Groundwater Research Report
WR08R004

ASSESSING LEVELS OF ENDOCRINE DISRUPTING CHEMICALS IN GROUNDWATER ASSOCIATED WITH KARST AREAS IN NORTHEAST WISCONSIN

Angela Bauer-Dantoin
Kevin Fermanich
Michael Zorn
Sarah Wingert

2011

Project Number WR08R004

Assessing Levels of Endocrine Disrupting Chemicals in Groundwater Associated with Karst Areas in Northeast Wisconsin

Angela Bauer-Dantoin, Ph.D
Department of Human Biology
UW-Green Bay

Kevin Fermanich, Ph.D
Natural and Applied Sciences
UW-Green Bay

Michael Zorn, Ph.D
Natural and Applied Sciences
UW-Green Bay

Sarah Wingert
Environmental Science & Policy
UW-Green Bay

2011

This project was supported, in part, by General Purpose Revenue funds of the State of Wisconsin to the University of Wisconsin System for the performance of research on groundwater quality and quantity. Selection of projects was conducted on a competitive basis through a joint solicitation from the University and the Wisconsin Departments of Natural Resources; Agriculture, Trade and Consumer Protection; Commerce; and advice of the Wisconsin Groundwater Research Advisory Council and with the concurrence of the Wisconsin Groundwater Coordinating Council.

Assessing Levels of Endocrine Disrupting Chemicals in Groundwater Associated with Karst Areas in Northeast Wisconsin

WRI Project Number WR08R004

Project Investigators:
Angela Bauer-Dantoin, Professor and Chair, Human Biology, UW-Green Bay
Kevin Fermanich, Associate Professor, Natural and Applied Sciences, UW-Green Bay
Michael Zorn, Associate Professor, Natural and Applied Sciences, UW-Green Bay
Sarah Wingert, Graduate Student, Environmental Science & Policy, UW-Green Bay

Table of Contents

List of Tables

Project Summary

Title: Assessing Levels of Endocrine Disrupting Chemicals in Groundwater Associated with Karst Areas in Northeast Wisconsin

Project I.D.: WRI Project Number WR08R004

Investigators: Angela Bauer-Dantoin, Professor and Chair, Human Biology, UW-Green Bay
Kevin Fermanich, Associate Professor, Natural and Applied Sciences, UW-Green Bay
Michael Zorn, Associate Professor, Natural and Applied Sciences, UW-Green Bay
Sarah Wingert, Graduate Student, Environmental Science & Policy, UW-Green Bay

Period of Contract: July, 2008 – January, 2010 (no cost extension granted in August, 2009)

Background/Need:
In recent years, concern has risen over the presence of various nonpoint source pollutants in drinking water, including a class of organic chemicals called endocrine disrupting chemicals (EDCs). The growing prevalence of EDCs in environmental systems has been linked to the disruption of aquatic endocrine systems and increased incidence of certain human cancers. Groundwater in the Silurian aquifer of northeastern Wisconsin may be particularly susceptible to nonpoint source contamination due to the existence of shallow soils, dolomite bedrock, and karst features, which combine to facilitate the transport of surface runoff to groundwater. Land application of manure containing synthetic and endogenous hormones may be a significant source of nonpoint source pollutants, including EDCs, to groundwater in the heavily farmed regions of northeast Wisconsin.

Objectives:
The specific objectives of the study were:
1. To test for indicators of livestock and/or human fecal contamination (E. coli, fecal coliform, nitrates) in groundwater near farmland in the northeast Wisconsin counties of Brown, Calumet, Fond du Lac and Kewaunee.
2. To assess levels of EDC activity in groundwater near farmland in the northeast Wisconsin counties of Brown, Calumet, Fond du Lac and Kewaunee.
3. To determine whether EDC activity and fecal waste indicators in groundwater near farmland change after major recharge periods (e.g., rainfall; spring thaw).
4. To discern whether levels of groundwater contamination by EDCs correlate with other water quality indicators (nitrates, fecal coliform, E. coli levels).
5. To measure estradiol levels in water samples through use of an enzyme-linked immunosorbent assay (ELISA).

