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January 12, 2018

Hon. John Shimkus
Chair, Subcommittee on Environment
Committee on Energy and Commerce
2125 Rayburn House Office Building
Washington, DC 20515

Chairman Shimkus,

Thank you for the opportunity to testify at the hearing entitled "H.R.____. Farm Regulatory Certainty Act" on Thursday, November 9, 2017. Per your request, please find attached my response to your questions for the record.

Sincerely,

/s/
Lynn Utesch

Enclosures

Responses to questions from the Honorable Frank Pallone:

1. **What are some of the health impacts you and your neighbors have experienced as a result of manure spraying?**

   It is hard to underestimate the negative health effects of living hear a CAFO. Many of our neighbors have suffered health impacts that research shows are often linked to exposure to CAFO wastes. For example, in our community:

   - A young boy went swimming in a local creek with a skinned knee and contracted a life-threatening infection. He had to have part of his knee cap removed as a result. Antibiotic resistant bacteria are associated with CAFO waste.
   - A young woman had a typical blemish on her face, but when she scratched it, her face became infected with antibiotic resistant MRSA, an opportunistic pathogen. The infection was so severe that she had to have a hole drilled in the top of her mouth to drain the infection.
   - Another community member, who relies on well water, has experienced ongoing, severe diarrhea. Microbial source tracking testing of water from his well showed that there were contaminants from a bovine source in the water.

   Many neighbors have suffered many other health problems associated with CAFO wastes.

   In 2014, John Hopkins Center for a Livable Future wrote a letter addressing our community and the increased risks associated with exposure to increasing volumes of manure. In the last 2 months our local health department has reported confirmed cases of salmonella, campylobacter, giardia, & cryptosporidium. While these were not traced to the source, due to the small size of our health department, USDA researcher Mark Borchardt found many of these same pathogens in well water in our county, likening the condition of our water quality "to that which one would expect to find in a third world country." In this same research, the majority of contaminants came from bovine sources.

   Here in the state of Wisconsin, antibiotic resistant infections such as MRSA, are not tracked by state and local health departments, therefore we cannot get true numbers on how prevalent these infections are in our community. With that being said, we are aware of multiple cases of MRSA in our community, some severe enough to require removal of appendages. Prevalence of these pathogens in our environment were shown in a study conducted by Marquette University which tested water and sediments from our local streams and rivers. This research showed the presence of multiple antibiotics, and antibiotic resistant bacteria. Samples indicated that some of the antibiotics are used exclusively in veterinary medicine. In 2013, Dr.

Angelia Bauer's research indicated "significant human health threats" found in water in northeastern Wisconsin, including the presence of endocrine disrupting compounds. We are highly concerned with air quality issues where we live, and toxic emissions known to be neurotoxins, but since emissions are not regulated we are offered virtually no protection against air tainted with considerable hydrogen sulfide, ammonia, methane, particulate matter and multiple volatile organic compounds. Attached: John Hopkins letter; Marquette University research; USDA Mark Borchardt research; Angelia Bauer research.

2. **What are some of the economic impacts you and your neighbors have Experienced?**

In November, 2017, Wisconsin's Department of Revenue conducted a study of home values in Kewaunee County. The study found that homes within a quarter mile of a large CAFO had a 13% reduction in property value. If the home was from a quarter mile to one mile from a large CAFO the property value was reduced by 8%. The study shows citizens are losing value on what is most residents largest asset –through no fault of their own, merely their proximity to CAFO activities within a certain radius. This study does not take into account other issues these homes may have such as toxic emissions, a contaminated well, or non-stop semi traffic in their neighborhoods. In the Town of Lincoln, when a large CAFO turned a pastoral field into a feed pad and 76 million gallon lagoon, 8 families immediately left, with some of the housing was bought at considerably reduced rates by the CAFO operator. It is not unusual for operators to buy homes cheaply after their operation has devastated families who seek to leave the health threats and diminished quality of life issues that arise due to CAFO activities and production areas, and the accompanying land, water, and air pollution.

Living in a county that has 34% of the tested wells contaminated with e. coli or nitrates makes it very difficult to attract new businesses and residents. While our county is attempting to increase tourism, it is difficult to get people to choose this as a travel destination since our Lake Michigan beaches are covered with dense algae mats that stink and cover the beach, fueled by excessive phosphorus from farm run-off into our tributaries which empty into Lake Michigan. Externalized costs from these mega dairies is untold to our environment; Our 3 major rivers, the Ahnapee, Kewaunee, and East Twin, are all on the EPA's impaired waters list. Many rivers in Kewaunee County are seriously impaired, although at one time held the status as class 1 trout streams.

Obviously, the biggest concern homestead owners have is a contaminated well, which in large part renders a home without water fit to drink, wash vegetables, brush one's teeth or bathe in. Costs to remediate problems are very costly for homeowners, well into thousands of thousands of dollars, and digging a new well has unfortunately failed for some residents who found in a very short time that their well had been re-contaminated. Remediation for well contamination through different technologies is extremely expensive, and buying multiple gallons of water at the store on a regular basis is also a financial burden for citizens. Water testing is also expensive for homeowners to incur costs on, and many are deterred from regular well testing due to costs associated.

The continued growth of CAFOs has highly impacted small dairy farmers by the surplus volumes of milk generated, which are driving milk prices down, having the greatest negative impact on small farmers. In 2017 Wisconsin lost 500 dairy farms. As of January 1, 2018, there were 8,801 licensed dairy herds. The number of dairy farms has dropped by 20% in Wisconsin in the last 5 years. The CAFO model has driven up land costs making it almost impossible for young and new farmers to purchase land. Land is valuable not in as so much for crop production, but for manure disposal.

3. **You, yourself are a farmer, so you have experience handing manure for farming purposes. In your experience, what are the big differences between the way you use manure as a farmer and the way Concentrated Animal Feeding Operations dispose of their manure?**

   Manure handling in our area is primarily through the use of liquid manure. What that means is the manure and urine are mixed with water for ease of handling. For the CAFOs this means all the wash water for dairy equipment, barns, leachate etc. is added to the manure, which is stored in a manure pit. These pits hold millions of gallons—the largest in our area a 76 million gallon lagoon. In addition, in the state of Wisconsin these pits can accept up to 10% industrial waste of the total volume of the pit, which is classified as “manure”. These pits are storing manure under anaerobic conditions [without oxygen], which starts the putrefaction process, which produces toxic gases such as hydrogen sulfide, methane, ammonia and up to 300 Volatile Organic Compounds. This liquid manure is then applied to the soil at what is termed agronomic rates. The typical rate for corn in our area is 22,000 gallons per acre, but it could be as high as 32,000 gallons per acre. These rates are based on the crop needs for the entire growing season. Unfortunately, liquid manure is applied in a single application. This application often takes place late in the fall when corn has been harvested on to bare soil that will not be planted until the next spring. This practice creates the potential for this liquid manure to migrate into the ground water or run off to surface waters. Even when this manure is applied in the spring at the volumes allowed, it creates the potential for the manure to leach below the root zone and enter the ground water.

On our farm, we use a rotational grazing system with 110 acres of pasture and 40 acres of woods and hedgerows. During the growing season our cows are moved to fresh pasture on a daily basis. Since our cows spend their time on grass we have a minimum of manure handling. The manure from our cows is in a solid form, and is deposited directly to the soil where the biology of the soil and living forms such as worms and dung beetles convert the waste to a bioavailable nutrient that can be used by the plant immediately. By using solid manure deposited on a carpet of plants we minimize the potential for our manure to migrate to our ground water or surface water. Even during the winter months our cows are moved on our pasture to round bales put out in different locations on the field, which reduces the concentration of manure from any one area. Any manure in the barns is cleaned out with a skid steer and composted, which reduces its volume by as much as 50%.

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March 27, 2014

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Disclaimer: The opinions expressed herein are our own and do not necessarily reflect the views of The Johns Hopkins University.

Re: Manure from intensive livestock operations: health and environmental concerns

To whom it may concern:

We are researchers at The Johns Hopkins Center for a Livable Future, based at the Bloomberg School of Public Health. The Center engages in research, policy analysis, education, advocacy, and other activities guided by an ecologic perspective that diet, food production, the environment, and public health are interwoven elements of a single complex system. We recognize the fundamental importance of food animal production in these issues as they relate to the U.S. food system.

We are writing to present some of the concerns associated with the generation and management of manure from intensive livestock operations, particularly regarding the health of Wisconsin's rural citizens. These health and environmental concerns include:

- The spread of infectious disease, including antibiotic-resistant bacteria, to nearby communities.
- Groundwater and surface water pollution, and associated health and ecological impacts.
- Air pollution, odors, and associated health and social impacts.

These are detailed below, with supporting evidence from the peer-reviewed scientific literature.

Background

According to the 2007 Census of Agriculture, Wisconsin is the second leading dairy-producing state in the country. The state is home to over 1.2 million milk cows, with an inventory of close to 3.4 million cattle and calves—the 9th largest in the nation. Wisconsin is also a significant contributor to U.S. pork, poultry and egg production (1,2).

Over half of Wisconsin's cattle and calves are on farms with reported inventories of over 200 head, and 27 percent are on farms with over 500 head (1). With regards to health and environmental concerns, it is critical to consider inventory size alongside other important factors such as feed inputs, stocking density, and the amount of available cropland for spreading manure.

Producing large numbers of animals over a relatively small land area presents the challenge of managing the quantities of manure they generate. A 1400 pound lactating cow, for example, produces an estimated 148 lbs of waste daily (3). Humans, by comparison, produce 2.5 lbs daily. An intensive dairy operation with several hundred animals, by extension, may produce as much excrement as a small city, concentrated over a tiny fraction of the land area and without the benefit of a wastewater treatment plant to eliminate biological and chemical contaminants. In large part because of these challenges, intensive livestock operations have emerged as a major source of pollution to ground and surface waters (4–9).

Any farmer can attest to the value of manure as a source of nutrients and organic matter for their soil. The quantity of manure generated at intensive operations, however, frequently exceeds the amount that can be utilized by surrounding cropland, and transporting manure further may not be economically feasible (10–12). When manure is over-applied, the excess—along with chemical (13–17) and microbial (4,18,19) contaminants associated with it—may be transported by runoff into surface waters and/or leach into groundwater. Results from a 2005 study, for example, suggest 71 percent of Wisconsin dairy farms generate manure in amounts that exceed the nutrient requirements of the cropland on which manure is applied (20). The potential health and ecological effects associated with these scenarios are detailed below.

Spread of infectious disease to nearby communities

Crowded conditions in intensive livestock operations present frequent opportunities for the transmission of viral and bacterial pathogens among animals, and between animals and humans. Many of these pathogens live in the digestive tracts of animals and may be passed in their waste (4,18,19).

The disease risks stemming from intensive livestock production are heightened by the potential for infection with antibiotic-resistant bacteria. The use of low doses of antibiotic drugs as a means to promote growth (often also called "disease prevention") in animals has become commonplace—an estimated 80 percent of antibiotics sold for human and animal uses in the U.S. are sold for use in food-producing animals (21). Administering antibiotics to animals at doses too low to treat disease fosters the proliferation of antibiotic-resistant pathogens, which can cause infections in humans. When a person is infected with antibiotic-resistant bacteria, these infections can be more difficult and expensive to treat (22).

A growing body of evidence points to the potential pathways by which pathogens (antibiotic-resistant or otherwise) might spread from intensive livestock operations into communities. Studies suggest, for example, that antibiotic-resistant pathogens may be transmitted by workers into their homes and communities (23,24), conveyed by runoff into ground and surface waters (19), blown out of ventilation systems (25–27), and spread to consumers via contaminated meat (28,29). Pathogens may also be transported by flies (30), wild birds (31,32), and animal transport vehicles (33). Further evidence for these pathways is documented in a 2013 study in which living closer to swine operations—and to fields where manure is spread—was significantly associated with elevated rates of infection with methicillin-resistant *Staphylococcus aureus* (MRSA), an antibiotic-resistant pathogen that can be challenging and expensive to treat (34). A similar study found similar associations between proximity to a swine operation and colonization with MRSA (35).

Health and ecological impacts of ground and surface water pollution

Manure from intensive livestock operations may introduce a range of waterborne contaminants into ground and/or surface waters, including nitrates (7,8), microbial pathogens (4,19,34), veterinary pharmaceuticals(14–18,36) and natural and synthetic hormones (37,38). Communities living downstream from these operations may be exposed to these agents via drinking or having skin contact with contaminated ground or surface waters.

Exposure to these waterborne contaminants can result in adverse health effects. Ingesting high levels of nitrate (naturally occurring in manure), for example, has been associated with increased risks for thyroid conditions (39,40), birth defects and other reproductive problems (39,41), diabetes (39), various cancers (39,42), and methemoglobinemia (blue baby syndrome), a potentially fatal condition among infants (43).

The risks of exposure to waterborne contaminants are particularly salient for the 70 percent of Wisconsin's population who depend on groundwater for their drinking water supply—the state ranks fourth in the nation for the percentage of households on private wells (44). Adding to these concerns, much of southern and eastern Wisconsin has karst geology—a feature that can readily channel surface contaminants into groundwater sources (45). Private wells are not subject to federal drinking water regulations, and while some states have minimal requirements for private wells, state-level action is usually only triggered during property transfer and rarely requires periodic monitoring of water quality (46). Further, most water treatment systems for private wells are designed to deal with heavy metals and other more common drinking water contaminants, and are not suited for removal of drug residues and hormonally-active compounds.

Nutrient runoff into surface waters may also have consequences for marine ecosystems and the people who depend on them for recreation and economic activity. Intensive livestock operations are a major source of nutrient runoff (6,7,47), contributing to algal blooms and subsequent hypoxic "dead zones" that may result from algal decomposition. Aquatic regions exposed to long periods of hypoxia often see dramatic reductions in fisheries, among other health, ecological, and economic harms (48). Nutrient runoff has also been implicated in the growth of harmful algal blooms (49), which may pose health risks for people who swim or fish in recreational waters, or who consume contaminated seafood. Exposure to algal toxins has been linked to neurological impairments, liver damage, stomach illness, skin lesions, and other adverse health effects (50).

In more severe cases, manure storage facilities may rupture, leak, or overflow during extreme weather events, releasing their contents into surrounding waterways. For example, in 1995 a large swine waste holding lagoon in North Carolina ruptured due to faulty management. Close to 26 million gallons of manure emptied onto fields and lawns of adjacent homes before draining into a nearby river. The pollution load led to the proliferation of toxic algal blooms and widespread fish kills, and fecal bacteria were detected in river sediment at levels over 15,000 times higher than state standards (51).

Air pollution, odors, and associated health and social impacts

Intensive livestock operations release a range of airborne pollutants, including ammonia, hydrogen sulfide, and other gases emitted from animal waste; and airborne particulates, which may be comprised of dried feces, animal dander, fungal spores, and bacterial toxins (52). Results from a two-year air monitoring study, jointly sponsored by the U.S. Environmental Protection Agency and representatives of the pork, poultry, dairy and egg industries, suggest intensive livestock operations produce several of these pollutants at levels well above federal standards.(53)

Much of the research on the health effects associated with exposure to airborne pollutants from confinement operations has focused on workers. At least one in four workers in these operations are estimated to suffer from respiratory illness (54).

A growing body of evidence suggests residents living near intensive livestock operations may also be at greater risks of respiratory illness. Results from a study of industrial-scale dairy operations in Washington State, for example, suggest intensive dairy operations are a significant source of particulate matter among nearby rural communities (55). Another study detected high concentrations of particulate matter downwind from swine confinement operations, which was linked to wheezing, breathing difficulties, and eye, skin, and nasal irritation among residents of downwind communities (56). Indicators of air pollution from swine confinement operations have also been linked to asthma symptoms among students at nearby schools (57). Additional studies have illustrated relationships between proximity to intensive livestock operations and respiratory effects (58–61) among other adverse health outcomes.

Odors associated with air pollutants from intensive livestock operations have been known to interfere with daily activities, quality of life, social gatherings, and community cohesion (62,63). In addition to the stigma and social disruption they often generate, odors from swine confinement operations have been associated with physiological and psychological effects, including high blood pressure, depression, anxiety, and sleep disturbances (64–66).

Despite the above concerns, all but the largest livestock operations—those designated as “Large CAFOs” (concentrated animal feeding operations)—are required by federal law to report hazardous airborne emissions, and then only if the levels are above certain thresholds. Even in cases when operations report emissions, such information may not be available to the public. For these reasons, the relationships between intensive livestock operations, air quality, and the health of rural residents are poorly understood. These data gaps speak to the need for better methods of estimating emissions, including more stringent reporting requirements and air monitoring stations at intensive livestock operations and communities (67).

Conclusion

For thousands of years, manure has been valued by farmers for its roles in building soil quality and increasing crop yields. Producing livestock such that they generate more manure than can be utilized by nearby cropland is not only a waste of this important resource, it is also a public health and environmental problem. A growing body of evidence has implicated the generation and management of manure from intensive livestock operations in the spread of infectious disease (including antibiotic-resistant strains), the introduction of microbial and chemical contaminants into ground and surface waters, impacts to air quality, and the wide range of adverse health, social, ecological and economic outcomes that result from these events.

We hope our letter is helpful in describing some of the public health and environmental concerns associated with the generation and management of manure from intensive livestock operations. Please do not hesitate to contact us if you have any questions.

Sincerely,

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Assessing Groundwater Quality in Kewaunee County, Wisconsin

Lead Scientists

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Marshfield Clinic Research Foundation
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UW-Extension Environmental Resources Center
Kevin Masarik
UW-Stevens Point Watershed Science and Education

Study Funding Sources

Wisconsin DNR

WI Groundwater Research Advisory Council

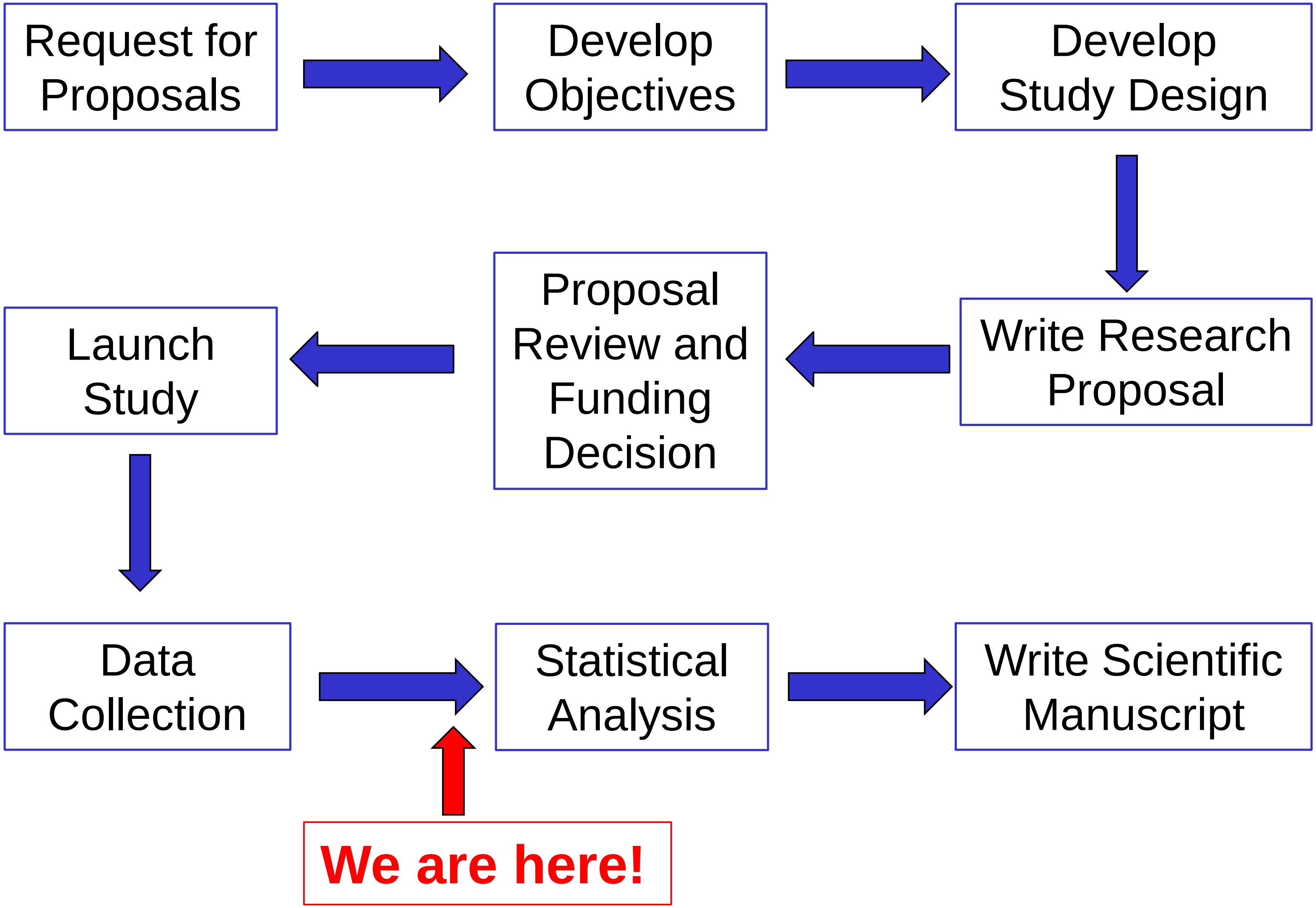
US Geological Survey

USDA - Agricultural Research Service

THANK YOU!

621 Kewaunee County households participated in the study

Study Steps



Request for Proposals

Develop Objectives

Develop Study Design

Write Research Proposal

Proposal Review and Funding Decision

Launch Study

Data Collection

Statistical Analysis

Write Scientific Manuscript

We are here!

