*** (Accessed 3-4-2013).

Glossary

Cyst. A microbial cyst is a resting or dormant stage of a microorganism, usually a bacterium or a protozoan, that helps the organism to survive in unfavorable environmental conditions outside a host. It can be thought of as a state of suspended animation in which the metabolic processes of the cell are slowed down and the cell ceases all activities like feeding and locomotion.

Fission. The subdivision of a cell into two or more parts and the regeneration of those parts into separate cells.

Flagellated. Describing an organism with one or more whip-like organelles called flagella. In microorganisms, flagella are generally used for propulsion or to create a current that brings in food.

Gram-negative. Gram-negative bacteria are bacteria that do not retain crystal violet dye in the Gram staining protocol. The test is widely used in classifying two distinct types of bacteria based on the structural differences of their bacterial cell walls.

Oocyst. An oocyst is a hardy, thick-walled spore able to survive for lengthy periods outside a host. The zygote develops within the spore, which acts to protect it during transfer to new hosts.

Spore-forming. Bacteria that form a spore that is resistant to heat, freezing, chemicals, and other adverse environments. Although the vegetative cell is killed by these conditions, the spores can survive and need harsher conditions to be inactivated.

Sporozoite. A cell form that infects new hosts.