Methods:
The MCF-7 breast cancer cell proliferation assay (E-screen) was used to determine if groundwater samples collected from four northeast Wisconsin counties, including Brown, Calumet, Fond du Lac, and Kewaunee, exhibited estrogenic behavior. Groundwater samples were collected four times between the summer of 2008 and the spring of 2009 (in August, November, Frebruary and March), and the samples were analyzed for estrogenicity, 17β-estradiol concentrations, nitrate, conductivity, total coliform, enterococci, and *E. coli.* The wells chosen for this study were located in agricultural areas of northeast Wisconsin, were cased into the Silurian aquifer, and were chosen in light of past contamination with bacteria and/or nitrate.

Results and Discussion:
Estrogenic activity was detected in a portion of the groundwater samples during all four sampling periods, despite apparent toxicity and/or anti-estrogenic effects in the E-screen assay. The estrogenic equivalency found in the samples used in the study are below the range known to cause endocrine disruption in wildlife and are within the range of levels found in other studies that utilized the E-screen to analyze water samples. Levels of estrogenicity were highest during the months of August and November. Specific 17β-estradiol concentrations in samples were not measurable with the ELISA, presumably due to cross-reactivity and/or matrix effects. Unsafe levels of bacteria and nitrate occurred during all four sampling periods. Average bacterial contamination increased following snowmelt events in February and March 2009. Coliform, enterococci, and *E. coli* were positively correlated throughout the study, with the strongest correlations occurring in the March 2009 sampling period. Correlations were not found between nitrate and bacteria, or nitrate and estrogenicity. One weak, positive correlation was found between *E. coli* and estrogenicity in the March 2009 sampling period.

Conclusions/Implications/Recommendations:
Results from the study indicate that groundwater contamination with EDCs, bacteria and nitrates is a common problem in karst areas of northeast Wisconsin. EDC contamination was greatest during the months of August and November, times at which land application of manure is frequent. Potential sources of EDC contamination within our study area (e.g., pharmaceuticals from leaky septic systems, land-applied manure, estrogenic pesticides) remain speculative based on the information provided in this study, and 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 quick transport of surface runoff to the groundwater. The impact of individual well characteristics (well depth, depth to bedrock, age, and soil type) on water quality parameters, likewise, is worthy of study. Finally, the specific contaminants exerting estrogenic activity within the water samples should be analyzed with a more reliable method of detection than the ELISA, such as liquid chromatography-mass spectrometry.

Related Publications:
Wingert, S.E.*;* Bauer-Dantoin, A. Fermanich, K.J.; Zorn, M.E. Assessing Levels and Potential Health Effects of Endocrine Disrupting Chemicals in Groundwater Associated with Karst Areas in Northeast Wisconsin. The 33rd Annual Meeting of the American Water Resources Association (AWRA) Wisconsin Section. March 5-6, 2009. Stevens Point, Wisconsin.

Wingert, S.E. Assessing Levels of Endocrine Disrupting Chemicals in Groundwater Associated with Karst Areas in Northeast Wisconsin. Master's Thesis in Environmental Science and Policy, University of Wisconsin – Green Bay, December, 2010.

Key Words: endocrine disruptors, groundwater, estrogenicity, E-screen, E.coli, nitrates, fecal coliform

Funding: State of Wisconsin Groundwater Research and Monitoring program through the University of Wisconsin Water Resources Institute (WRI)

Introduction

There is widespread concern over the presence of organic compounds within groundwater that have the ability to mimic or interfere with the activities of endogenous hormones within the body. These endocrine disrupting chemicals (EDCs) come from a variety of sources (National Research Council, 1999), including industrial effluent (polychlorinated biphenyls, plasticizers), human waste (synthetic hormones from contraceptives), and animal waste (endogenous as well as synthetic hormones injected into livestock to induce growth). 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, there exists great potential for EDCs to cause physiological abnormalities in exposed organisms (reviewed in Colburn et al., 1996).

Of particular concern for humans is the possible association between EDC exposure and endocrine-related cancers, such as breast cancer. Cumulative exposure to estrogen is a major risk factor for the development of breast cancer (Toniolo et al., 1995; Dorgan et al., 1996), and thus there is concern that exposure to estrogenic EDCs may increase one's risk for developing breast cancer. Indeed, not only have laboratory studies linked EDC exposure with the development of breast cancer in mice (Murray et al., 2006), but human studies likewise have found a correlation between elevated levels of EDCs such as DDT 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 developing androgen-sensitive cancers, including testicular cancer (Skakkebaek et al., 2001; Weir et al., 2000) and prostate cancer (Fleming et al., 1999; Ho et al., 2006).