Presentation Outline

- Introduction
- Hydrogeology of Kewaunee County
- Private well contamination and sources
- Contamination timing and variability
- What a private well owner can do
- Study next steps

INSERT MOE’S HYDROGEOLOGY PRESENTATION

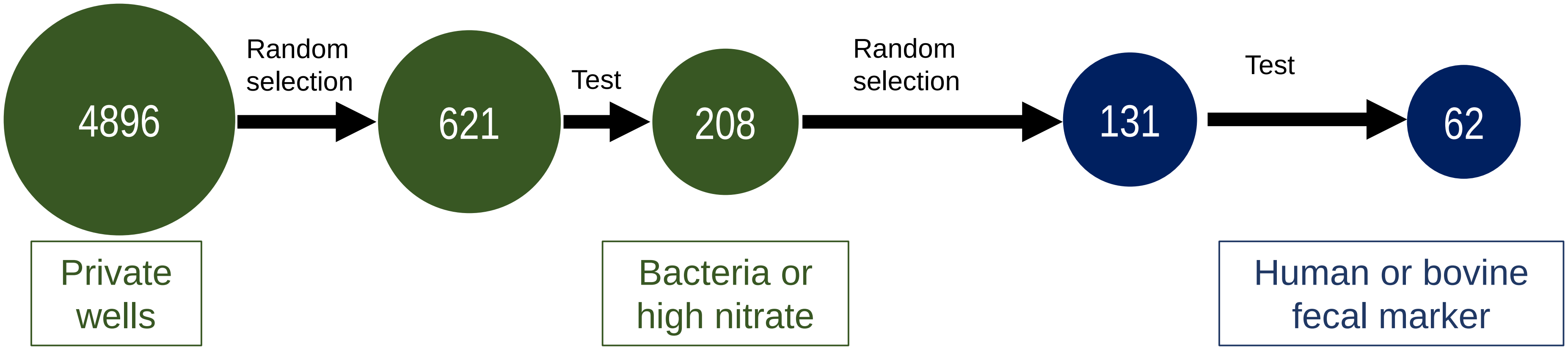
Research Objectives

1. Design a county-wide randomized sampling plan, stratified by depth-to-bedrock, for nitrate and indicator bacteria
2. Sample once per season a subset of wells for viruses and fecal markers capable of distinguishing septic versus bovine sources of contamination
3. Install automated sampling systems on one or two wells to determine the timing of peak transport for viruses and indicator bacteria
4. Identify spatial and temporal patterns of contamination

Project objectives & study design

1. Measure total coliform, *E. coli*, nitrate → 2. Determine fecal source

Given contamination


4896 → Random selection → 621 → Test → 208 → Random selection → 131 → Test → 62

Private wells

Bacteria or high nitrate

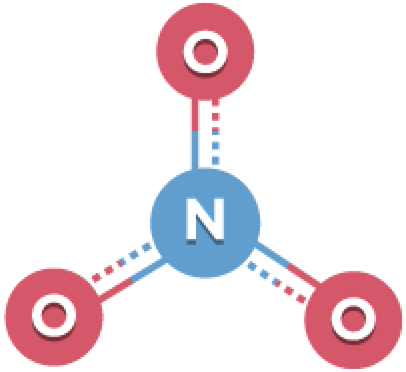
Human or bovine fecal marker

Outcome: County-wide occurrence as % wells contaminated

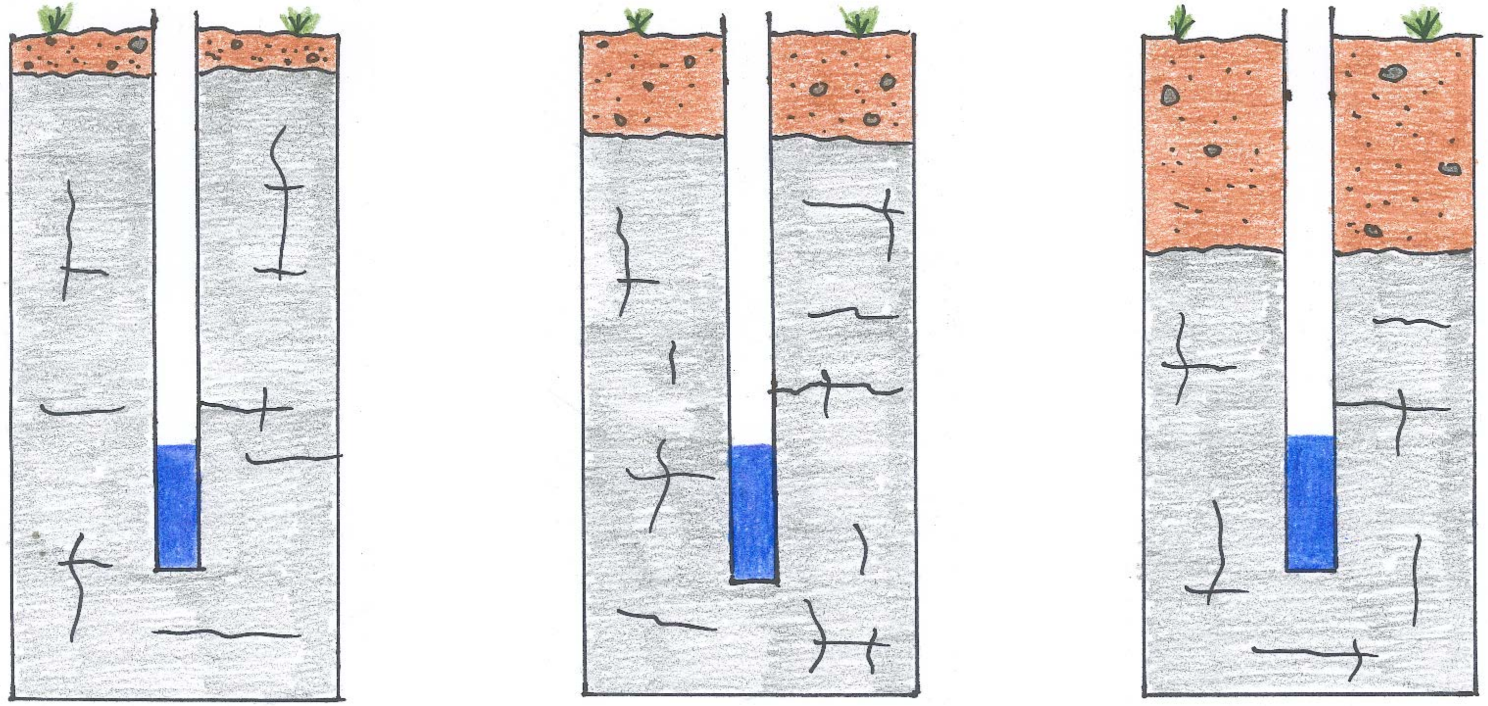
Outcome: Number of wells with human or bovine fecal markers

Objective 1: Total Coliform, *E. coli*, Nitrate

- County-wide randomized sampling of private wells stratified by depth-to-bedrock: 0-5 ft, 5-20 ft, 20+ ft
- Participation rate ~ 50%
- Several day "Synoptic" sampling
- Recharge
    - November 2015
    - 317 wells in analysis
- No recharge
    - July 2016
    - 400 wells in analysis



Total coliform in private wells by depth to bedrock



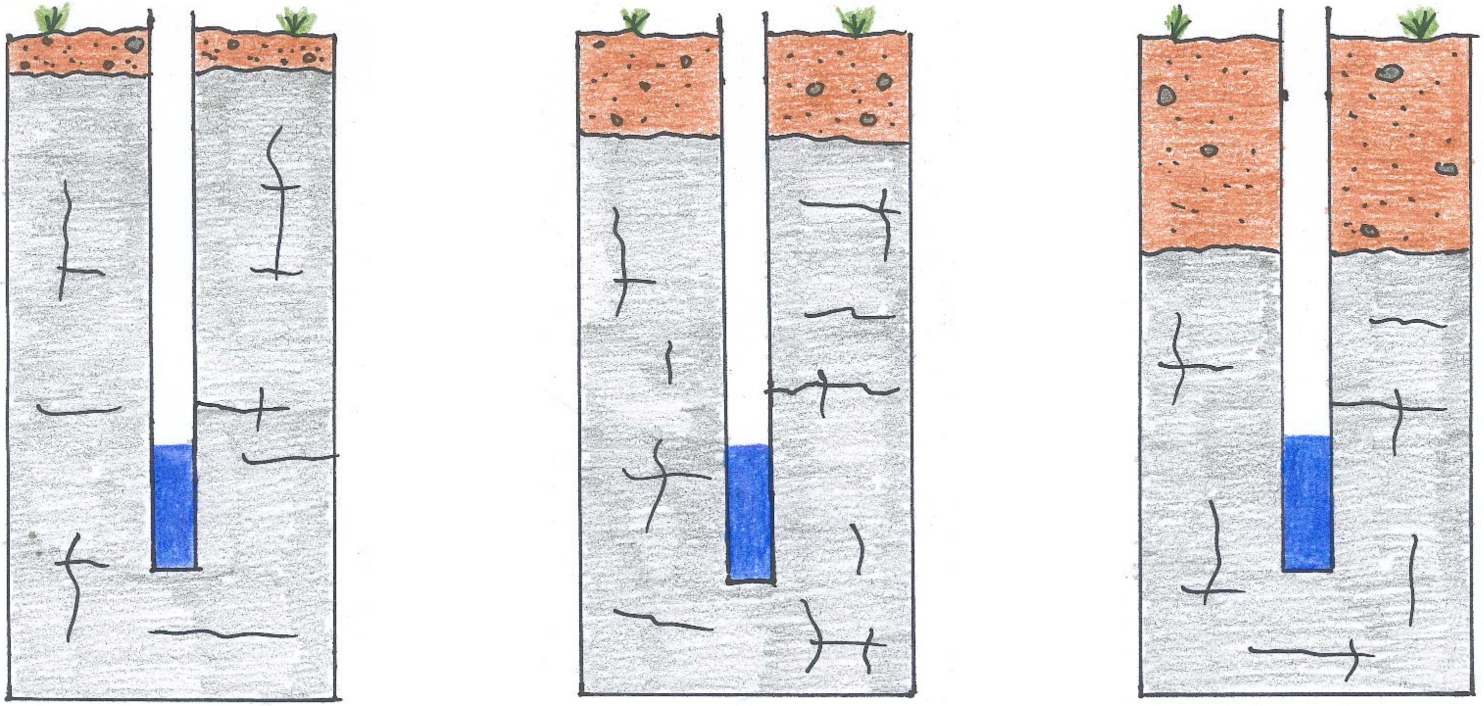
Total coliform (% positive wells)

	0 – 5 feet	5 – 20 feet	20 + feet
Recharge	46	28	19
No recharge	23	29	21

Wisconsin (all depths): 22.8

US General Accounting Office 1997

E. coli in private wells by depth to bedrock



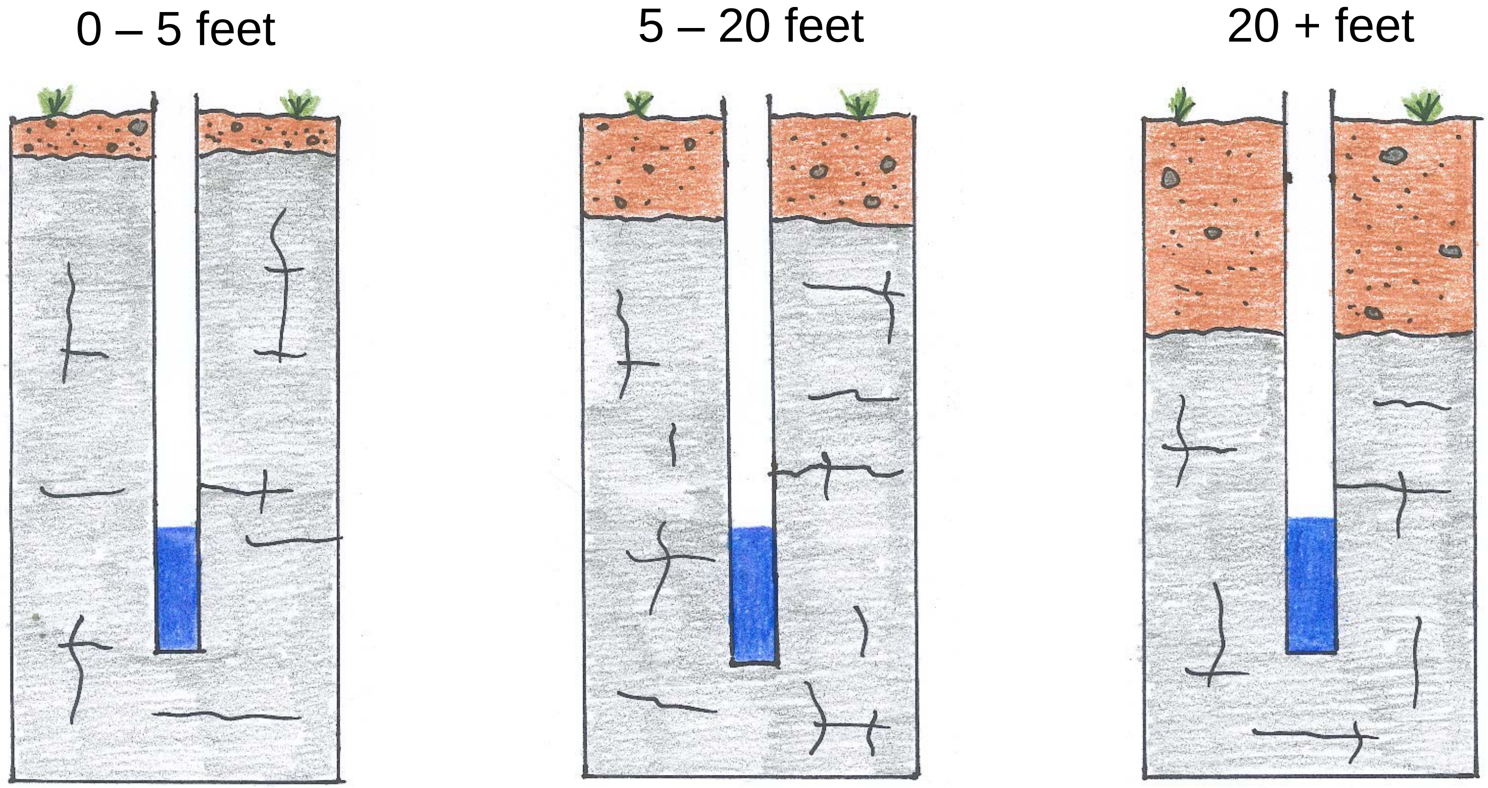
E. coli (% positive wells)

	0 – 5 feet	5 – 20 feet	20 + feet
Recharge	4	1	0.3
No recharge	7	1	1

Wisconsin (all depths): 2.6

US General Accounting Office 1997

High nitrate in private wells by depth to bedrock



High nitrate (% positive wells)

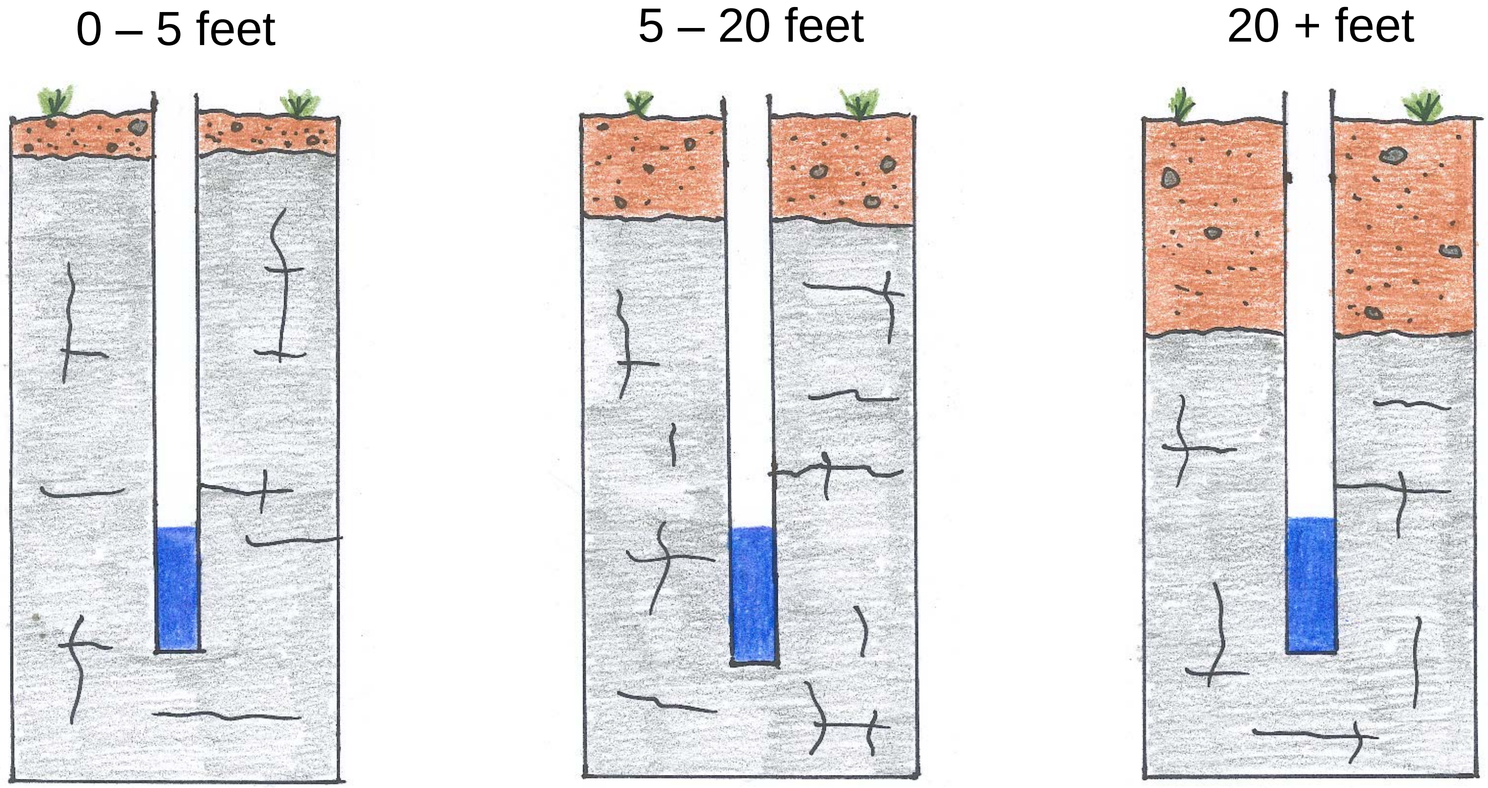
Recharge	7	20	6
No recharge	10	19	5

Wisconsin (all depths): 6.6

US General Accounting Office 1997

High nitrate: exceeds health standard; N-NO_3^- > 10 ppm

Total coliform, *E. coli*, or high nitrate in private wells by depth to bedrock



	Total coliform, *E. coli*, or high nitrate (% positive wells)		
Recharge	50	42	23
No recharge	33	40	26

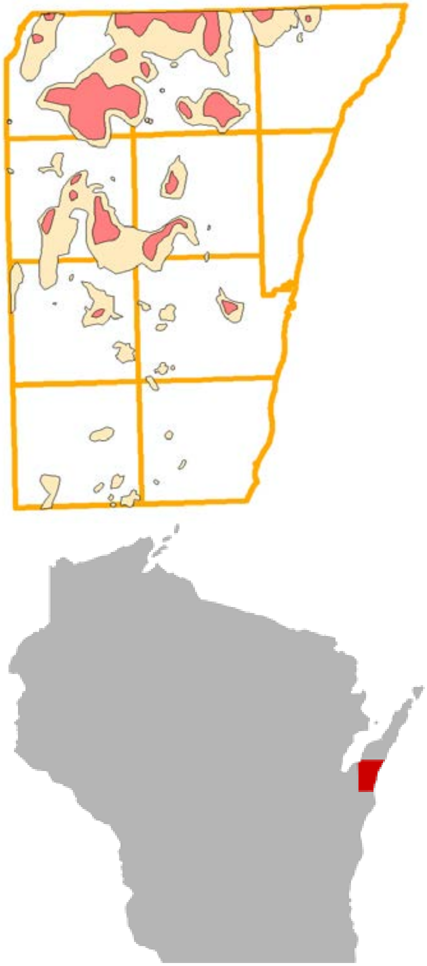
High nitrate: exceeds health standard; $N\text{-}NO_3^-$ *> 10 ppm*

County-wide contamination rate; weighted by depth to bedrock

Percent wells contaminated

	Kewaunee County		Wisconsin*
	Recharge (n = 317)	No Recharge (n = 400)	(n = 534)
Total coliform	20.8	22.2	22.8
E. coli	0.4	1.2	2.6
High nitrate	7.4	6.8	6.6
Any of the 3 contaminants	26.4	27.6	NA

High nitrate: exceeds health standard; N-NO_3^- > 10 ppm

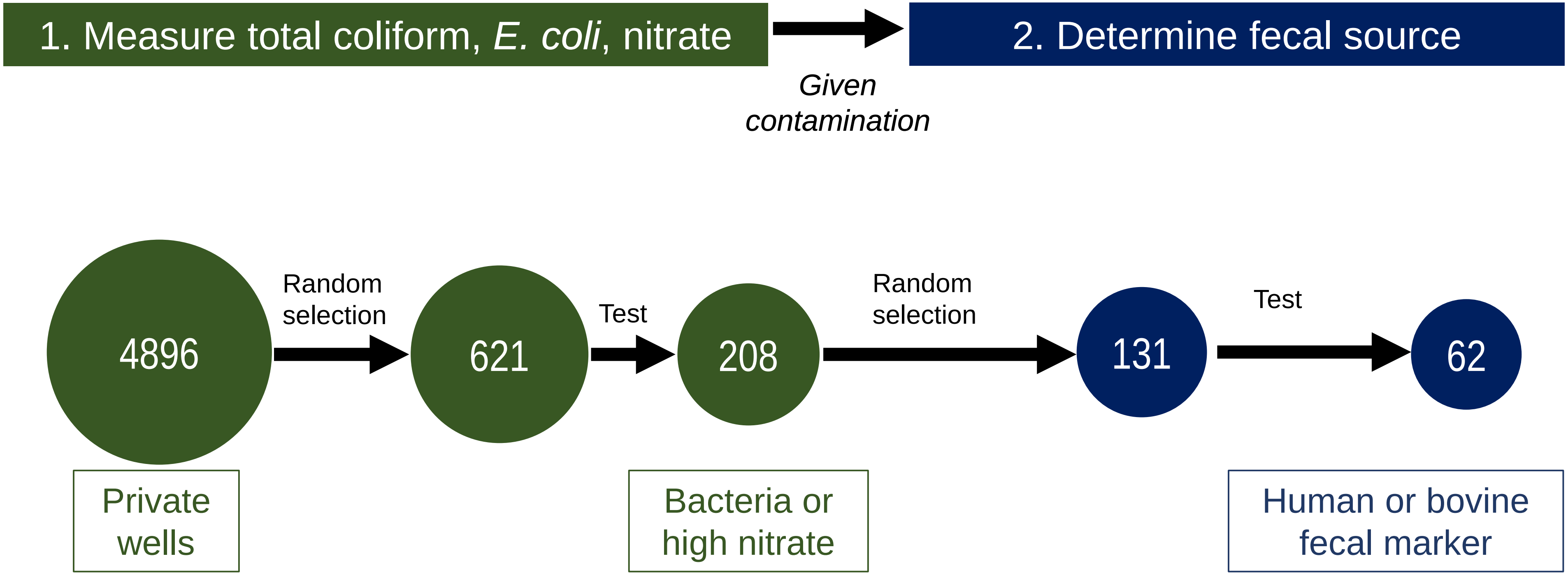


*private wells sampled; *Information on the quality of water found at community water systems and private wells. United States GAO/RCED-97-123, June 1997*

Objective 2: Determine fecal source

- Randomized stratified sampling from 208 wells positive for total coliform, *E. coli*, or high nitrate (N-NO_3^- > 10 ppm)
- Five sampling rounds, all completed:
  - April, August, November, 2016
  - January and March, 2017

Project objectives & study design



1. Measure total coliform, *E. coli*, nitrate

Given contamination

2. Determine fecal source

4896

Private wells

Random selection

621

Test

208

Bacteria or high nitrate

Random selection

131

Test

62

Human or bovine fecal marker

Outcome: County-wide occurrence as % wells contaminated

Outcome: Number of wells with human or bovine fecal markers

Kewaunee County Cattle

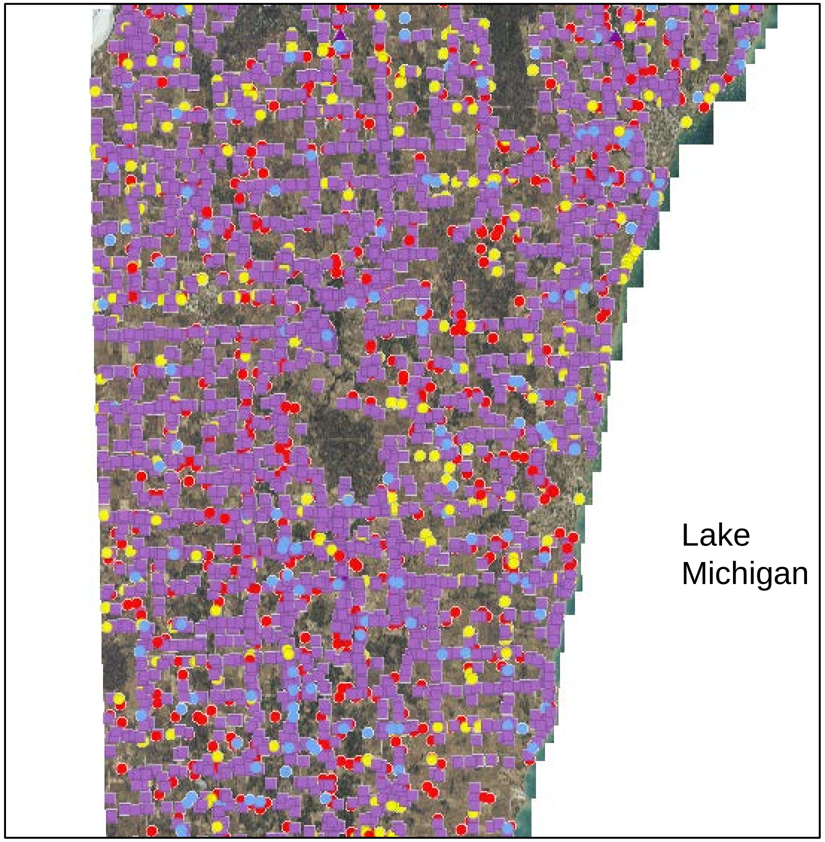
- All cattle & calves in 2016 = 97,000
- Milk cows in 2013 = 45,500
- Milk cow herds in 2016 = 167
- Concentrated Animal Feeding Operations (CAFOs) 15 dairy, one beef
- Approximately 700 million gallons cattle manure per year

Kewaunee County Septic Systems

- 4822 septic systems in the county
- 540 holding tanks, 155 abandoned

Personal comm. Lee Luft, Kewaunee County Supervisor, March 7, 2017

<u>Legend</u>
Purple = replaced or inspected
Red = not inspected
Yellow = holding tank
Blue = abandoned system



Kewaunee County septic systems

Approximately 200 million gallons septic effluent per year released to the subsurface

Study Sampling and Analyses

- Collected 138 samples from 131 household wells in Kewaunee County
- Pump ~800 L through hemodialysis filters
- Laboratory tests for genetic sequence unique to microorganism
  - Human-specific microbes
  - Bovine-specific microbes
  - Non-specific microbes (pathogens of both people and cattle)

Microbes: Identifying the Fecal Source

(n = 138 samples from 131 wells) (red font indicates pathogenic)

Host	Microorganism	Wells	Concentration (gene copies/L)
Human-specific	Adenovirus A	1	1
	Bacteroidales-like Hum M2	7	< 1 – 1050
	Human *Bacteroides*	27	< 1 – 34
	Cryptosporidium hominis	1	qualitative
	All	**29**	
Bovine-specific	*Bacteroidales*-like Cow M2	2	29 - 915
	Bacteroidales-like Cow M3	4	3 – 49818
	Bovine *Bacteroides*	36	< 1 – 42398
	Bovine polyomavirus	8	< 1 – 451
	Bovine enterovirus	1	2
	All	**40**	

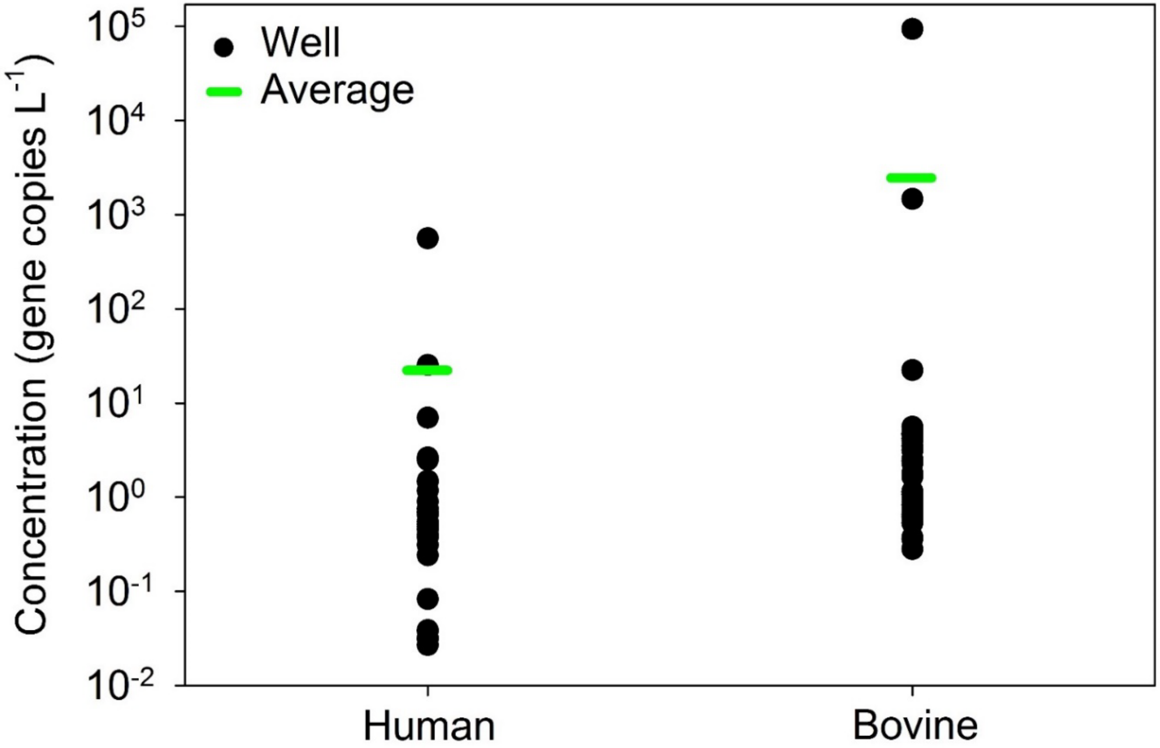
Not detected: [human-specific] adenovirus B & C, D, F, enterovirus, human polyomavirus, norovirus GI & GII
[bovine-specific] coronavirus, bovine diarrheal virus 1 & 2

Wells with human or bovine microorganisms (62 of 131 tested)

Number of wells with human or bovine microorganisms:

Human: **29** *Both*: **7** *Bovine*: **40**

Concentrations of microorganisms in wells



Concentration is sum of human- or bovine-specific microorganisms in a positive well; displayed on a $\log_{10}$ scale

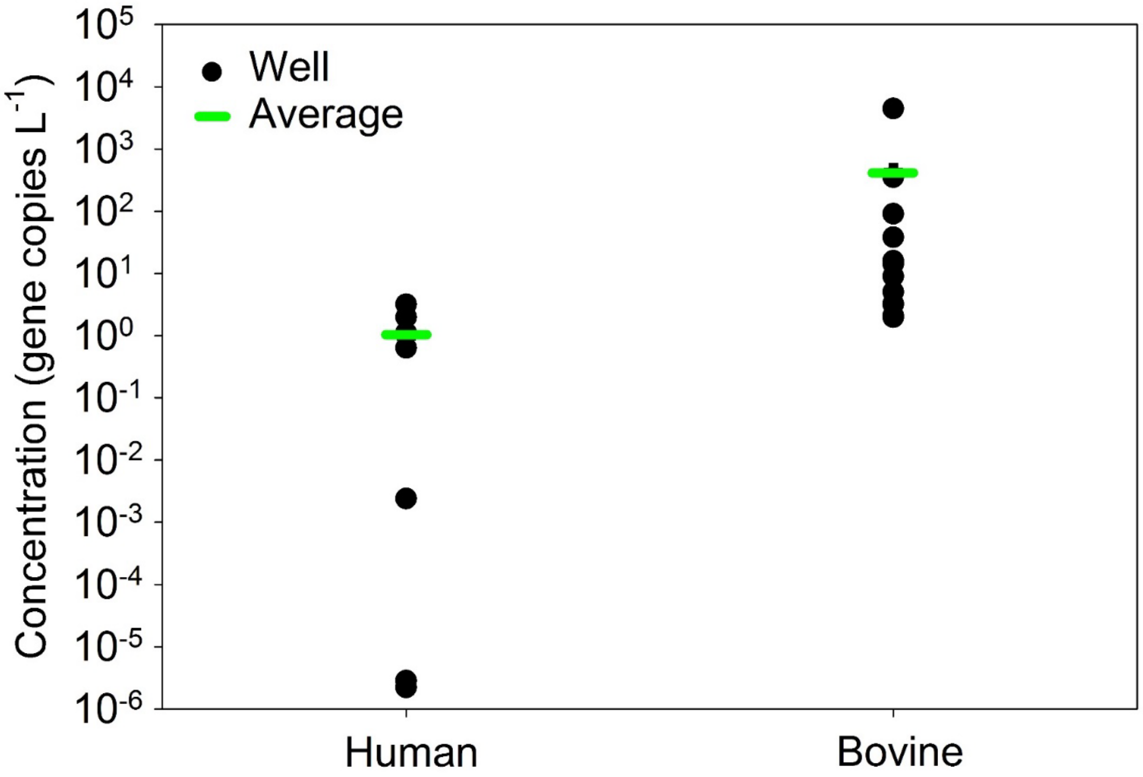
Host	Microorganism	Wells	Concentration (gene copies/L)
Non-specific	*Campylobacter jejuni*	1	< 1
	Cryptosporidium parvum	13	qualitative
	Cryptosporidium spp.	16	< 1 – 3
	Giardia lamblia	2	< 1
	Pathogenic *E. coli* (*eae* gene)	1	4
	Pathogenic *E. coli* (*stx1* gene)	1	16
	Pathogenic *E. coli* (*stx2* gene)	1	1
	Pepper mild mottle virus	13	2 - 3811
	Rotavirus A (*NSP3* gene)	17	< 1 – 4481
	Rotavirus A (*VP7* gene)	7	< 1 – 732
	Rotavirus C	3	45 – 1301
	Salmonella (*invA* gene)	3	< 1 – 13
	Salmonella (*ttr* gene)	5	5 – 59
	All	**44**	
	Total positive wells	**79**	**< 1 - 49818**

Wells with human or bovine rotavirus group A (17 of 131 tested)

Number of wells with human or bovine rotavirus group A:

Human: **7** *Both*: **2** *Bovine*: **12**

Concentrations of rotavirus group A in wells



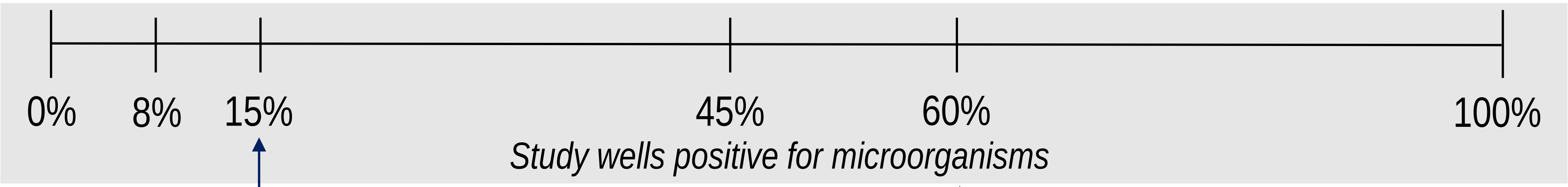
Concentration is displayed on a $\log_{10}$ scale

Pathogens & fecal markers in Kewaunee County: Comparison to other studies

Wisconsin 2003:
Private wells
50 tested

Ontario 2017:
Private wells
11 tested

Pennsylvania 2017
Private wells
5 tested



Canada & USA 1990 – 2013
Public & private wells
12,616 tested

Kewaunee County
Private wells
131 tested

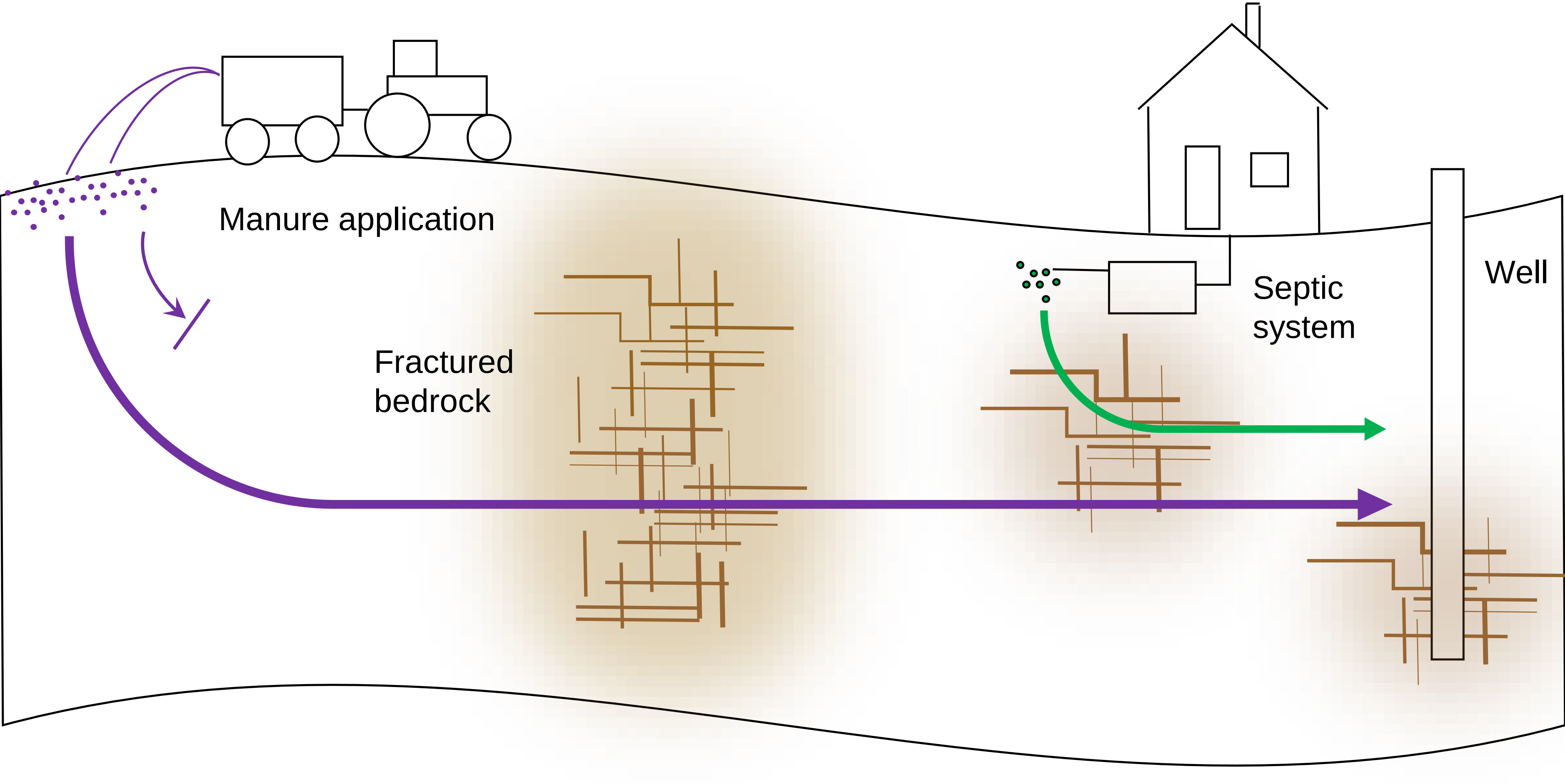
Conceptual Model of Fecal Contamination in Kewaunee County - 1

Bovine pathogen source

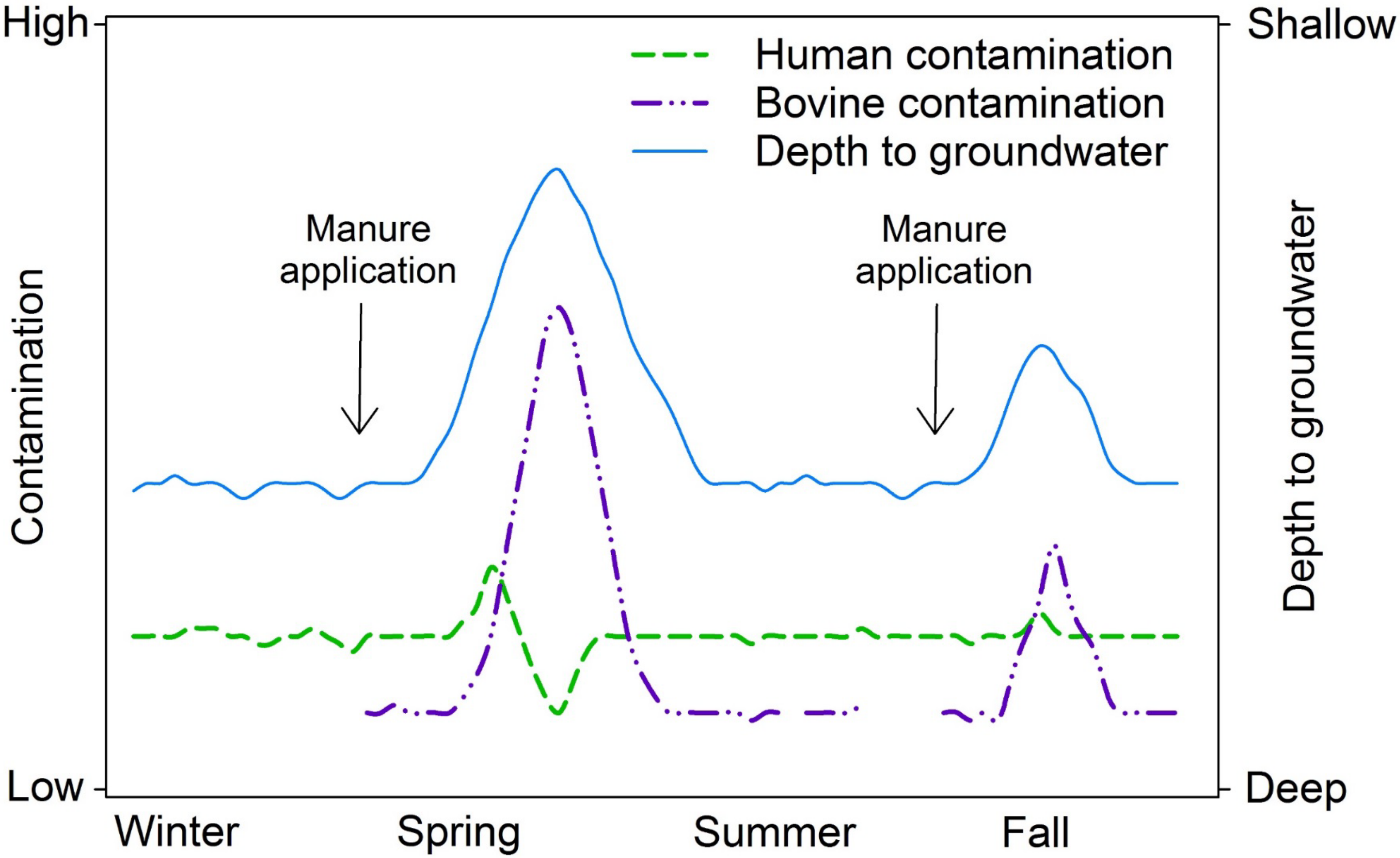
- Large fecal source
- Surface applied periodically
- Episodic infiltration