In addition to posing cancer risks, EDCs are thought to interfere with reproductive function in both males and females (reviewed in Colburn, 1996). Animal studies that have documented a negative impact of EDCs on germ cell production in both sexes (Sakaue et al., 2001; Susiarjo et al., 2007); as a result, it has been proposed that EDC exposure is responsible for the decline in sperm counts observed in males in industrialized countries (Toppari et al., 1996; Toppari et al., 2002). EDCs also impair fertility in laboratory animals by interfering with the signaling of endogenous sex hormones during development of the reproductive system (Gray et al., 1999; Fisher et al., 1999). Thus, EDC exposure is thought to be responsible for the marked increase in disorders of human sexual development such as hypospadias and testicular dysgenesis that has been observed in industrialized countries (Toppari et al., 1996; Toppari et al., 2002).

A critical step toward minimizing exposure to EDCs and thus decreasing the 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, since it contains not only endogenous estrogens from cattle (estradiol, estriol and estrone; Hanselman et al., 2006; Peterson et al., 2000) but also synthetic steroids administered to livestock as growth-enhancing agents (Herschler et al., 1995). Manure-borne EDCs are introduced into the environment as a result of the standard practice of applying animal wastes to pastures and croplands as fertilizers. 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) with concentrations of EDCs that are known to exert biological effects (Irwin et al., 2000; Panter et al., 1998).

Groundwater contamination by manure runoff is of particular concern to the residents of northeast Wisconsin, given the unique geology of the region. Northeast Wisconsin is characterized by carbonate bedrock areas, shallow soil depths, and karst features (sink holes and bedrock openings) that allow ready access of surface contaminants to well water. A recent report of the Northeast Wisconsin Karst Task Force (2007) indicates that a significant proportion of water supply wells in northeast Wisconsin have been contaminated by bacteria or high levels of nitrate. Numerous incidences of contamination have been linked to manure runoff within recent years, particularly during the spring thaw. Indeed, when the Calumet County Land and Water Conservation Department conducted voluntary well water testing in spring of 2007, they found that 32% of the samples tested positive for some level of coliform bacteria (an indicator of contamination by livestock and/or human waste) and high nitrate levels (Calumet County, 2007). These results are consistent with previous data collected in Calumet County during 2002-2006. Similar findings were obtained by the Brown County Land Conservation Department in an analysis of well water samples collected from the town of Morrison (Brown County Land Conservation Department, 2007).

The majority of coliform-positive well water samples identified in the aforementioned studies came from areas in northeast Wisconsin that are heavily utilized as farmland and have relatively shallow soils over fractured dolomite. Thus, it is likely that groundwater contamination in these counties is due to the 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 arise that manure-born EDCs are also contaminating well water. Thus, in the present study, we conducted experiments to assess whether coliform- and E. coli-positive groundwater samples obtained from the northeast Wisconsin counties of Brown, Calumet, Fond du Lac and Kewaunee contain measurable levels of manure-born EDCs (e.g., 17β estradiol, estriol and testosterone), through use of the MCF-7 breast cancer cell proliferation assay (also known as the E-screen assay). Levels of EDCs were measured at four time points to determine seasonality and possible changes associated with recharge periods (heavy rainfall or spring thaw). Finally, estradiol concentrations in water samples were assessed through use of the enzyme-linked immunosorbent assay.

Procedures and Methods

Well Selection and Sample Collection

The study area consisted of rural land in northeast Wisconsin with known instances of past contamination of the uppermost Silurian aquifer. Private groundwater wells within five counties, including Brown, Calumet, Dodge, Fond du Lac, and Kewaunee Counties, were selected to investigate the potential for groundwater contamination with estrogenic chemicals. Besides the fact that each of these counties has areas that are susceptible to contamination, these counties were chosen because we were able to identify representatives from local environmental agencies that were willing to help us contact well owners and sample the wells. Ten wells per county were chosen for sampling in Brown, Calumet, and Kewaunee counties. Eight wells were selected from Fond du Lac County and two wells were chosen from Dodge County immediately south of the Fond du Lac wells. For sample collecting and analysis purposes, the Dodge County wells were included with the Fond du Lac wells due to their close proximity.