Human pathogen source

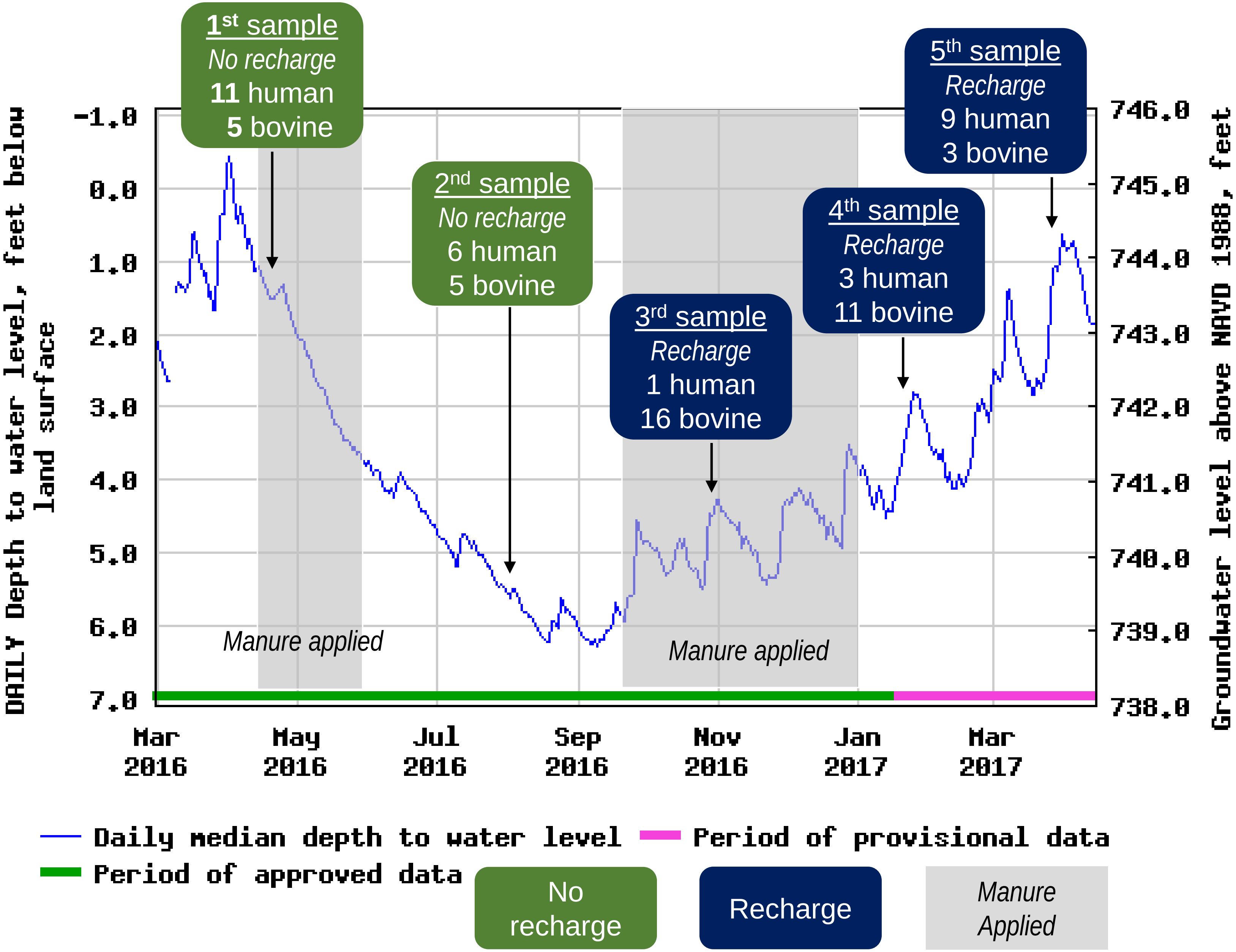
- Small fecal source
- Sub-surface release continuously
- Continuous infiltration



Conceptual Model of Fecal Contamination in Kewaunee County - 2



Groundwater levels during sampling for pathogens & fecal indicators



1st sample
No recharge
11 human
5 bovine

2nd sample
No recharge
6 human
5 bovine

3rd sample
Recharge
1 human
16 bovine

4th sample
Recharge
3 human
11 bovine

5th sample
Recharge
9 human
3 bovine

Manure applied

Manure applied

No recharge

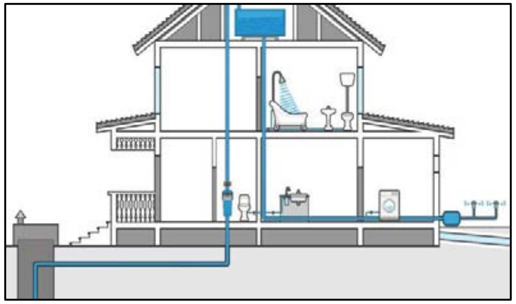
Recharge

Manure Applied

From Farm Field to Household Well

Manure applied Oct 25, 2016

> 1 inch rain Oct 26, 2016



House near field

Farm field sampled Oct 27, 2016

Bovine Bacteroides
Bovine enterovirus
Bovine polyomavirus
M2 Bacteroides-like
M3 Bacteroides-like

Rotavirus A NSP3
Rotavirus A VP7
Rotavirus C

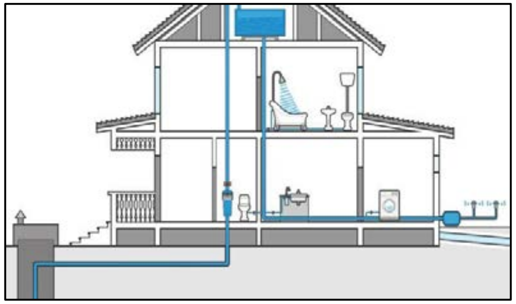
Tap water Oct 27, 2016

Bovine Bacteroides
Bovine enterovirus
Bovine polyomavirus
M2 Bacteroides-like
M3 Bacteroides-like
Campylobacter jejuni
Cryptosporidium
Rotavirus A NSP3
Rotavirus A VP7
Rotavirus C

From Farm Field to Household Well

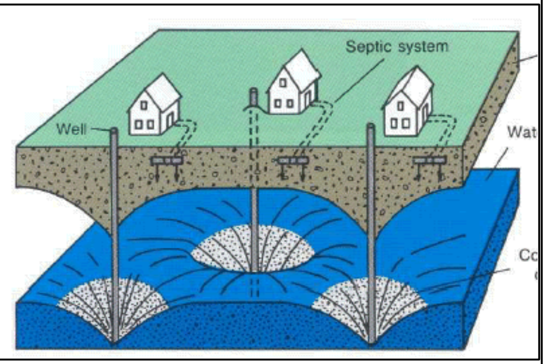
Manure applied Oct 25, 2016

> 1 inch rain Oct 26, 2016



House near field

Neighbor's well sampled Oct 31, 2016



Bovine Bacteroides

Bovine polyomavirus
M2 Bacteroides-like
M3 Bacteroides-like

Rotavirus A NSP3
Rotavirus A VP7
Rotavirus C

Tap water Oct 27, 2016

Bovine Bacteroides
Bovine enterovirus
Bovine polyomavirus
M2 Bacteroides-like
M3 Bacteroides-like
Campylobacter jejuni
Cryptosporidium
Rotavirus A NSP3
Rotavirus A VP7
Rotavirus C

Do people get sick from drinking contaminated private well water?

- Consider one pathogen: *Cryptospordium parvum*
- Confirmed cryptosporidiosis cases in Kewaunee County reported to State:
    - 2 to 9 cases per year (2010 to 2016)
- Under-reporting of cryptosporidiosis cases in the USA is estimated to be 100-fold (Centers for Disease Control and Prevention, 2012)
- Therefore, in Kewaunee County there are likely 200 to 900 cryptosporidiosis cases per year

How many of these cases are from private wells?

Estimate of Kewaunee County *Cryptosporidium parvum* infections from private wells

	People	**Calves**
Population using private wells	12,200	17,300
Wells contaminated by *C. parvum*	3.1%	3.1% (assumed)
Population exposed per day	380	540
Infections per exposure	10 infections per 10,000 people	85 infections per 10,000 calves
Total infections per year	140	1,700

Summary

- On a county-wide basis 26% to 28% of private wells are positive for total coliforms, E. coli, or nitrate-N > 10 ppm.
- At depths to bedrock less than 20 feet contamination rates generally exceed statewide averages.
- Well contamination results from both human and bovine fecal sources.
- The primary source of fecal contamination in the wells, bovine or human, appears to vary with groundwater recharge and the timing of manure application.
- Wells are contaminated with pathogens of significant concern: *Salmonella*, EHEC, *Cryptosporidium*, rotavirus.
- We estimate contaminated private wells are responsible each year in Kewaunee County for 140 people and 1,700 calves infected with *Cryptosporidium parvum*.

INSERT MOE’S AUTO-SAMPLER PRESENTATION

Living in Kewaunee County with a private well

- Water treatment by reverse osmosis or ultraviolet light
- Maintain water treatment equipment
- Be aware of heavy rainfall and snowmelt as times when wells are most vulnerable to contamination
- Monitor the USGS monitoring well in Kewaunee County for groundwater recharge
- Be careful to avoid contaminated drinking water exposure to young children, elderly, and people with altered immune systems

Study Next Steps

- Determine how fecal source, pathogen types, and pathogen concentrations are associated with well construction, hydrogeological, and environmental variables
- Prepare scientific manuscript for peer-review

Wish List

- Estimate risk of illness from private well water by risk assessment or epidemiological methods
- Develop health-risk based well vulnerability tool
- Develop early warning system for pathogen contamination of private wells
- Use nano-scale pathogen transport models to predict well contamination

Kewaunee County: Using research to help determine contaminants and risks to human health

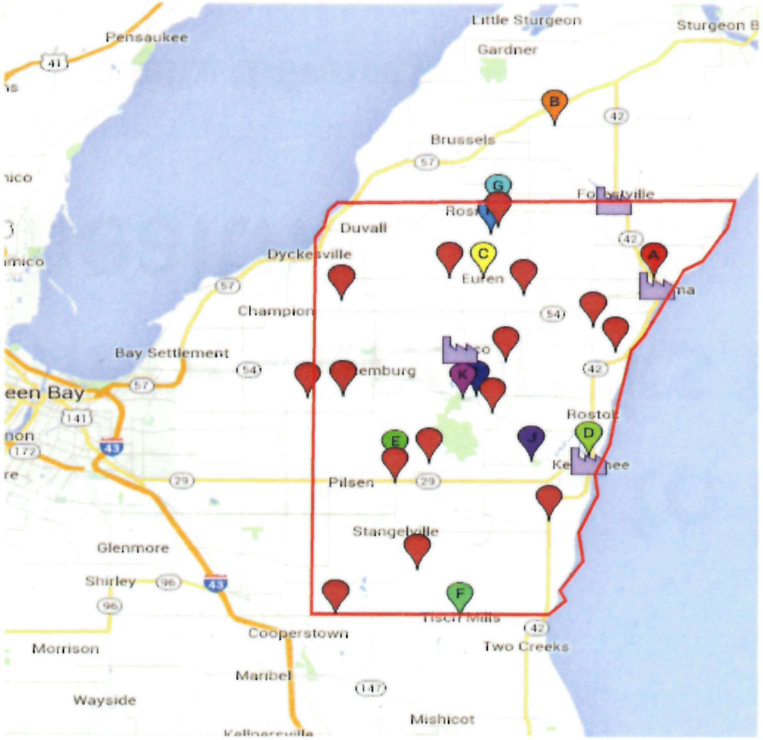
Dr. Krassimira Hristova, Marquette University

September 5th, 2017

MARQUETTE
UNIVERSITY

Surface and Groundwater Contamination in Kewaunee County, WI

- Impaired Rivers in KC
- Unsafe drinking water wells based on presence of nitrate, *E. coli*, *Salmonella* and viruses
- Point and Non-point source of pollution
- KC is home to 16 CAFOs + ~200 smaller farms; 4 Wastewater Treatment Plants (WWTPs)



Non-Point Source Pollution

- EPA Definition: “Discernible, confined and discrete conveyance, including but not limited to any … concentrated animal feeding operation … from which pollutants are or may be discharged.”
- Practical Definition: Pollution that does not come from a single source, such as a sewage pipe.
- Non-point Source Pollution is becoming an increasing threat to our water ways across the world.

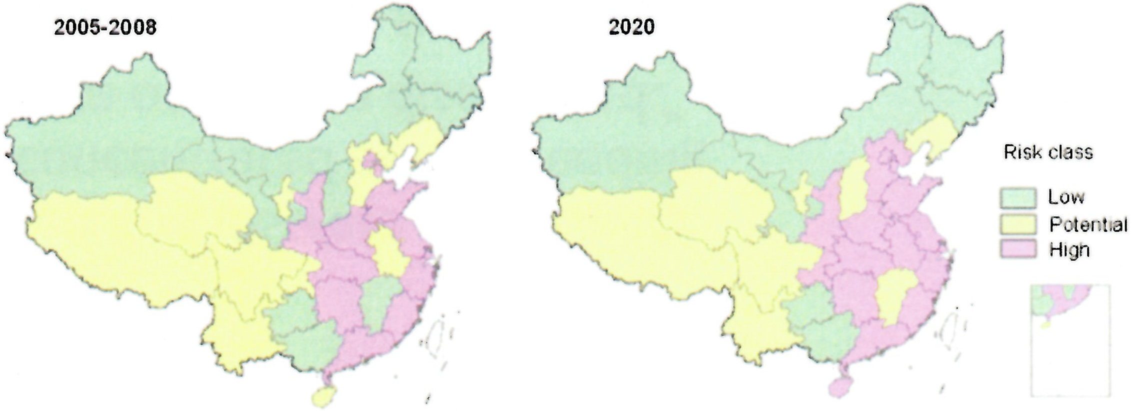
Figure 1 - Point Sources versus NPS Sources - Indiana County Conservation District

Sun, Bo et al. “Agricultural Non-Point Source Pollution in China: Causes and Mitigation Measures.” *Ambio* 41.4 (2012): 370–379. *PMC.* Web. 27 Mar. 2017.

Global Cases of NPS Pollution

Des Moines, Iowa Legal battle involving counties around central Iowa and their role in polluting water sources. Picture shows citizen sampling for nitrate measurements.

Threat assessment of waterways throughout China caused by non-point source pollution

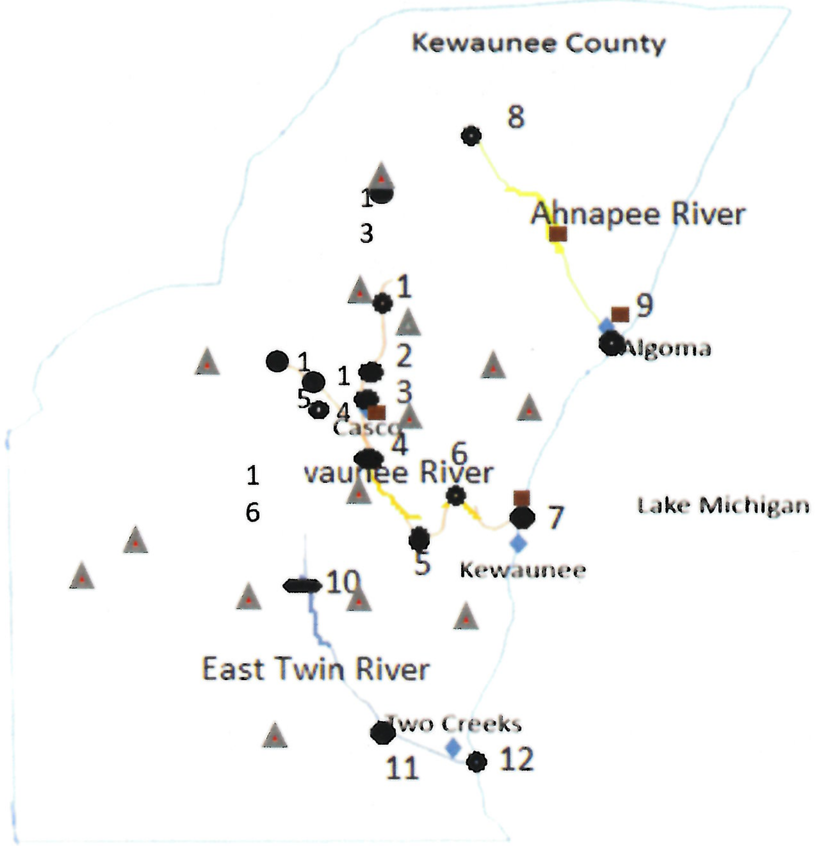


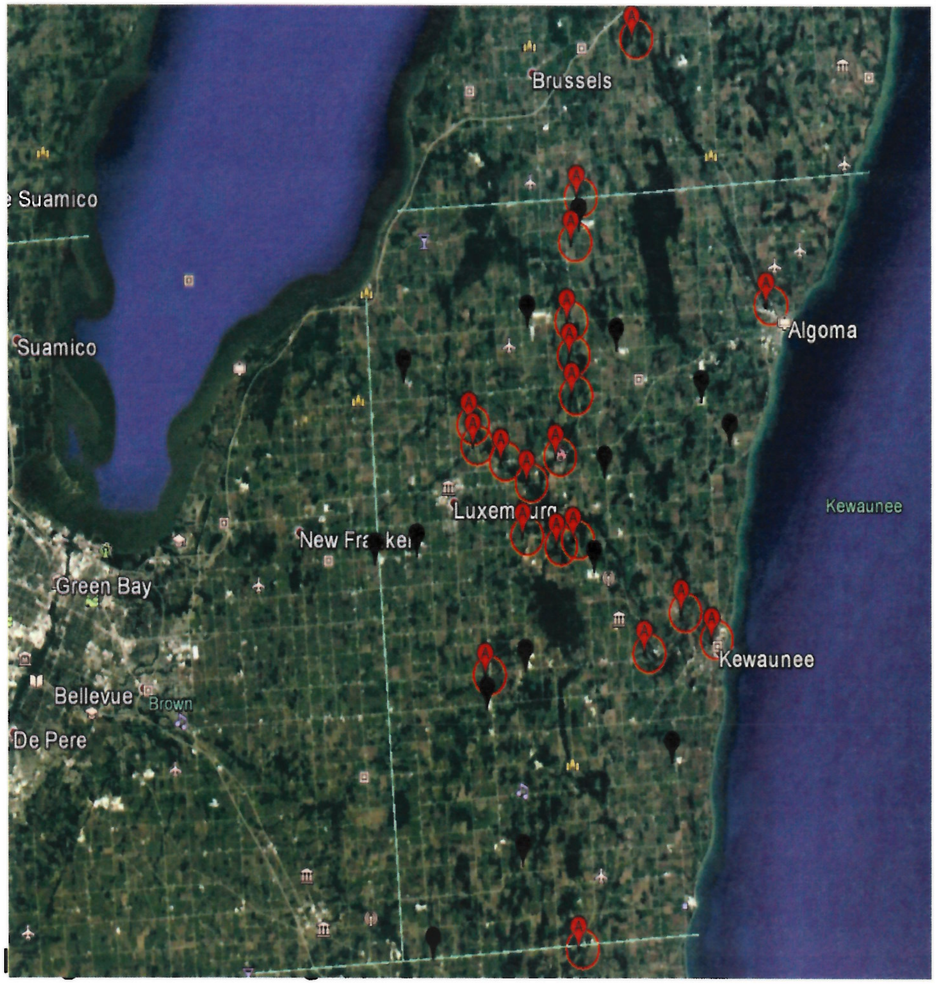
Sun, Bo et al. "Agricultural Non-Point Source Pollution in China: Causes and Mitigation Measures." *Ambio* 41.4 (2012): 370–379. *PMC*. Web. 27 Mar. 2017.