The wells chosen for the study were not selected in a statistically rigorous manner, and were not chosen with the intent to represent county-level water quality trends. Rather, the wells were selected based on five characteristics: they were cased into the Silurian aquifer; they were shallow in depth; historical sampling data for bacteria and nitrate existed; the well owners agreed to participate in the study; and the wells were located in areas with suspected or known sources of agricultural contamination. Eight wells from each county were designated "susceptible" to contamination based on past high levels of contamination, while two wells from each county were deemed "control" wells based on low levels of past contamination (no or low bacteria counts and less than 2 mg/L NO3--N). 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 UW-Green Bay researcher.

Bacterial and Nitrate Analyses

Bacteria samples were analyzed within 24 hours of collection at the UW-Oshkosh Halsey Science Center's Environmental Microbiology Laboratory. *Escherichia coli* (*E. coli*) and total coliform were measured using the Colilert procedure, and enterococci were measured using the Enterolert procedure (IDEXX 2010). Nitrate samples were analyzed for nitrate-nitrite levels within 48 hours of collection in our laboratory using a Lachat QuickChem 8500 Flow Injection Analysis System and the Lachat Instruments QuikChem Method 10-107-04-1-A (Wendt 2000). Results were reported as mg/L N, with a lower limit of detection of 0.1 mg/L N.

Sample Extraction for Biological Assays

The organic compounds from the samples collected for the estrogenicity tests were extracted at the UW-Green Bay lab within 48 hours of collection. One sample from each well was extracted following the Wisconsin State Laboratory of Hygiene's Aquatic Life Toxicity Testing Laboratory protocol for the extraction of organic compounds from water (ESS Bio Method 108.0) utilizing C-18 disks (3M Empore high

performance extraction disk #2215). Samples were stored in 15 mL vials in a 4-degree Celsius refrigerator until the nitrogen dry-down procedure could be performed. During the nitrogen dry-down procedure, a sample extract was dried almost completely with ultra high purity nitrogen, and the 15 mL vial was 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 were run through the extraction procedure for quality assurance purposes. For each sampling period, four duplicates (one per county) and two spiked samples were chosen randomly from the refrigerator and extracted for use in the biological assays. In the spiked samples, 1 mM 17β-estradiol was used to achieve a concentration of 2×10^{-11} M (20 pM) estradiol in the one liter sample. The spiked samples were extracted using the procedure described above and then concentrated to 2×10^{-8} M (20,000 pM) in the sample extracts using the nitrogen evaporation procedure.

Before use in the biological assays, 500 µL of each sample extract was transferred to a new, clean 1.5 mL amber vial, evaporated with nitrogen, and re-suspended in 500 µL of diluted extraction buffer. The extraction buffer was obtained from Oxford Biomedical Reseach, Inc.'s Estradiol Enyzme Immunoassay Kit (EA 70) and diluted five times with high-purity water prior to use. Sample extracts in the diluted extraction buffer were stored in a freezer until use in the E-screen and ELISA assays.

E-screen Assay

The E-screen assay was used to measure the general estrogenic activity of groundwater samples. The human breast cancer cells used in the assay, the MCF-7 BOS cells, were obtained from the laboratory of Dr. Ana Soto and Dr. Carlos Sonnenschein at the Tufts University School of Medicine in Boston, Massachusetts. The cells were grown in the UW-Green Bay lab and cared for following a procedure obtained from the Soto laboratory.

To harvest the cells for the E-screen assay, tissue culture flasks were rinsed with phosphate buffered saline and trypsinized with 1.5 mL of trypsin-EDTA solution. Cells were counted with a hemocytometer and diluted to a concentration of 7,000 cells per mL with DMEM and seeded in 24-well tissue culture plates (1 mL/tissue culture well). After 24 hours of incubation, the DMEM was removed and an estradiol standard dose response curve and the groundwater samples were added to the plates in experimental media. DMEM without the pH indicator phenol red was used as the experimental media due to phenol red's estrogenic properties (Shappell 2006). The experimental media was supplemented with 1% antibiotic/antimycotic solution and 5% charcoal-dextran stripped FBS (CD-FBS).

The standard curve for each assay contained 16 concentrations of 17β-estradiol, ranging from 5×10^{-14} M (0.05 pM) to 1×10^{-8} M (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 wells were included in the assay that included – along with each dilution of experimental sample – the estrogen receptor antagonist, ICI 182,780, in order to determine if any proliferative effects generated by samples could be attributed to actions exerted specifically via the estrogen receptor. After an incubation period of five days, the assay was assessed for cell proliferation using the sulforhodamine B (SRB) protein assay. The absorbance of each sample, after staining with SRB dye, was read at a wavelength of 515 nm with a Molecular Devices microplate reader. The standard curve was fit with a four-parameter logistic equation using the Softmax PRO v. 2.6 analytical software package, and 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.

The limit of quantification varied for each assay, ranging from 0.4 to 1 pM. The least sensitive assay had a lower limit of quantification (LOQ) of 1 pM (1.0 x 10-12 M) EEq in the sample extracts. For consistency, 1 pM EEq was chosen as the lower LOQ for use across all assays. Only groundwater samples exhibiting an estrogenic response above the lower LOQ of 1 pM were analyzed statistically.

ELISA

Attempts were made to measure concentrations of 17β-estradiol in the groundwater sample extracts using enyzme-linked immunosorbent assay (ELISA) kits obtained from Oxford Biomedical Research, Inc. (Product Number EA 70). Specific 17β-estradiol concentrations in samples were not measurable with the ELISA, due to cross-reactivity and/or matrix effects.

Statistical Analyses

Statistical analyses were employed using SAS statistical software to determine if any trends existed between estrogenicity and other parameters, including nitrate, total coliform, *E. coli*, enterococci, and conductivity. Spearman's rank correlation test was used to examine potential correlations between the results of all seven tests (PROC CORR; Cody and Smith 2006; Peterson et al. 2000). Seasonality was also assessed by comparing the results of the four sampling periods. For nitrate results, a repeated measures analysis for a repeated measure on one factor was conducted to examine seasonality, with the well identification number as the random effect and the sampling period as the fixed effect (PROC MIXED; Cody and Smith 2006; Shappell 2006). For the remaining five parameters, seasonality was analyzed using the Signed Rank Test, a non-parametric test for non-normal, paired data sets (PROC UNIVARIATE; 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; Cody and Smith 2006). 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; Cody and Smith 2006), and the Kruskal-Wallis test for the remaining five parameters (PROC NPARIWAY; Cody and Smith 2006). County-level differences were not expected since the groundwater wells were chosen based on similar characteristics, but differences could occur due to sampling technique (each county was sampled separately by different people) or differences in the geologic make-up of an area. All statistical results were analyzed for significance at the 0.05 level.

Results and Discussion

Weather Conditions

Groundwater samples were collected on the following dates: August 11 and 12, 2008 (first sampling period), November 17 and 18, 2008 (second sampling period), February 13, 17, 24, and March 2, 2009 (third sampling period), and March 18 and 19, 2009 (fourth sampling period). Precipitation data from the National Oceanic and Atmospheric Administration's (NOAA) National Weather Service (NWS) station in Green Bay was obtained prior to each sampling period (NOAA 2009). The largest rain event prior to the first sampling period occurred 26 days before sampling, with a precipitation total of 1.32 inches. No other major rain events occurred prior to the first sampling period, and no significant rain events occurred within 16 days of the second sampling period. Precipitation data were not available from the Green Bay station from October 22 to October 31, 2009, so only the two weeks prior to sampling are included for the second sampling period. Due to the lack of significant rain events prior to both the first and second sampling periods, it was assumed that groundwater levels in the study area were at low-flow or base-flow conditions during the first and second sampling periods.

The third and fourth sampling periods were executed with the 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, while daily maximum temperatures hung above freezing from the 6th to the 12th, and topped out at 50 degrees Fahrenheit on the 10th. No major precipitation events occurred between February 1st and 10th, but the Green Bay area had a foot of snow accumulated from past precipitation events. The record temperatures caused half of the snow to melt by February 10th, and only one inch of snow remained on February 12th.