Regulations concerning water

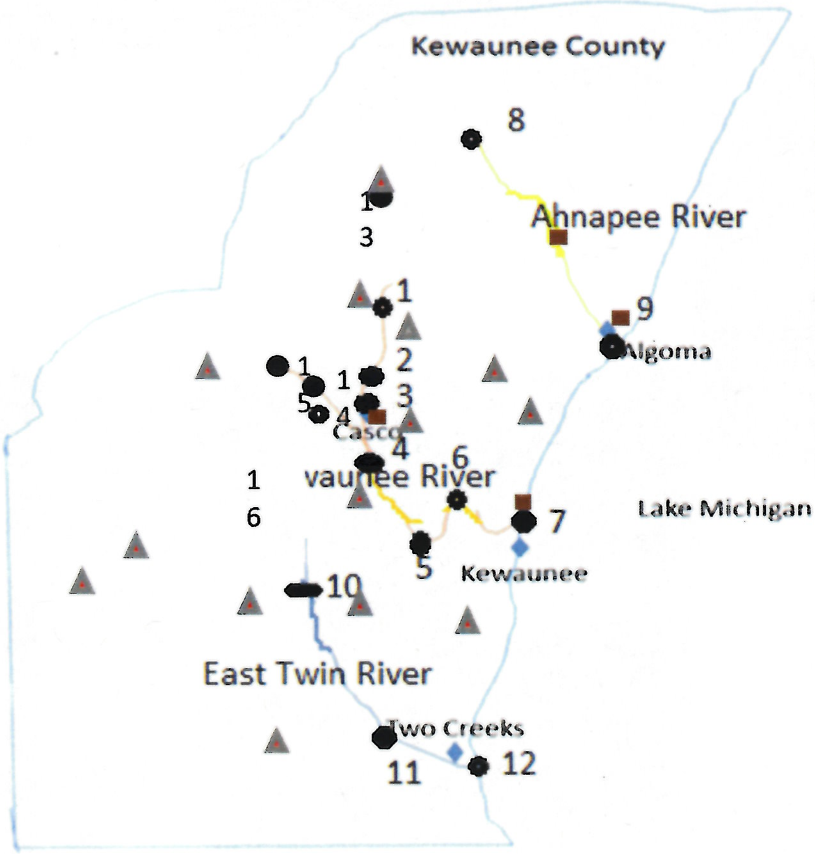
- Clean Water Act
  - EPA jurisdiction over point source pollution and dumping into surface water sources
  - Point source pollution focused
  - Does not cover ground water
- Safe Drinking Water Act
  - Covers all public drinking water sources to EPA regulation
  - Does not cover private wells
- National Pollutant Discharge and Elimination Services
  - Detailed Clean Water Act
  - Set testing standards for outflow

Assessing contamination in the Kewaunee River Watershed





Kewaunee Sampling



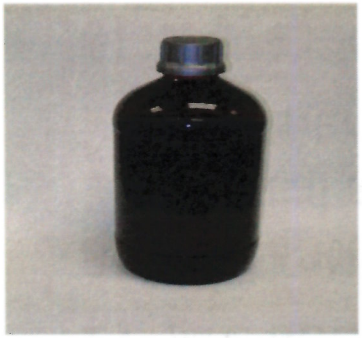
Top: Casco maple (Left),
Kewaunee river Source (Right)
Center: Kewaunee Rockledge
Bottom: Kewaunee River Mouth
(Left), Casco Crevice (Right)

Research Questions

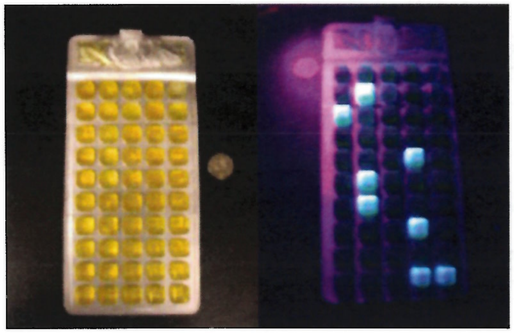
1) What is the level of nutrients and fecal pollution in Kewaunee surface waters?
2) Are hormones and PPCPs present?
3) Are antibiotic resistance genes (ARGs), coding for resistance to clinical antibiotics, present in Kewaunee County surface waters and sediment?
4) If present, does proximity to CAFO operations impact ARG levels?
5) Does seasonal manure application impact the dissemination of ARGs in Kewaunee County?

Sampling

Chemical Methods

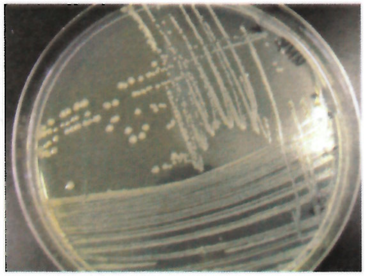


Water Chemistry

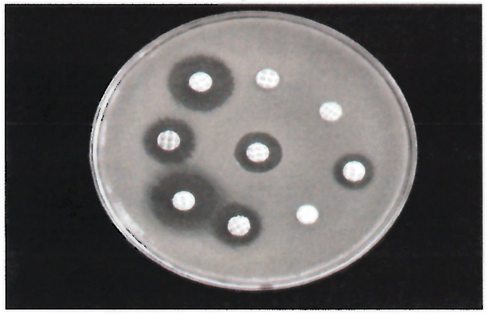


Total Coliforms

Isolation Methods



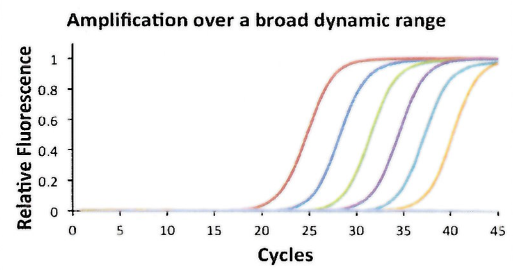
Bacterial Isolation



Antibiotic Disk Diffusion

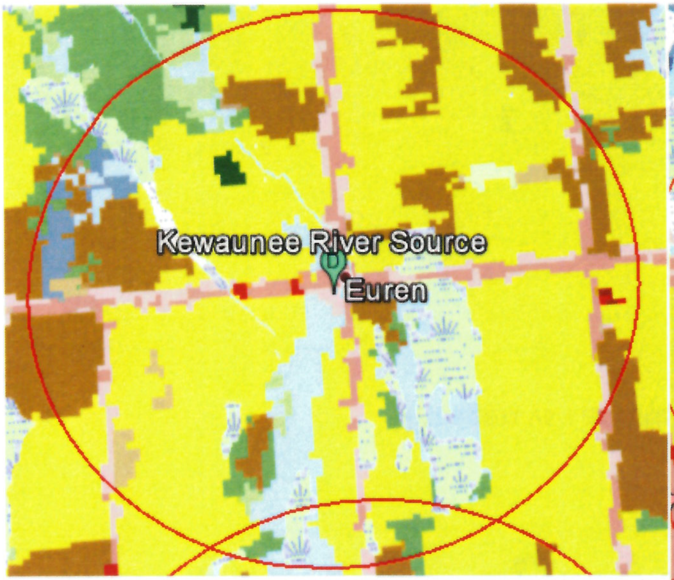
Molecular Methods

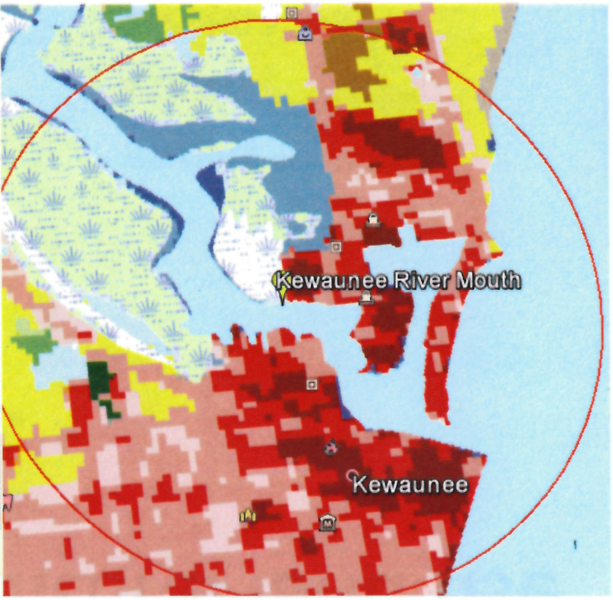
DNA extraction

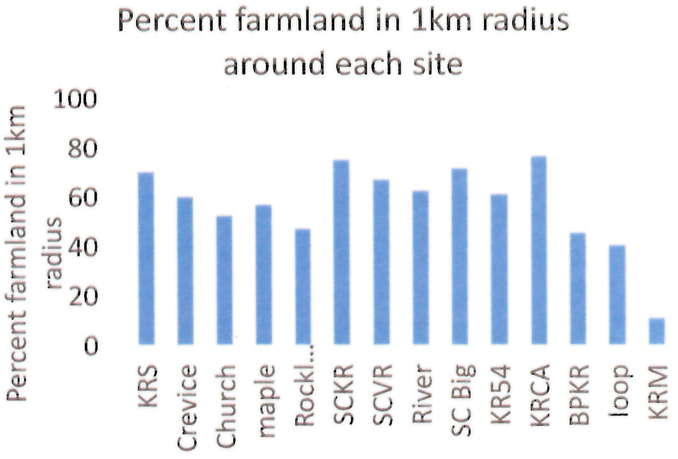


qPCR of antibiotic and *Bacteriodes* genes

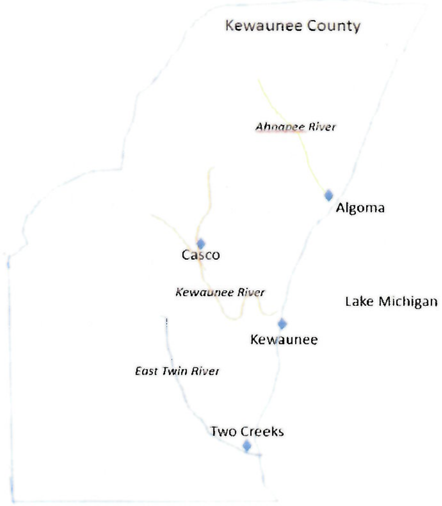
Map of land use around Kewaunee River source and mouth sites

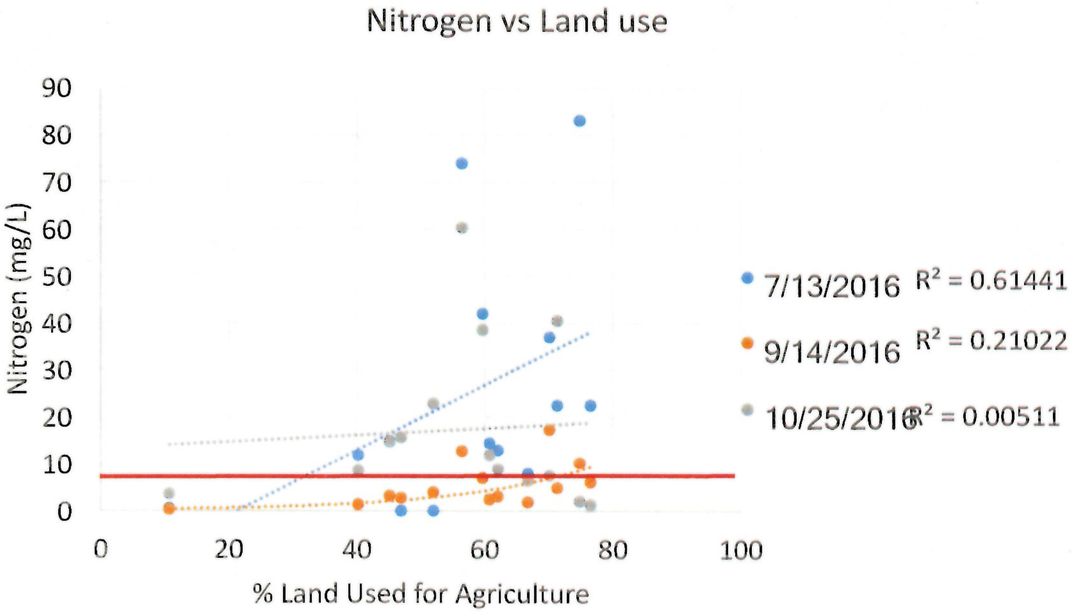






Nitrogen positively correlates with percent of agricultural land use



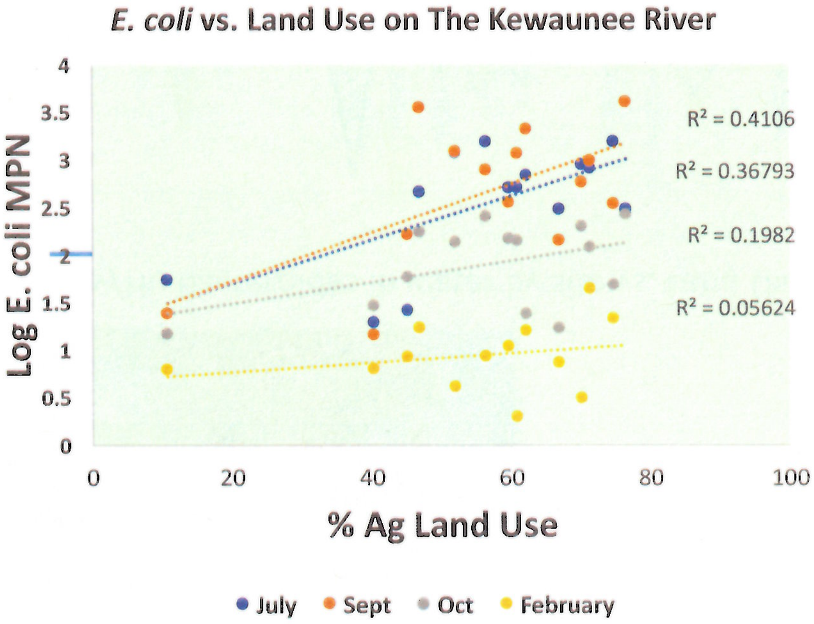


Red line:
EPA Limit: Nitrogen=10mg/L

Detected PPCPS and Hormones

- caffeine
- carbamazepine
- fluoxetine
- naproxen
- triclocarban
- gemfibrozil
- sulfamethazine
- triclosan
- **estrone-3-sulfate**

E. coli correlates with Agricultural Land Use

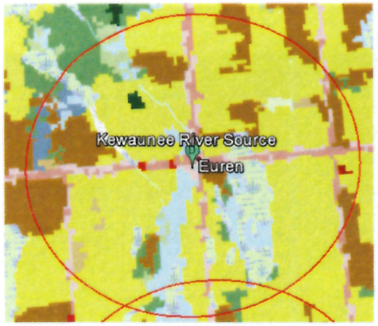


EPA Limit:

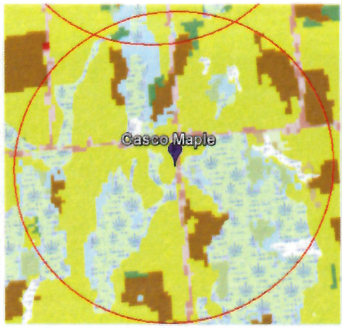
E.coli = 126MPN/100ml(LOG 2.1)

Bovine/Human Fecal Markers Detected in Water

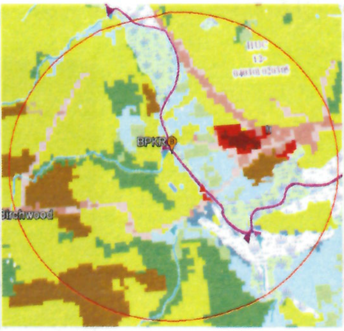
Kewaunee River Source 70%



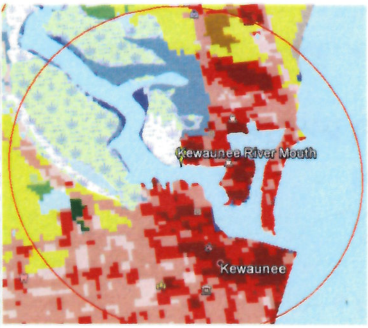
Casco Maple 56%

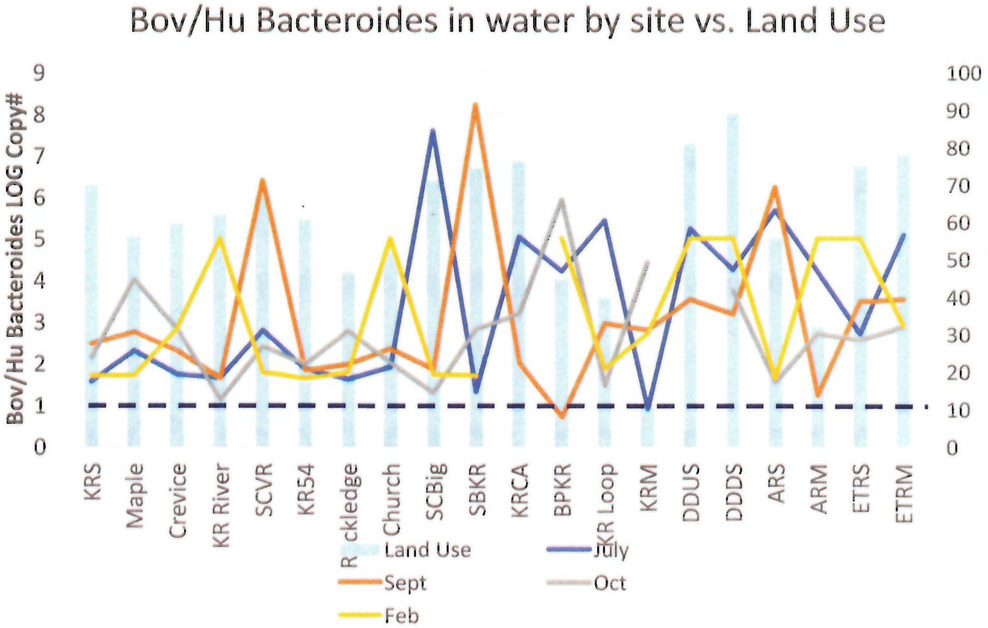


Bruemmer Park 40%



Kewaunee River Mouth 10%





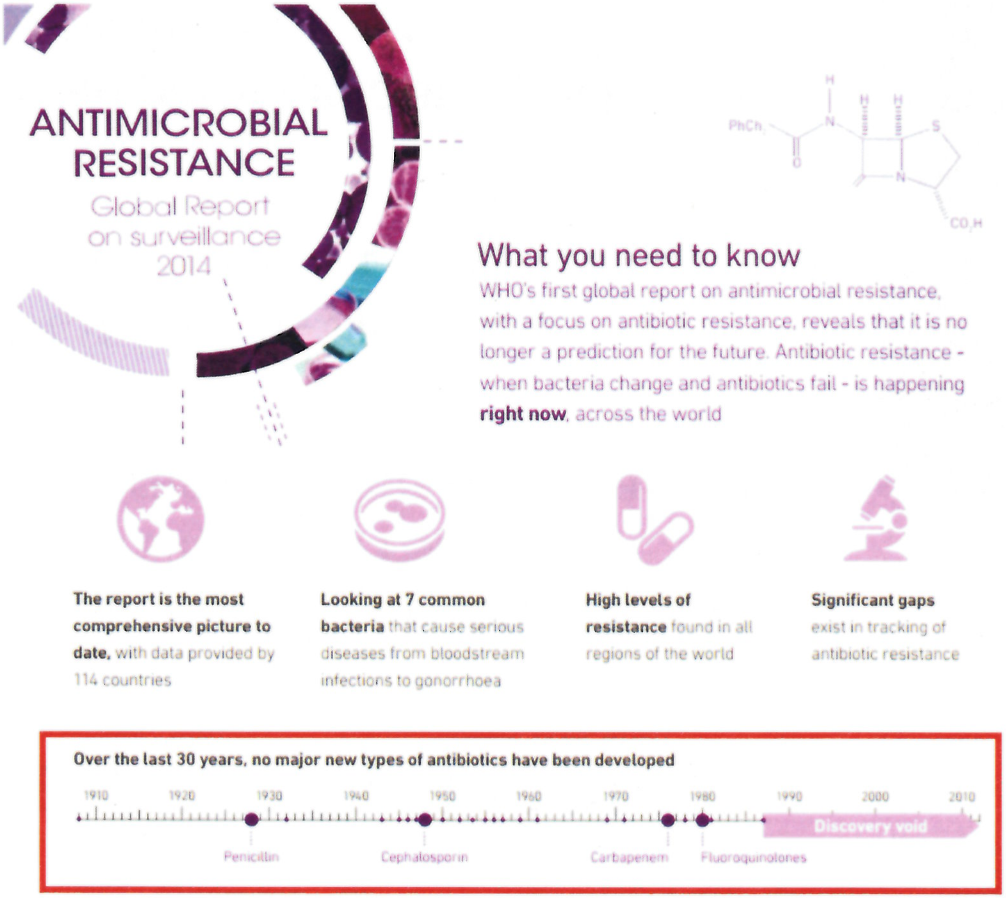
- Sites with ratios greater than 1 showed greater bovine contamination than human
- Sites less than 1 showed greater human

Antibiotic Use: The Problem

- Overuse and misuse of antibiotics in agricultural and healthcare settings increases the risk of development of multidrug resistant bacteria (CDC, 2017)
- MRSA
  - Methicillin-resistant *Staphylococcus aureus*
  - Resistant to most clinically relevant antibiotics
- Other common human pathogens are quickly gaining multidrug resistance
  - *E. coli, Pseudomonas sp., Aeromonas sp.*

Google Image, 2017

Antibiotic Resistance: A Worldwide Health Problem



ANTIMICROBIAL RESISTANCE

Global Report on surveillance 2014

What you need to know

WHO's first global report on antimicrobial resistance, with a focus on antibiotic resistance, reveals that it is no longer a prediction for the future. Antibiotic resistance - when bacteria change and antibiotics fail - is happening **right now**, across the world

The report is the most comprehensive picture to date, with data provided by 114 countries

Looking at 7 common bacteria that cause serious diseases from bloodstream infections to gonorrhoea

High levels of resistance found in all regions of the world

Significant gaps exist in tracking of antibiotic resistance

Over the last 30 years, no major new types of antibiotics have been developed

WHO. Global Report on Surveillance. 2014.

Antibiotic Resistance: The Facts

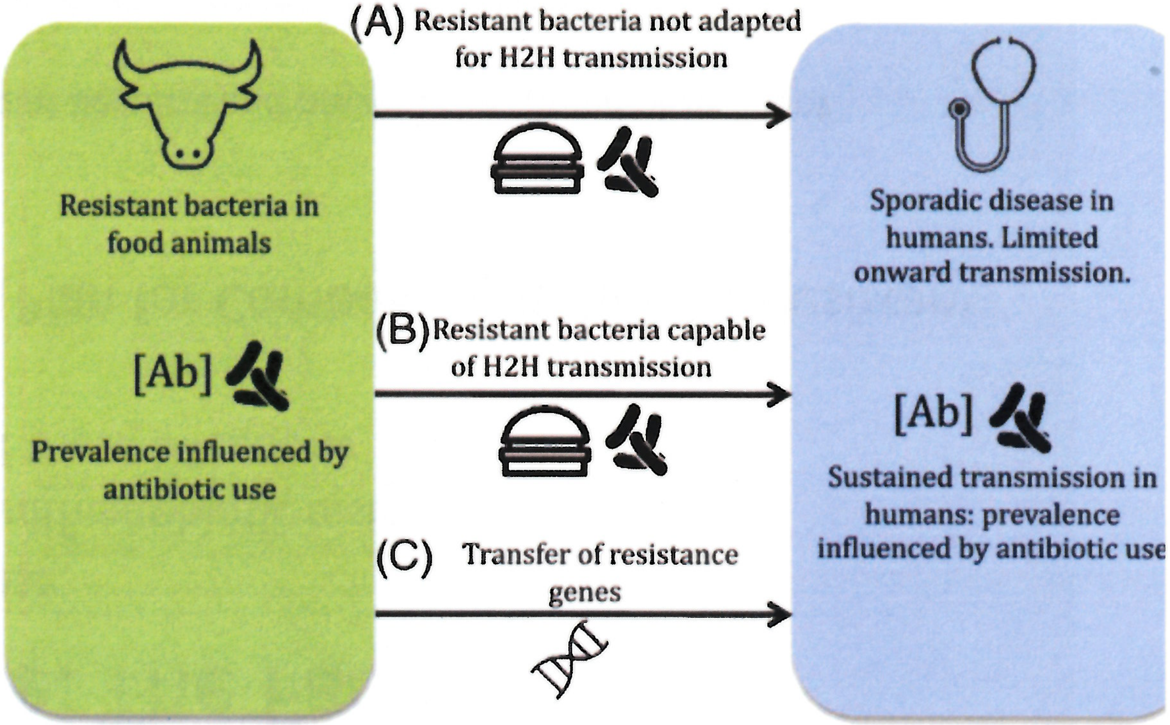
- Each year in the United States:
  - 2 million people are infected with antibiotic resistant bacteria
  - 23,000 die as a direct result of these infections

- In 2015, The National Action Plan for Combatting Antibiotic Resistant Bacteria was introduced
  - Goals include:
    1. Slow the emergence of resistant bacteria and prevent the spread of resistant infections.
    2. Advance development and use of rapid and innovative diagnostic tests for identification and characterization of resistant bacteria.
    3. Improve international collaboration and capacities for antibiotic-resistance prevention, surveillance, control, and antibiotic research and development.

CDC, 2015

Antibiotic Resistance: Environmental and Clinical Connections

- Antibiotic use in humans and livestock selects for antibiotic-resistant bacteria
- 80% of all antibiotics in US are received by livestock (Hollis and Ahmed 2013)
- 25-75% of antibiotics are excreted unchanged in animal feces (Sarmah *et al*. 2006)



Resistant bacteria in food animals

[Ab]

Prevalence influenced by antibiotic use

(A) Resistant bacteria not adapted for H2H transmission

(B) Resistant bacteria capable of H2H transmission

(C) Transfer of resistance genes

Sporadic disease in humans. Limited onward transmission.

[Ab]

Sustained transmission in humans: prevalence influenced by antibiotic use

Chang *et al.* 2015. Evol Appl, 8(3): 240-247.

Common Agricultural Practices Linked to Antibiotic Resistance

- Environment is a natural reservoir of antibiotic resistant bacteria and genes (Finley *et al.* 2013)
  - Enhanced by common agricultural practices such as:
    - Antibiotic use for disease treatment, prevention, and growth promotion
    - Manure fertilization of field crops
    - Concentrated Animal Feeding Operations (CAFOs)

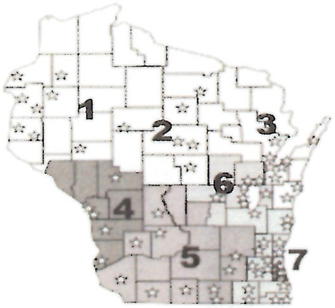
- CAFOs
  - More than 1,000 animal units confined on site for more than 45 days
  - Disease prevention and control needs increase
  - Waste management needs increase

Clean Wisconsin, 2017

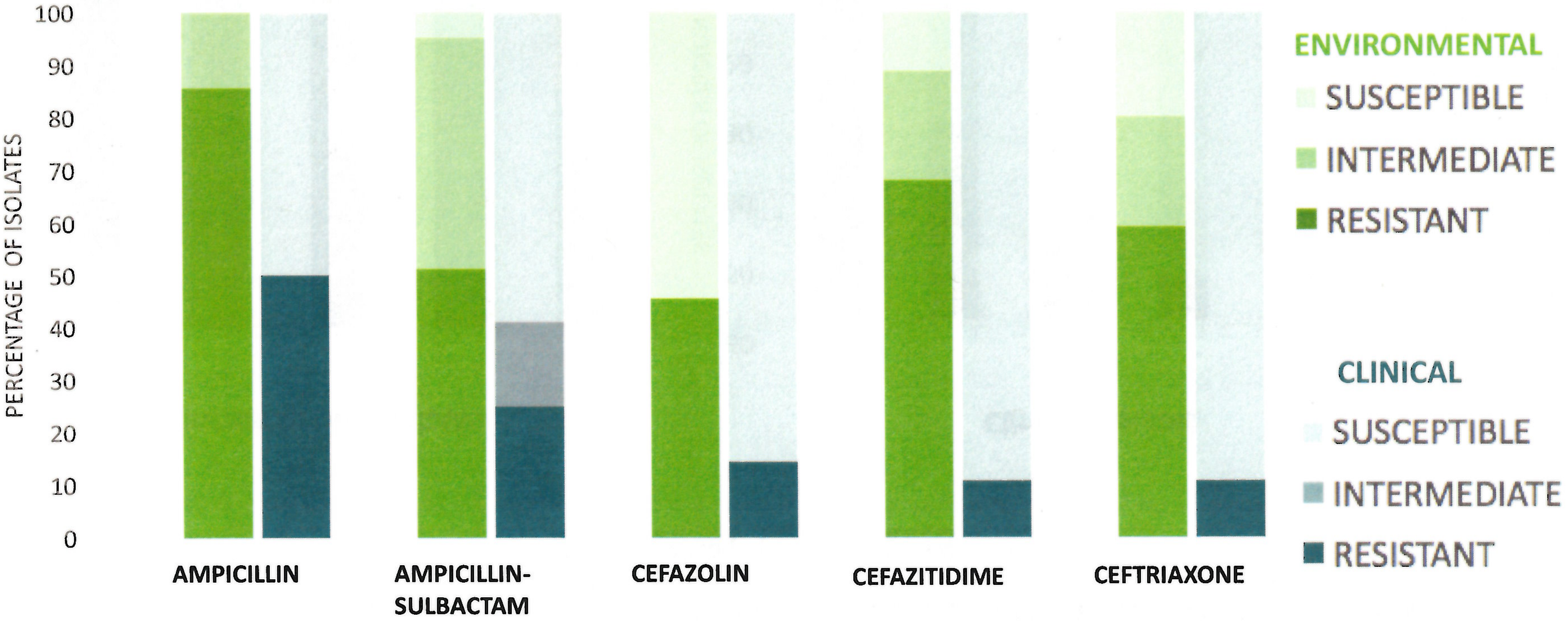
Multi-drug Resistant Bacterial Isolates from Kewaunee County

- Multi-drug resistance (MDR) is defined as resistance to 3 or more classes of antibiotics
- *Pseudomonas sp.*
  - 3/6 Isolated Strains MDR
- *E. coli*
  - 6/10 Isolated Strains MDR
- *Aeromonas sp.*
  - 28/80 of Isolated Strains MDR

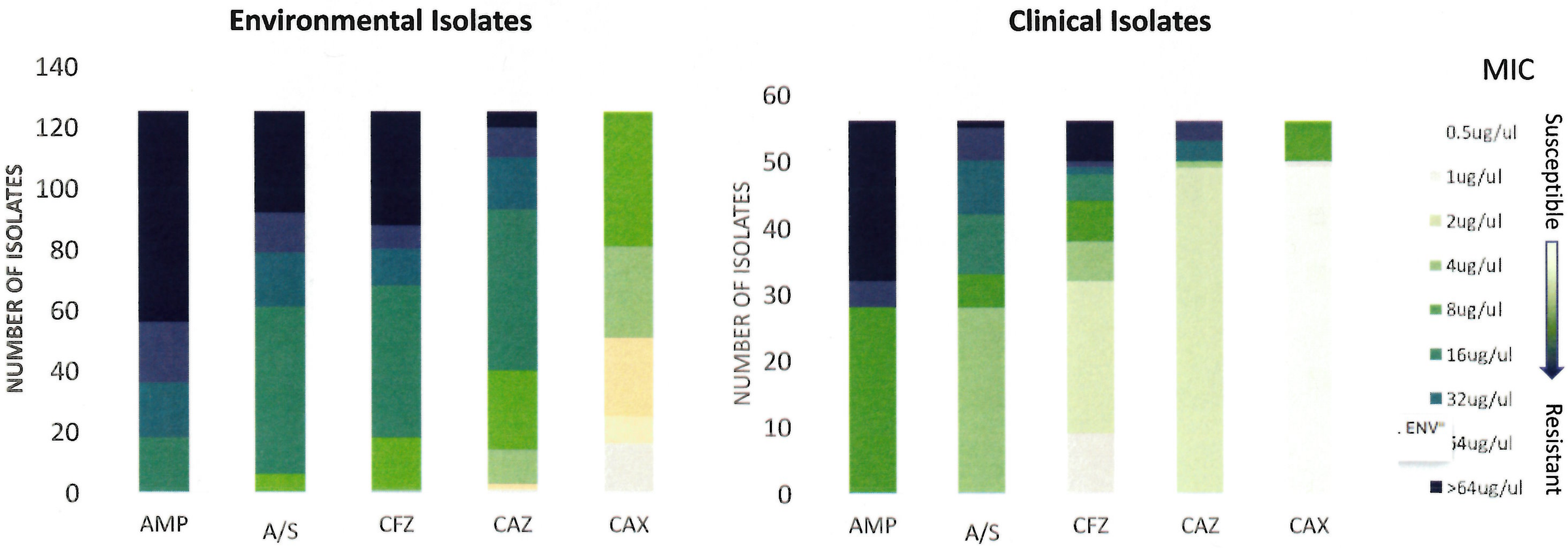
- *Aeromonas hydrophila*
  - Known human pathogen commonly transmitted through fecal contamination
  - 75% (9/12) of isolated strains are Multi-drug resistant in Kewaunee Isolates



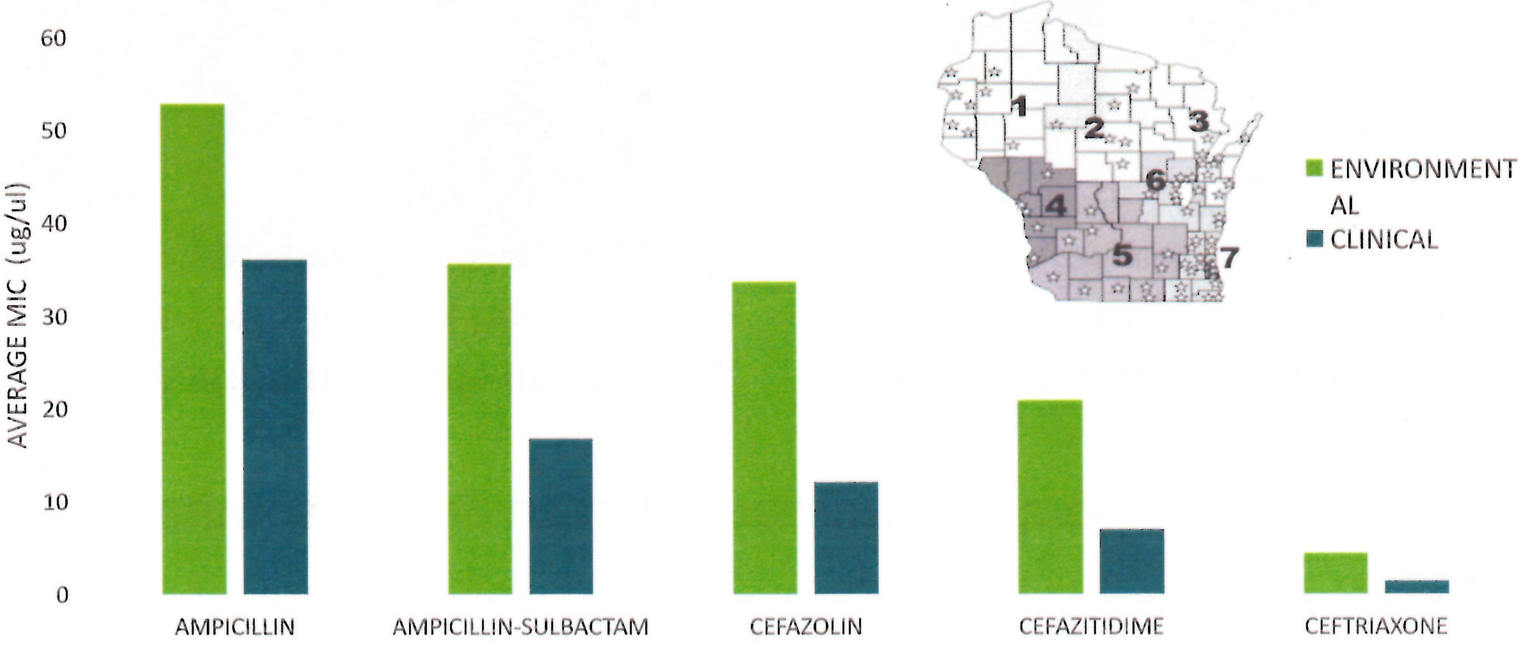
Environmental *E.coli* have higher abundance of AB resistance than clinical *E.coli*



Environmental *E.coli* isolates have higher MICs than clinical isolates



Environmental *E.coli* isolates have higher MICs than clinical isolates



Molecular Methods for Detecting Antibiotic Resistance

- Multiple methods can be used to measure antibiotic resistance

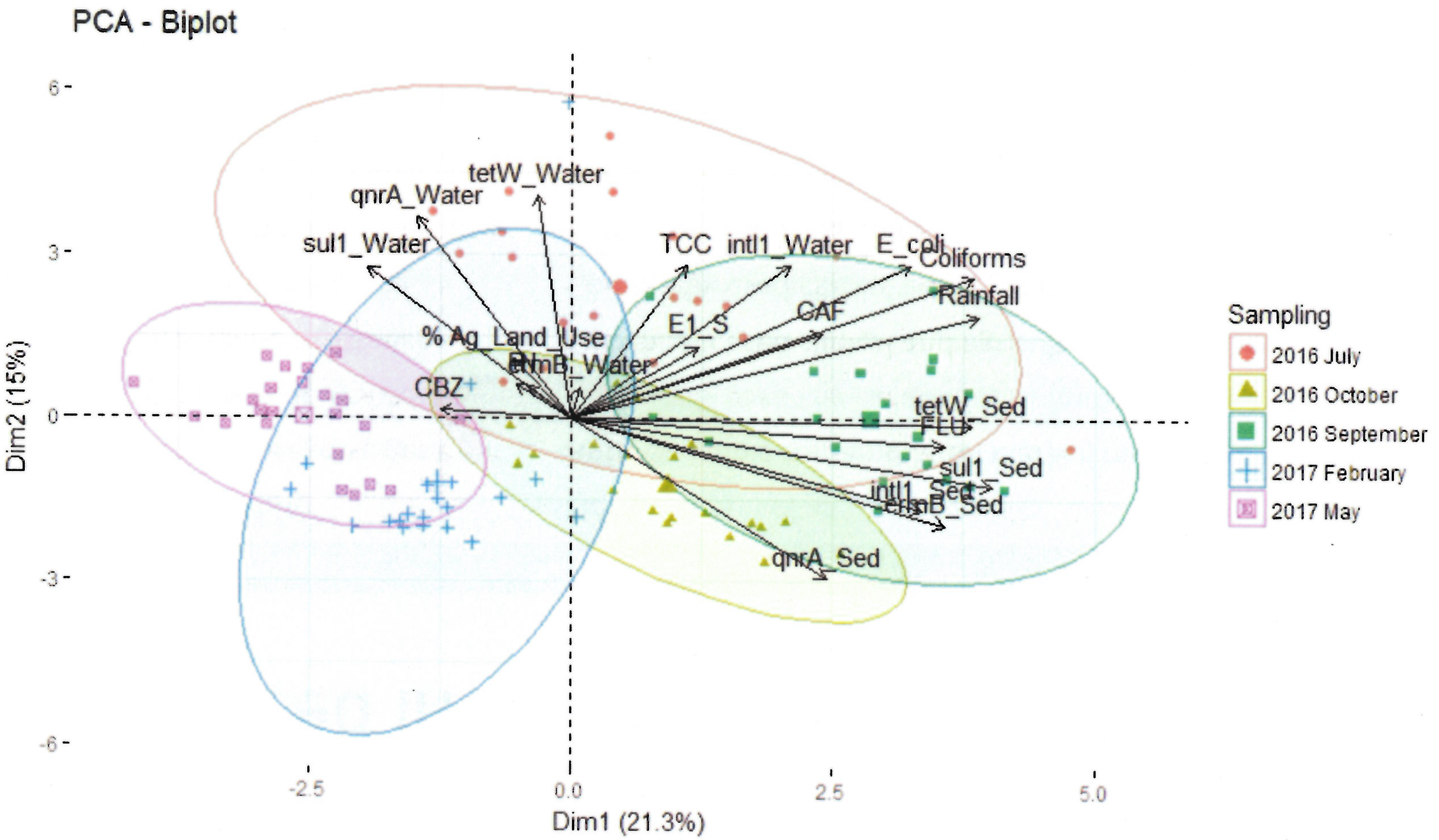
Isolation and cultivation methods measure resistance profiles for individual bacteria

Molecular methods can measure the number of antibiotic resistance genes present in the entire sample

ARG analyzed in this study

Gene	Measurement	Significance
16S rRNA	Total Bacteria	Normalization of all other genes
intI1	Integron-integrase gene	Anthropogenic contamination marker, multi-drug resistance
sul1	sulphonamide resistance	Commonly used clinical and agricultural antibiotic
tet(W)	Tetracycline resistance	Commonly used clinical and agricultural antibiotic
erm(B)	Macrolide resistance	Commonly used clinical and agricultural antibiotic
qnrA	Fluoroquinolone resistance	Restricted use in agriculture

PCA on all measured variables

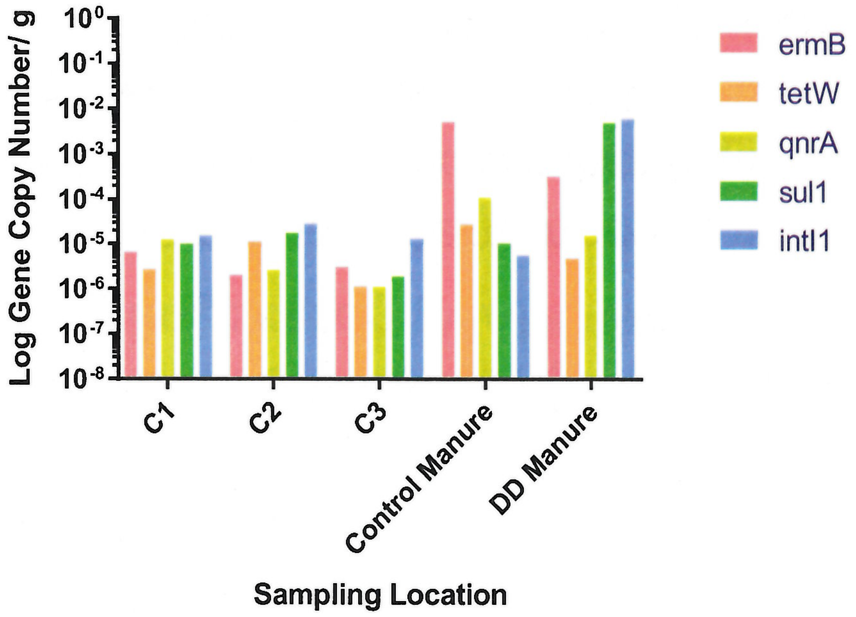


Seasonal gene variation is statistically significant

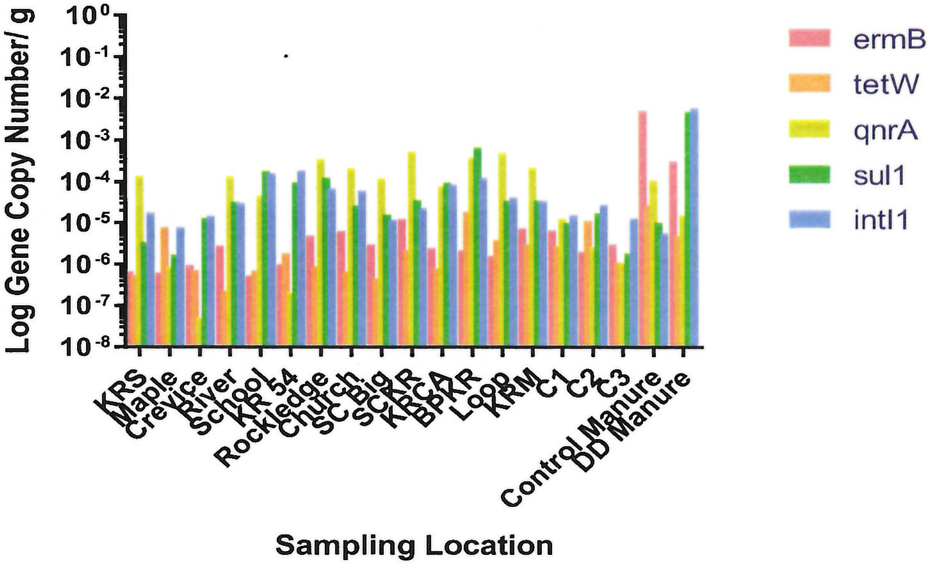
- Significant differences ($p<0.05$) in gene copy number between sampling season (MANOVA analysis)
- Post-hoc tests determined significant differences between:
    - February 2017 and October 2016
    - May 2017 and October 2016
        - **October contained the highest mean gene copy numbers**

    - Accumulation effect of growing season manure application

Manure from Small and a Big farm Contain Elevated ARGs



May 2017 Gene Abundance in Control and Manure Samples



May 2017 Gene Abundance

Conclusions from this work

- E. coli and coliforms are present above EPA standard for recreational water in Kewaunee Rivers
- Nitrate is above drinking water standard at multiple sites
- Presence of low concentrations of hormones (estrone) and pharmaceuticals (fluoxetine) are a threat for chronic exposure to aquatic life and humans
- Multidrug resistant, pathogenic bacteria are residing within river sediment

Conclusions

- ARGs and Class 1 integrons are present in Kewaunee County surface waters and sediment
- Gene copy numbers are strongly influenced by season

  Connected to manure application
- Manure from antibiotic treated cattle contains significantly higher sul1 and intl1 genes than untreated manure
- All sites contain elevated ARGs compared to background

  - Higher gene copy numbers were present in sediment samples
  - Highest numbers were found in October
- **Why is this a concern?**
  - Sediment is an indicator of long-term contamination
  - Sediment provides space for bacterial attachment and cell-to-cell contact
    - Facilitates Horizontal Gene Transfer (Janzon *et al*, 2012; Marti *et al*, 2013)
  - October copy numbers represent year-long accumulation after seasonal manure application
  - Bacteria trapped in sediment can re-suspend in the water column after rain events
    - Provides a constant reservoir of antibiotic resistance genes

Conclusions

Together, the chemical and biological assessment confirms impairment of Kewaunee County surface water and poses concerns for fishing, recreation, and drinking water wells of local residents

Farming practices in Kewaunee County impact ARG abundance on a seasonal scale

What we could do to mitigate the problem of antibiotic resistance on a global scale?