Objective 1: To test for indicators of livestock and/or human fecal contamination (E. coli, fecal coliform, nitrates) in groundwater near farmland in the northeast Wisconsin counties of Brown, Calumet, Fond du Lac and Kewaunee

During each sampling period, a number of groundwater wells were found to be contaminated with each of the three types of bacteria: coliform, enterococci, and *E. coli* (see Table 1). A bacterial detection of 1 MPN (most probable number) units or greater is unsafe by public water drinking standards. Total coliform levels ranged from below detection (<0.1 MPN) to above detection (>2,419.6 MPN), enterococci levels ranged from below detection to 579.4 MPN, and *E. coli* levels ranged from below detection to 816.4 MPN (See Tables 1-4 of Appendix B for individual well data during the four sampling periods). The highest average coliform and enterococci levels and the highest number of *E. coli* detections occurred during the fourth sampling period (during the spring thaw). Coliform was detected most frequently, followed by enterococci. In the first, third, and fourth sampling periods, coliform was detected in more than 50% of our wells, and enterococci was detected in more than 25% of the wells. *E. coli* was detected the least frequently, with two contaminated wells in the first sampling period, one in the second sampling period, three in the third sampling period, and ten in the fourth sampling period.

Table 1: Percentage of groundwater wells contaminated with coliform, enterococci, and *E. coli* during each sampling period.

Sampling	Coliform			E. coli			Enterococci		
Period	Unsafe	Safe	N	Unsafe	Safe	N	Unsafe	Safe	N
1	62.5%	37.5%	40	12.5%	87.5%	40	27.5%	72.5%	40
2	40.5%	59.5%	37	2.7%	97.3%	37	10.8%	89.2%	37
3	59.0%	41.0%	39	7.7%	92.3%	39	29.7%	70.3%	37
4	64.9%	35.1%	37	27.0%	73.0%	37	46.0%	54.1%	37

E. coli and enterococci are both indicators of animal or human waste and hence could be from the same source. Fecal coliform bacteria (*E. coli*) have been shown to be less resistant in the environment than fecal enterococci and are also found at a lower ratio in animal feces than fecal enterococci (Celico et al. 2004). This might explain why *E. coli* was found less frequently than enterococci. In 59 spring water samples from a fractured limestone aquifer in Italy, Celico et al. (2004) found that approximately 52% of their samples were contaminated with enterococci, while only 22% were contaminated with *E. coli*. This aquifer is known to be impacted by manure from grazing cattle. These percentages are similar to the results we found in the fourth sampling period.

With the exception of the first sampling period, the control groundwater wells exhibited less bacterial contamination than the susceptible wells. Four control wells (C03, B07, K04, and K13) had detectable levels of total coliform twice during this study. Three of these wells (B07, K04, and K13) also had at least one enterococci detection. No *E. coli* hits were recorded for the control wells in any of the sampling periods, and no coliform or enterococci detections occurred in the control wells during the fourth sampling period.

The nitrate results were relatively consistent among the four sampling periods (see Table 2), with the average concentration of the control groundwater wells slightly above 1 mg/L N for each sampling period and the average concentration of the susceptible wells ranging between 11 mg/L to 14 mg/L N (see Tables 1-4 of Appendix B for individual well nitrate data during the four sampling periods). Results ranged from below detection (<0.1 mg/L N) to 31.1 mg/L. For each sampling period, there was a significant difference between the average concentration of the control groundwater wells and the average concentration of the susceptible wells: the control wells had much lower concentrations than the susceptible wells. No significant differences were found between the average nitrate concentrations of each county for any of the four

sampling periods, though Brown County consistently had the highest average concentrations and Fond du Lac County consistently had the lowest.

Table 2: Percentage of wells falling in different nitrate pollution categories during each sampling period.