- Stop the use of antibiotics in feed for farm animals
- Do not dispose unused medications
- Develop alternatives to treat multidrug-resistant bacteria – phage therapy

What we could do to mitigate the problem of water pollution on a local scale?

- Work on changing environmental policy
  - Task force on alternative strategies for manure management

- New technologies for manure treatment before land application

Acknowledgements

- Hristova Lab
    - Mike Walsh
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- Kewaunee residents for help with sampling

- Environmental Analyses
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- Marquette University Innovation Grant

MARQUETTE
UNIVERSITY

BE THE DIFFERENCE.

Well Water in Karst Regions of Northeastern Wisconsin Contains Estrogenic Factors, Nitrate, and Bacteria

Angela C. Bauer[1*], Sarah Wingert[2], Kevin J. Fermanich[3], Michael E. Zorn[3]

ABSTRACT: Well water in karst regions is particularly susceptible to contamination by various nonpoint source pollutants such as nitrate, fecal bacteria, and endocrine disrupting chemicals (EDCs). This study analyzed 40 wells in heavily farmed karst areas of northeastern Wisconsin to determine whether these and other pollutants are present, and if so, whether their presence is (1) correlated with other contaminants and (2) exhibits seasonal variation. Nitrate, bacteria, and estrogenicity (indicating the presence of EDCs) were present in at least some of well water samples collected over the course of four time periods between the summers of 2008 and 2009. Although estrogenicity was greatest during the summer months, bacterial contamination was most prevalent during snowmelt. Levels of estrogenicity present in some well water samples approached a threshold concentration that is known to exert endocrine disruption in wildlife. Strong correlations between estrogenicity and other water quality parameters were not found. *Water Environ. Res.*, **85,** 318 (2013).

KEYWORDS: groundwater, nitrate, coliform, *Escherichia coli*, enterococci, endocrine disruptors (EDCs), estrogenicity, E-screen assay.

doi:10.2175/106143012X13373575831358

Introduction

Groundwater in karst regions moves rapidly into the subsurface through conduits (sink holes, sinking streams, and springs) or porous, fractured bedrock where relatively little filtration of water contaminants occurs (Vesper et al., 2001). Thus, karst aquifers are particularly vulnerable to groundwater pollution. The susceptibility of karst aquifers to pollution is particularly problematic in rural, agricultural areas where residents commonly rely on private wells and springs for their drinking water. Studies in northeastern Wisconsin (e.g., Erb and Stieglitz, 2007) have documented that well water in agricultural regions possessing karst topography frequently contains contaminants (e.g., bacteria and nitrate [NO_3-]) that pose a significant human health threat. Potential sources of these contaminants include land spread manure and sewerage sludge, and sewage effluent from improperly constructed septic systems.

Consumption of well water contaminated with fecal bacteria and nitrate from human or animal waste is associated with a variety of adverse health effects, some of which can be life-threatening or even lethal. For example, consumption of water containing verotoxin- (Shiga toxin) producing strains of *Escherichia coli* such as *E. coli* O157:H7 (an enteric pathogen commonly found in livestock manure) can produce symptoms ranging from mild gastrointestinal illness to hemorrhagic colitis to renal failure and death (Pell, 1997). Consumption of water containing high concentrations of nitrate, such as from cropland runoff of synthetic fertilizers, can cause methemoglobinemia (*blue baby syndrome*) in infants (Karr, 2012). The significant human health threat posed by the consumption of well water contaminated with bacteria and nitrate has led many U.S. counties and states to enact legislation that regulates the land application of animal and human waste (Brown County Wisconsin, 2007; Illinois Department of Agriculture, 2012; Kewaunee County Wisconsin, 2010; Wisconsin Department of Natural Resources, 2002).

Public health concern continues to grow over the presence of another class of organic compounds found within groundwater that might pose a human health threat. These chemicals—called endocrine disrupting chemicals or EDCs—originate from a wide variety of sources (NRC, 1999), including human waste (e.g., synthetic hormones from contraceptives), animal waste (e.g., endogenous or synthetic hormones injected into livestock to induce growth), and pesticides commonly applied to croplands (Hodges et al., 2000). Many EDCs have been shown to mimic or block the actions of endogenous sex hormones (estrogens and androgens) within the body. Given that sex hormones are the principal regulators of the development and function of a wide variety of tissues, a great potential exists for EDCs to cause physiological abnormalities in exposed organisms (Colburn et al., 1996).

Of particular concern for humans is the possible association between EDC exposure and endocrine-related cancers. For example, cumulative exposure to estrogen is a known risk factor for the development of breast cancer (Dorgan et al., 1996; Toniolo et al., 1995). In addition to laboratory studies linking EDC exposure with the development of breast cancer in mice (Murray et al., 2007), research has also found a correlation between elevated levels of EDCs such as DDT (dichlorodiphenyltrichloroethane) and the development of breast cancer in young women (Cohn et al., 2007). Additional concerns have been raised about EDC exposure and a male's risk for infertility

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(Sakaue et al., 2001; Susiarjo et al., 2007; Toppari et al., 1996, 2002) and for developing androgen-sensitive cancers such as testicular (Skakkebaek et al., 2001; Weir et al., 2000) and prostate cancer (Fleming et al., 1999; Ho et al., 2006).

A critical step toward minimizing exposure to EDCs and thereby decreasing associated health risks is identifying routes of contamination within the environment. Recently, attention has turned to livestock waste as a source of EDCs. Manure is a rich source of EDCs because it contains both endogenous estrogens from cattle (estradiol, estriol, and estrone) (Hanselman et al., 2006; Peterson et al., 2000) and synthetic steroids administered to livestock as growth-enhancing agents (Herschler et al., 1995). One important route for manure-borne EDCs to enter the environment results from the common practice of applying animal wastes to pastures and croplands as fertilizer. Several studies have suggested that land application of animal wastes results in EDC contamination of agricultural drainage water and groundwater (Hanselman et al., 2006; Peterson et al., 2000) at concentrations that are known to elicit biological effects (Irwin et al., 2001; Panter et al., 1998).

Groundwater contamination by manure runoff is of particular concern to residents of northeastern Wisconsin given the unique geology of the region, much of which is characterized by carbonate bedrock areas, shallow soil depths, and karst features (sink holes and bedrock openings). These allow ready access of surface contaminants to well water. A 2007 report of the Northeast Wisconsin Karst Task Force (Erb and Stieglitz, 2007) found that a significant portion of water supply wells in northeastern Wisconsin are contaminated by bacteria or high levels of nitrate. Numerous instances of contamination have been linked to manure runoff in recent years, particularly during the spring thaw. When the Calumet County Land and Water Conservation Department conducted voluntary well water testing in spring of 2007, they found that 32% of samples tested positive for some level of coliform bacteria (an indicator of fecal contamination by livestock, humans, or other animals), and contained high nitrate levels (Calumet County Wisconsin, 2007). Similar findings were reported by the neighboring Brown County Land Conservation Department in an analysis of well water samples collected from the Town of Morrison (Erb and Stieglitz, 2007).

The majority of coliform-positive well water samples identified in the above county studies originated from areas in northeastern Wisconsin that are heavily farmed with relatively shallow soils over fractured dolomite. Thus, it is likely that groundwater contamination in this region is a result of land application of livestock manure as fertilizer to pastures and croplands. Given that livestock manure contains appreciable amounts of steroid hormones (Hanselman et al., 2006; Peterson et al., 2000), concerns have arisen that manure-born EDCs are also contaminating well water.

In this study, well water samples were collected from drinking water wells in five northeastern Wisconsin counties and analyzed for nitrate and bacteria (including total coliforms, *E. coli*, and enterococci). In addition, levels of estrogenicity (indicating the presence of EDCs) were measured through use of the MCF-7 breast cancer cell proliferation assay, which is commonly referred to as the E-screen assay (Soto et al., 1995). Well water samples were collected during four time periods to examine seasonality trends as well as potential changes associated with recharge periods (i.e., heavy rainfall or spring thaw).

Methodology

Well Selection and Sample Collection. The study area consisted of rural land in northeastern Wisconsin with known instances of past groundwater contamination of the uppermost Silurian aquifer. Forty private wells within five counties (Brown, Calumet, Dodge, Fond du Lac, and Kewaunee Counties) (Figure 1) were selected to investigate the potential for groundwater contamination with estrogenic chemicals, fecal bacteria, and nitrate. A significant portion of each of these counties is underlain by the Silurian bedrock aquifer, has extensive areas where the unconsolidated surficial sediment and soil is <15 m deep, and contains karst features (i.e., swalets, sink-holes, fractures). In addition to their susceptibility to groundwater contamination, these counties were chosen because representatives from local environmental agencies were willing to help contact well owners and sample the wells. Ten wells per county were selected for sampling in Brown, Calumet, and Kewaunee Counties. Eight wells were selected from Fond du Lac County and two from Dodge County that were immediately south of the Fond du Lac wells. For sample collection and analysis purposes, the Dodge County wells were included with those of Fond du Lac because of their close proximity. Figure 1 shows the distribution and approximate locations of the study wells.

Note that the 40 wells chosen for this study were not selected in a statistically rigorous manner, nor were they chosen with the intent to represent county-level water quality trends. Rather, well selection was based on following characteristics: (1) they were cased into the Silurian aquifer, (2) they were shallow (all but two wells were <60 m in depth), (3) historical sampling data for fecal bacteria and nitrate were available, (4) the well owners agreed to participate in the study, and (5) the wells were located in areas with suspected or known sources of agricultural contamination.

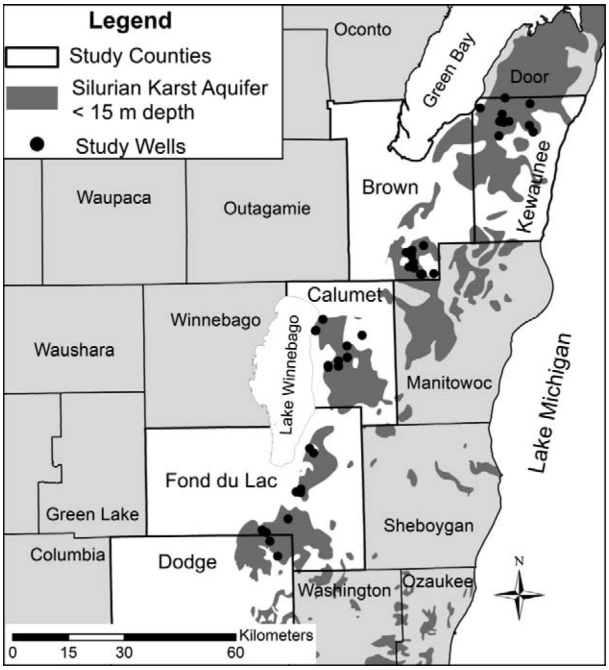

Figure 1—Location of study counties in northeastern Wisconsin and approximate locations of drinking water wells. The relationship to areas dominated by the shallow Silurian karst aquifer is also shown.

A total of eight wells from each county were designated *susceptible* to contamination; two wells from each county were deemed *control* wells based on low levels of past contamination (no or low fecal bacteria counts and <2 mg/L of nitrate measured as NO_3-N). Groundwater samples were collected from each well in mid-August 2008, mid-November 2008, mid-to-late February 2009, and mid-March 2009 by a county representative or University of Wisconsin-Green Bay researcher.

Bacteria. Samples were analyzed for fecal bacteria within 24 h of collection at the University of Wisconsin-Oshkosh Halsey Science Center's Environmental Microbiology Laboratory. *Escherichia coli* and total coliforms were measured using the IDEXX Laboratories (Westbrook, Maine) Colilert reagent; enterococci was measured using the IDEXX Enterolert test kit.

Nitrate. Samples were analyzed for nitrate within 48 h of collection at the University of Wisconsin-Green Bay using a Lachat (Loveland, Colorado) QuickChem 8500 Flow Injection Analysis system, and following Lachat QuikChem Method 10-107-04-1-A (Wendt, 2007). Nitrate results were reported as mg/L NO_3-N with a detection limit of 0.1 mg/L NO_3-N.

Conductivity. The conductivity of each sample was measured using a Hydrolab Quanta G water quality sonde (Hydrolab Corporation, Loveland, Colorado) within 48 h of collection and reported in mS/cm.

Sample Extraction for Biological Assays. One sample from each well was extracted for estrogenicity testing within 48 h of collection at the University of Wisconsin-Green Bay per Drewes et al., (2005). The extraction procedure involved vacuum filtering a 1 L sample through an Empore C-18 extraction disk (Product No. 2215, Fisher Scientific, Pittsburgh, Pennsylvania) that was preconditioned using a 1:1 solution of ethyl acetate/methylene chloride, methanol, and high-purity water. Materials remaining in the sample bottle and on the extraction disk were eluted into a 15-mL glass vial using the following solvent series: 5 mL of ethyl acetate, 5 mL of 1:1 ethyl acetate/methylene chloride, and 5 mL of methylene chloride. Samples were stored at 4 °C pending further processing and analysis. Next, a nitrogen dry-down procedure was performed in which a sample extract was dried almost completely with ultra-high purity nitrogen (99.999% purity, <1 ppm oxygen, and <0.5 ppm hydrocarbons) before being rinsed with methanol three times. The remaining sample extract and methanol rinses were transferred to a 1.5 mL amber vial and evaporated with nitrogen to 1 mL. The extracts in methanol were stored in a freezer.

Field blanks, duplicates, spikes, and a high-purity water blank underwent the above extraction procedure for quality assurance purposes. For each sampling period, four duplicates (one per county) and two spiked samples were selected at random and extracted for use in the biological assays. In the spiked samples, 1 mM 17β-estradiol was used to achieve a concentration of 20 pM estradiol in the 1 L sample. The spiked samples were extracted as described above and concentrated to 20 000 pM using the nitrogen dry-down procedure.

Five hundred microliters of each sample extract were transferred to a new 1.5 mL amber vial, evaporated with nitrogen, re-suspended in 500 μL of diluted extraction buffer, and frozen until used in the E-screen assay.

Prior to the extraction procedure, all glassware was cleaned to eliminate organic compounds. In addition, the 15 mL glass vials, 1.5 mL glass amber vials, and glass Pasteur pipets were heated at 450 °C for 4 h in a furnace prior to use.

E-Screen Assay. The E-screen assay was used to measure the estrogenic activity of groundwater samples. The MCF-7 BOS human breast cancer cells used in the assay were obtained from the laboratory of Dr. Ana Soto and Dr. Carlos Sonnenschein at the Tufts University School of Medicine in Boston, Massachusetts. Additional cells were grown and maintained at the University of Wisconsin-Green Bay using a Soto Laboratory procedure.

To harvest cells for use in the E-screen assay, tissue culture flasks were rinsed with phosphate buffered saline and trypsinized with 1.5 mL of trypsin-EDTA (ethylenediaminetetraacetic acid) solution. Cells were counted with a hemocytometer, diluted to a concentration of 7000 cells per mL with DMEM (Dulbecco's modified eagle medium), and seeded in 24-well tissue culture plates (1 mL/tissue culture well). After 24 h of incubation, DMEM was removed and an estradiol standard dose-response curve and groundwater samples were added to the plates in experimental media. Note that DMEM without a pH indicator (phenol red) was used as the experimental media because of phenol red's known estrogenic properties (Shappell, 2006). The experimental media was supplemented with 1% antibiotic-antimycotic solution and 5% charcoal dextran stripped fetal bovine serum (CD-FBS).

The standard curve for each assay contained 16 concentrations of 17β-estradiol and ranged from 0.05 pM to 10 000 pM 17β-estradiol. A dilution series was created for each groundwater sample included in an assay. A total of five different dilutions were used for each individual groundwater sample, 1:100, 1:200, 1:400, 1:800, and 1:1600. Standards and experimental samples were plated at a volume of 500 μL/tissue culture well. Additional plate wells contained, along with each dilution of experimental sample, the estrogen receptor antagonist ICI 182,780. This was done to determine if any proliferative effects generated by the samples could be attributed to actions exerted specifically via the estrogen receptor. After an incubation period of 5 days, the assay was assessed for cell proliferation using the sulforhodamine B (SRB) protein assay. After staining with SRB dye, the absorbance of each sample was read at a wavelength of 515 nm with a Molecular Devices (Sunnyvale, California) Versa Max Tunable Microplate Reader. The standard curve was fit using a 4-parameter logistic equation and the Softmax PRO v. 2.6 analytical software package (Molecular Devices, Sunnyvale, California). Estradiol equivalency (EEq) was determined by inserting the absorbance readings into the equation generated by the standard curve (Soto et al., 1995); results were reported as pM EEq. A representative standard curve for the E-screen assays used in this study is shown in Figure 2.

The limit of sensitivity for the E-screen varied for each assay and ranged from 0.4 to 1 pM in the sample extracts. In reporting EEqs, only groundwater samples exhibiting an estrogenic response above the limit of sensitivity (1 pM) were counted as *detects* and analyzed statistically.

Statistical Analyses. Statistical analyses employed SAS statistical software (SAS Institute, Inc., Cary, North Carolina) to determine if any trends existed between estrogenicity and other parameters, including nitrate, total coliforms, *E. coli*, enterococci, and conductivity. Spearman's rank correlation test was used to examine potential correlations between the results of all six tests (PROC CORR) (Cody and Smith, 2006; Peterson et al., 2000). Seasonality was assessed by comparing the results of the four sampling periods. For nitrate and conductivity results, a

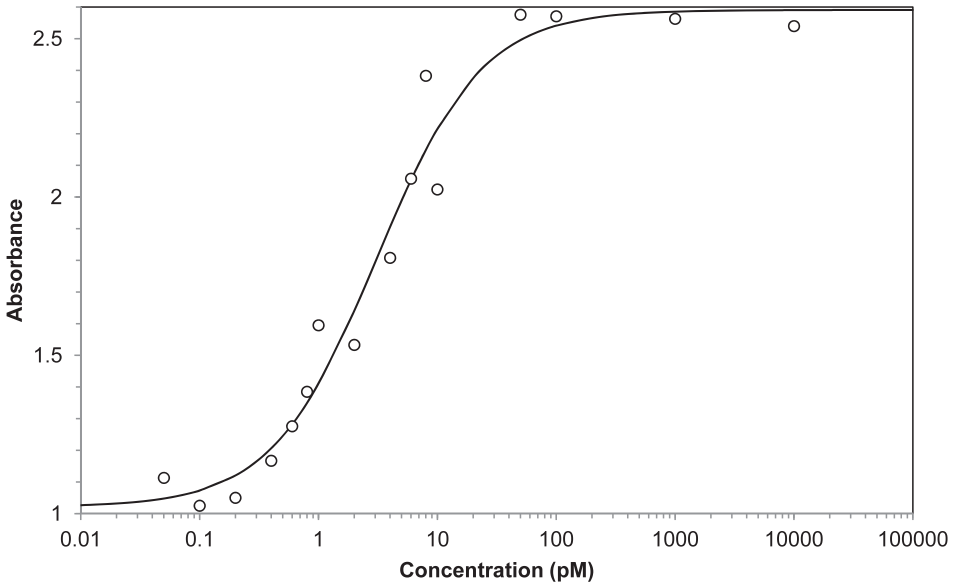

Figure 2—A representative standard curve for the E-screen assays (absorbance versus concentration) used in this study.

repeated measures analysis on one factor was conducted to examine seasonality using the well identification number as the random effect and the sampling period as the fixed effect (PROC MIXED) (Cody and Smith, 2006; Shappell, 2006). The Tukey multiple comparison adjustment for *p*-values was used in the repeated measures analysis. For the remaining four parameters, seasonality was analyzed using the Wilcoxon Signed Rank test—a nonparametric test for non-normal, paired data sets (PROC UNIVARIATE) per Cody and Smith (2006). A nonparametric statistical test (comparison of mean Wilcoxon scores using the *T* approximation test) was used to determine if the results of the control wells differed significantly from the susceptible wells (PROC NPAR1WAY). The results were also analyzed for county-level differences using a one-way analysis of variance test for the nitrate and conductivity results (PROC GLM). The Kruskal-Wallis test was used for the remaining parameters (PROC NPARIWAY). Although county-level differences were not expected because the groundwater wells were selected based on similar characteristics, differences could occur as a result of sampling technique (i.e., each county was sampled separately by different individuals) or geological variations in an area. All statistical results were analyzed for significance at the $p < 0.05$ level.

Results and Discussion

Weather Conditions. Groundwater samples were collected on the following four sampling periods: (1) August 11 to 12, 2008; (2) November 17 to 18, 2008; (3) February 13, 17, 24 and March 2, 2009; and (4) March 18 to 19, 2009. Precipitation data from the National Oceanic and Atmospheric Administration's National Weather Service station in Green Bay were obtained prior to each sampling period (NOAA, 2009). The largest and only significant rain event prior to the first sampling period occurred 26 days before sampling with a precipitation total of 34 mm. No significant rain events occurred within 16 days of the second sampling period. Because of the lack of significant rain events, it was assumed that groundwater levels in the study area were at low or baseflow conditions during the first and second sampling periods.

The third and fourth sampling periods were conducted with the express intent of capturing potential groundwater recharge events following instances of snowmelt. In February 2009, record temperature highs occurred in the Green Bay area on the 7th, 8th, and 10th day of the month, whereas daily maximum temperatures were above freezing from the 6th to the 12th. A maximum temperature of 10 °C was record on February 10, 2009. Although no major precipitation events occurred between February 1st and 10th, the Green Bay area already had accumulated 0.31 m of snow. The record high temperatures caused much of the snow to melt by February 10, 2009, and only 25 mm remained on the 12th. The Fond du Lac/Dodge (February 13, 2009) and Kewaunee (February 17, 2009) wells were sampled within 1 week of the majority of snow melt. However, wells in the remaining three counties were not sampled until February 24, 2009, following minor melting of new snow and about 12 mm of rain. Prior to the fourth sampling period (March 18 to 19, 2009) two additional warming periods from March 4 to 17, 2009, led to the melting of about 330 mm of existing and new snow.

Nitrate. Nitrate concentrations were relatively consistent for a given well across all four sampling periods. Individual results ranged from below detection (<0.1 mg/L NO_3-N) to 31.1 mg/L NO_3-N. The average nitrate concentration of the control groundwater wells slightly exceeded 1 mg/L NO_3-N for each sampling period, and the average concentration of the susceptible wells ranged from 11 to 14 mg/L NO_3-N. For each sampling period, there was a significant difference between the average nitrate concentration of the control wells and susceptible wells (i.e., the control wells had much lower nitrate concentrations). Although no significant differences were found between the average nitrate concentrations of wells in individual counties for any of the four sampling periods, Brown County consistently had the highest average nitrate concentrations and Fond du Lac/Dodge Counties the lowest.

A concentration of 10 mg/L NO_3-N is both the maximum contaminant level for public drinking water systems (U.S. EPA, 2009) and the groundwater quality enforcement standard in Wisconsin (Wisconsin Department of Natural Resources, 2010). Table 1 shows that 22 to 55% of groundwater wells in this study exceeded this health standard across the four sampling periods. Moreover, the percentage of wells exceeding 10 mg/L NO_3-N would have likely increased for the second, third, and fourth sampling periods if all 40 wells had been sampled during each sampling period. A total of seven wells were not sampled (once each) over the course of the study. One well was in Brown County in the fourth sampling period; five wells were in Calumet County in the second (3 wells), third (1 well), and fourth (1 well) periods; and one well was in Fond du Lac County in the fourth period. Six of the seven wells not sampled had average nitrate concentrations >10 mg/L NO_3-N for the other three sampling periods. One additional Calumet County well not sampled also had a relatively high average nitrate concentration (8.4 mg/L NO_3-N) for the other three sampling periods.

These elevated nitrate levels are indicative of anthropogenic sources such as agricultural fertilizers and manure (Panno et al., 2006). The relatively consistent nitrate concentrations in conjunction with the largely unchanging number of contaminated wells (>2.0 mg/L NO_3-N) over the four sampling periods suggests widespread contamination of the shallow carbonate aquifer in these areas of northeastern Wisconsin.

Bacteria. Table 2 shows a high percentage of sampled groundwater wells were contaminated with one or more types of fecal bacteria (coliforms, *E. coli*, and enterococci) over the course of the study. A bacterial detection of 1 MPN (most probable number)/100 mL or greater is unsafe by public drinking water standards (U.S. EPA, 2009). Coliform bacteria levels ranged from below detection (<0.1 MPN/100 mL) to >2420 MPN/100 mL, enterococci levels from below detection to 579 MPN/100 mL, and *E. coli* levels from below detection to 816 MPN/100 mL. The highest average coliform bacteria and enterococci levels as well as the highest number of *E. coli* detections occurred during the fourth sampling period (during the spring thaw). Coliform bacteria were detected most frequently, followed by enterococci. In the first, third, and fourth sampling periods, coliforms and enterococci were detected in more than 50 and 25% of the wells, respectively. *Escherichia coli* was detected the least frequently, with 2 contaminated wells in the first, 1 in the second, 3 in the third, and 10 in the fourth sampling period. *Escherichia coli* and enterococci were highly correlated (data not shown); enterococci were present in all wells with detectable *E. coli*.

Because *E. coli* and enterococci are both indicators of animal or human waste, they could originate from the same source. Fecal *E. coli* have been shown to be less resistant in the environment than enterococci and occur at a lower ratio in animal feces than enterococci (Celico et al., 2004). This could explain why *E. coli* was found less frequently than enterococci. In 59 spring water samples from a fractured limestone aquifer in Italy, Celico and colleagues reported that approximately 52% of their samples were contaminated with enterococci, whereas only 22% were contaminated with *E. coli*. That aquifer was known to be influenced by manure from grazing cattle. Those contamination percentages are similar to the results found in the fourth sampling period in this study.

With the exception of during the first sampling period, the control groundwater wells exhibited less bacterial contamination than the susceptible wells. A total of four control wells had detectable levels of coliform bacteria twice during this study; three wells also had at least one enterococci detection. No *E. coli* detections were recorded for the control wells in any of the sampling periods, and no coliforms or enterococci detections occurred in the control wells during the fourth sampling period.

EDC Activity. Estrogenic activity was found in groundwater during all four sampling periods. Based on the number of wells run through the E-screen in each sampling period, 50, 27, 14, and 5% of groundwater samples exhibited estrogenicity in the first, second, third, and fourth sampling periods, respectively (Table 3). Groundwater extract-induced cell proliferation was determined to be estrogen receptor-dependent through use of the estrogen receptor antagonist ICI 182,780. Estradiol equivalency ranged from 0.0114 to 12.87 pM (0.003 to 3.51 ng/L) in the actual well water samples.

The EEqs levels found in this study are within the range of levels reported in other studies that employed the E-screen. For example, Shappell et al. (2007) reported EEqs between 0.1 and 858 pM in lagoons, manure pits, and wetlands receiving swine wastewater. Water samples collected from 20 ponds and wetlands located in agricultural areas near Fargo, North Dakota, yielded EEqs within approximately one order of magnitude (0.1 to 1 pM) (Shappell, 2006). By comparison, approximately 62% of the EEqs in this groundwater study fell within an order of magnitude range; the remaining 27 and 10% fell between 0.01 and 0.1 pM, and 1 and 10 pM, respectively. Note that most groundwater samples in this study were either lower than or near the bottom of the range reported by Shappell et al. (2007), which can be attributed to the different types of water sampled in each study. That is, Shappell and colleagues evaluated water bodies directly affected by pollution whereas this study assessed groundwater that might be affected by pollution. One might expect the concentrations of estrogenic chemicals originating at the surface to decrease as they enter the water table, whether by filtration through the unsaturated zone, degradation by microbes, or dilution through mixing with other water sources. During transport through an aquifer, concentrations can become

Table 1—Percentage of groundwater wells in different nitrate (NO_3-N) concentration ranges during each sampling period.

Sampling period	Number of wells sampled	Concentration (mg/L NO_3-N)			
		0–2	2–5	5–10	>10
1	40	17.5%	7.5%	20.0%	55.0%
2	37	21.6%	8.1%	21.6%	48.7%
3	39	18.0%	12.8%	18.0%	51.3%
4	37	11.1%	33.3%	33.3%	22.2%

Table 2—Percentage of groundwater wells with unsafe levels of coliform bacteria, enterococci, and *Escherichia coli* during each sampling period. Number of wells sampled is same as in Table 1.

Sampling period	Coliform bacteria	*Escherichia coli*	Enterococci
1	62.5%	12.5%	27.5%
2	40.5%	2.7%	10.8%
3	59.0%	7.7%	29.7%
4	64.9%	27.0%	46.0%

Table 3—Percentage of sampled groundwater wells with detectable estradiol equivalents (EEq) in the E-screen assay during each sampling period.

Sampling period	EEq detections[a]
1	50.0 %
2	27.0 %
3	13.9 %
4	5.4 %

[a] Limit of sensitivity = 1 pM EEq in sample extracts.

even more diluted before reaching a groundwater well, depending on the distance from the source of the estrogenic chemicals.

No public drinking water health standard exists for estrogenicity. However, several studies have shown that low concentrations of estradiol in surface waters (37 to 370 pM or 10 to 100 ng/L) can disrupt the endocrine systems of aquatic species including fish, turtles, and frogs (Hanselman et al., 2003). In a study analyzing the reproductive capacity of a fish population and with the goal maintaining population sustainability, the Environment Agency of England and Wales estimated 36.7 pM estradiol (10 ng/L) as the *lowest observable effect concentration*, and 3.7 pM (1 ng/L) as the threshold concentration yielding no effect on the fish (Shappell et al., 2007). Other researchers have predicted that the *no-observed-effect-concentration* for 17β-estradiol is between 18.4 to 91.8 pM (5 to 25 ng/L) (Harper and Sinh, 2006). Although the vast majority of groundwater samples in this study were well below the 3.7 pM *no effect* threshold identified by Shappell et al. (2007), the E-screen results in this study show that some wells had concentrations near or above this level. Three wells in Calumet County exhibited EEqs >0.37 pM during the first sampling period, whereas one well in Brown County, two wells in Calumet County, and two wells in Fond du Lac/Dodge County exhibited EEqs >0.37 pM during the second sampling period. In addition, two wells recorded values exceeding 3.7 pM during the second sampling period, one in Brown County (12.9 pM) and the other in Calumet County (7.2 pM). No groundwater samples exceeded an EEq of 0.37 pM in the third or fourth sampling periods.

Seasonality. As noted previously, the third and fourth sampling periods followed groundwater recharge events; because of little rainfall in late summer and fall, the first and second sampling periods were mostly representative of baseflow conditions within the aquifer. No significant differences in conductivity existed between control and susceptible wells for all four sampling periods. A comparison of the least squares means with the Tukey adjustment showed that susceptible wells during the first and second sampling periods had significantly greater conductivity values relative to the fourth sampling period ($p = 0.0006$ and $p = 0.0005$, respectively.) This reduction in average conductivity in the fourth sampling period corresponded with the recharge that occurred following snowmelt events in February and March 2009. These results are consistent with those reported by Muldoon and Bradbury (2010) in shallow carbonate aquifer monitoring wells adjacent to agricultural fields in Brown, Calumet, Manitowoc, and Kewaunee Counties, Wisconsin. Those researchers found that most groundwater recharge occurring between September 2007 and August 2008 followed snowmelt events in January and March/April 2009. In addition, rapid declines in conductivity in response to recharge were observed in all four of their continuously monitored wells, which indicated that low conductivity recharge water traveled from the soil surface to the saturated zone within 1 to 2 days of the event.

Table 4 summarizes the significance of conductivity and the other five parameters discussed below among the four different sampling periods. When the dataset was analyzed as a whole, a significant difference was found among the four sampling periods for nitrate ($p = 0.0151$). Similar to conductivity, the Tukey adjustment indicated that this was a result of the significant difference between the first and fourth sampling periods ($p = 0.0086$). When the control and susceptible wells were analyzed separately, the control wells did not differ significantly among the four sampling periods ($p = 0.6543$). Thus, the difference between sampling periods was a result of a difference in contamination of the susceptible wells, which had significantly greater nitrate contamination in the first sampling period compared to the fourth ($p = 0.0081$).

Several significant seasonal differences in bacteria levels were observed in susceptible wells across the four time periods of this study. Average coliform bacteria contamination was significantly greater in the fourth sampling period compared to the first, second, and third sampling period, as indicated by the Wilcoxon Signed Rank test ($p = 0.0017$, $p \leq 0.0001$, $p = 0.0014$, respectively). Average coliform levels in the third sampling period were also significantly greater than those of the second sampling period ($p = 0.0019$). In other words, the second sampling period had less average microbial contamination than the fourth and third sampling period, but was not significantly different from the first ($p = 0.0554$).

Similar to coliform bacteria, the susceptible wells had significantly less average enterococci contamination in the second sampling period than the other three periods ($p = 0.0469$, $p = 0.0059$, $p \leq 0.0001$). Enterococci contamination of the susceptible wells in the fourth sampling period was also significantly greater than the third sampling period ($p = 0.0249$). Although the fourth sampling period had greater average enterococci values compared to the first sampling period, the difference was not significant ($p = 0.6993$). Differences between the average *E. coli* results of the susceptible wells were similar to the coliform and enterococci parameters. *Escherichia coli* contamination in the fourth sampling period was significantly greater than in the first ($p = 0.0164$) and second ($p = 0.002$) sampling periods.

Collectively, the seasonality results indicate that bacteria levels were greatest during winter/spring thaw compared to summer and fall months (Table 4). The fourth sampling period exhibited the largest bacterial contamination, the third period had the

Table 4—Summary of significant differences in average concentrations between sampling periods for susceptible well water samples.

Parameter	Sampling periods					
	1 and 2	1 and 3	1 and 4	2 and 3	2 and 4	3 and 4
Nitrate	–	–	1 > 4	–	–	–
Conductivity	–	–	1 > 4	–	2 > 4	–
Coliform bacteria	–	–	4 > 1	3 > 2	4 > 2	4 > 3
Enterococci	1 > 2	–	–	3 > 2	4 > 2	4 > 3
E. coli	–	–	4 > 1	–	4 > 2	–
E-screen	–	1 > 3	1 > 4	–	2 > 4	–

second largest, and the second had the least. Sample timing relative to snowmelt recharge events likely influenced the frequency and level of bacteria found in wells during the third and fourth sampling periods. All 37 wells in the fourth period were sampled over the course of 2 days immediately following complete snow melt. In contrast, half of the 39 wells sampled during period three were sampled within 1 week of the major thaw; the remaining wells were sampled 12 days after the thaw. For the wells sampled soon after melt events for both periods, 37 to 53% and 15 to 21% of the wells had detectable levels of enterococci and *E. coli*, respectively. For wells sampled more than a week after the snow melt in period three, 21 and 0% tested positive for enterococci and *E. coli*. But when these same wells were sampled within 2 days of the period four melt, 39 and 17% tested positive for enterococci and *E. coli*. Because of the rapid nature of groundwater recharge processes in the vicinity of the study well, it is possible that the period three observations underestimated bacterial frequency and concentrations resulting from the February 2009 thaw.

Generally, monitored fecal bacteria groups were significantly correlated for all sampling periods. In addition, bacterial levels were inversely correlated with conductivity in sample periods three and four (data not shown). During the March 2009 recharge event (period four), 14 of 17 wells testing positive for enterococci had lower conductivity values than those measured under baseflow conditions (period one). The average reduction in fluid conductivity was 22% with the largest changes in conductivity (49 to 52%) corresponding to wells with the two highest enterococci levels (579 and 248 MPN/100 mL, respectively). This dramatic change implies that a large volume of low conductivity surface water carrying fecal bacteria, pathogens, and potentially other constituents penetrated rapidly from land surface to the well intake.

As stated previously, the presence of enterococci or *E. coli* in a groundwater well indicates contamination with some type of human or animal waste. Because of the nature of *E. coli* and enterococci, both of which are found in the intestines of warm-blooded animals, these results suggest that as many as 46% of tested wells were contaminated with animal or human waste in the fourth sampling period. Unlike the fecal bacteria and nitrate results, EEqs were significantly lower in the fourth sampling period compared to sampling periods one ($p = 0.0006$) and two ($p = 0.002$). No significant differences were found between the first and second sampling periods, which had both the greatest average EEqs and most estrogenicity detections ($p = 0.6995$). Sampling period three also had significantly less contamination than period one ($p = 0.001$). No differences were found between sampling periods three and four, which had the fewest E-screen detections ($p = 0.2188$), or between sampling periods two and three ($p = 0.25$).

Overall, fewer detections of estrogenicity were found in the groundwater wells compared to fecal bacteria and nitrate detections across all four sampling periods. This result can be attributed to one or more factors. First, estrogen contamination might simply occur less frequently in the test wells than fecal bacteria and nitrate contamination events. Perhaps there are fewer sources of estrogenic contamination in the study area than fecal bacteria or nitrate sources. Second, some samples might have had estrogenic activity below the measurement sensitivity of the assay (1 pM) that prevented detection. Third, higher levels of estrogenicity during the summer and fall sampling periods might reflect the presence of estrogenic pesticides (e.g., methoxychlor and dieldrin; Hodges et al., 2000) that were applied to crops in the spring. Fourth, and perhaps most importantly, the E-screen is a biological assay that depends on the consistent response of a living cell line. If the groundwater extracts contained chemicals that were toxic to cell growth, the ability of the E-screen assay to properly measure estrogenicity would be compromised. In samples containing both estrogenic and toxic chemicals, toxicity could inhibit an estrogenic response (cell proliferation). This would affect the estrogenicity results by (1) lowering EEqs, or (2) reducing values below the sensitivity of the assay and thus preventing detection.

Toxicity, which was assessed by comparing the difference in cell proliferation in response to a given sample of groundwater extract in the presence or absence of a known concentration of estrogen, occurred very frequently in the assays—especially during the third and fourth sampling periods. Although limitations in extract volume prevented an assessment of toxicity for individual well water samples included in this study, a limited analysis revealed an average toxicity level of 31% during sampling periods one and two compared to 99% during sampling periods three and four. Therefore, it is possible that the estrogenicity of the groundwater samples might be greater than these study results indicate, particularly during the third and fourth sampling periods. This possibility exists because cell death resulting from the presence of toxic chemicals in the sample prevented or lowered EEq detection by the E-screen. Thus, it is possible that wells with apparent toxicity and that registered below detection in the E-screen might have contained estrogenic chemicals, but the dose-dependent response of the cells was masked by the toxic components of the sample.

The above limitations of bioassays such as the E-screen highlight the need for a method that allows the identification and detection of specific estrogenic chemicals in complex water samples containing unknown compounds, such as LC-MS (liquid chromatography-mass spectrometry) or GC-MS (gas chromatography-mass spectrometry) (Chen et al., 2006; Drewes et al., 2005; Soliman et al., 2007). Although an initial goal of this study was to analyze samples with high EEqs by LC-MS to determine the presence of specific EDCs, this was not possible because the entire sample from each well was required for the E-screen assay procedures.

EDC Correlation with Nitrate, Coliform Bacteria, and Escherichia coli. Strong correlations between estrogenicity (i.e., E-screen data) and the other water quality parameters were not found. However, one significant, albeit weak correlation was found—a positive correlation between the *E. coli* results and the E-screen results in the fourth sampling period. The weakness of this correlation ($r = 0.364$) makes it difficult to draw any conclusions. This relationship was driven by two samples collected from Fond du Lac/Dodge Counties. Both samples tested positive for estrogenicity, *E. coli*, coliform bacteria, and enterococci.

Several possible explanations exist for the lack of correlation between measured water quality parameters and estrogenicity. For example, and as noted above, toxicity of groundwater samples during the fourth sampling period (which could have led to low or undetectable EEqs) might have prevented the detection of a correlation of fecal bacteria and estrogenicity data. Also, sources of groundwater contamination are widespread and estrogenic activity could have originated from non-bacterial contamination sources such as land application of estrogenic pesticides.

Implications. The study results indicate that areas susceptible to groundwater contamination by fecal bacteria and nitrate can also exhibit elevated levels of estrogenicity. Contaminant sources are likely to be land-applied animal or human wastes, underground septic systems, or land-applied agrochemicals such as fertilizers and pesticides. In addition to the karst areas of northeastern Wisconsin evaluated in this study, other areas with shallow depth-to-bedrock or areas with sandy soils over shallow unconfined aquifers could also be susceptible to similar drinking water contaminants. Groundwater in areas containing high organic content and that have sufficient soil depths and textures (i.e., longer retention time) would presumably be less affected by EDCs because the contaminants would be less mobile and have a longer period of time to degrade.

Conclusions

Results from this study indicate that groundwater contamination with EDCs, fecal bacteria, and nitrate is a common problem in karst areas of northeastern Wisconsin. Contamination by waterborne pathogens can occur rapidly during winter and spring groundwater recharge events. Endocrine disrupting chemicals contamination was greatest during the months of August and November 2008. Although potential sources of EDC contamination within the study area (e.g., pharmaceuticals from leaky septic systems, land-applied manure, estrogenic pesticides) remain speculative, their identification provides an intriguing avenue for future research. It will also be worthwhile to identify fracture zones, bedrock openings, and other potential hazardous areas that allow for rapid transport of surface runoff to groundwater. Local and state resource management agencies (including Calumet County, Brown County, and the State of Wisconsin) have begun to collect and compile these and related types of data. The effect of individual well characteristics (well depth, depth to bedrock, age, and soil type) on water quality parameters is also worthy of additional research. Finally, the specific contaminants exerting estrogenic activity within the water samples should be identified through the use of LC-MS or other technique.

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