Sampling Period	0-2 mg/L N	2-5 mg/L N	5-10 mg/L N	>10 mg/L N	# Wells Sampled
1	17.5%	7.5%	20.0%	55.0%	40
2	21.6%	8.1%	21.6%	48.7%	37
3	18.0%	12.8%	18.0%	51.3%	39
4	11.1%	33.3%	33.3%	22.2%	39

Objective 2: To assess levels of EDC activity in groundwater near farmland in the northeast Wisconsin counties of Brown, Calumet, Fond du Lac, and Kewaunee

We detected estrogenic activity in groundwater during all four sampling periods. Based on the number of wells run through the E-screen in each sampling period, 58%, 31%, 14%, and 5% of our groundwater samples exhibited estrogenicity in the first, second, third, and fourth sampling periods, respectively (Table 3). Cell proliferation was determined to be estrogen-dependent through use of the estrogen receptor antagonist, ICI 182,780, which inhibited cell growth in the presence of our samples. Estradiol equivalency ranged from 0.0114 pM to 12.87 pM (0.003 ng/L to 3.51 ng/L or 1.14 x 10-14 M to 1.29 x 10-11 M) (see Tables 1-4 of Appendix B for individual EEq well data during the four sampling periods).

Table 3: Percentage of sampled groundwater wells with detectable estradiol equivalents in the E-screen during each sampling period (lower LOQ of 1 pM EEq in sample extracts). Unknowns = samples in which estrogenicity was below the level of detectability in the E-screen assay.

Sampling Period	Below Detection	Detections	Unknown	N
1	35.0%	50.0%	15.0%	40
2	59.5%	27.0%	13.5%	37
3	80.6%	13.9%	5.6%	36
4	94.6%	5.4%	0.0%	37

The EEqs found in our study are within the range of levels found in other studies that utilized the E-screen. For instance, Shappell et al. (2007) found EEqs between 0.1 pM 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 produced EEqs within approximately one order of magnitude: 1 x 10^{-13} M (0.1 pM) to 1.0 x 10^{-12} M (1 pM) (Shappell 2006). In comparison, approximately 62% of the EEqs in our groundwater study fell within this order of magnitude; the remaining 27% and 10% fell between 1 x 10^{-14} M (0.01 pM) and 1.0 x 10^{-13} M (0.1 pM), and 1 x 10^{-12} M (1 pM) and 1.0 x 10^{-11} M (10 pM), respectively. The fact that most of our samples were either lower than the range found by Shappell et al.

(2007) or near the bottom of the range can be attributed to the fact that Shappell was looking at surface water bodies directly impacted by pollution, and we were looking at groundwater that may or may not be impacted by pollution. One would expect the concentrations of estrogenic chemicals originating at the surface to be somewhat reduced as they enter the water table, whether it be by filtration through the unsaturated zone, degradation by microbes, or dilution through mixing with other water sources. During transport through the aquifer, concentrations may become 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 estradiol equivalency. However, studies have shown that low concentrations of estradiol in surface waters (10-100 ng/L or 36.7 - 367 pM) 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, with the goal being population sustainability, the Environment Agency of England and Wales estimated 36.7 pM (10 ng/L) estradiol as the "lowest observable effect concentration", and 3.67 pM (1 ng/L) as the threshold concentration yielding no effect on the fish (Shappell et al. 2007). Others have predicted that the "no-observed-effect-concentration" for 17β-estradiol is between 5-25 ng/L (Harper and Sinha 2006). While the vast majority of our samples tested well below the 1 ng/L "no effect" threshold identified by the Environment Agency of England and Wales, our E-screen results show that some wells may have fallen within this range. Wells C02, C03, and C04, and wells F05, C03, F07, C04, and B12 exhibited EEqs above 0.1 ng/L (0.367 pM) during the first and second sampling periods, respectively, while samples C04-2 and B12-2 recorded values above the 1 ng/L threshold. No groundwater samples had an EEq greater than 0.1 ng/L in the third or fourth sampling period.

Objective 3: To determine whether EDC activity and fecal waste indicators in groundwater near farmland change after major recharge periods (e.g., rainfall; spring thaw).

Several significant, seasonal differences in bacterial levels were observed in susceptible wells across the four time points examined. Average coliform contamination was significantly greater in the fourth sampling period as compared to the first, second, and third sampling period as indicated by the Signed Rank Test ($p=0.0017$, $p=<0.0001$, $p=0.0014$, 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 contamination than the fourth and third sampling period, but was not significantly different from the first ($p=0.0554$).

Similar to coliform, the susceptible wells had significantly less average enterococci contamination in the second sampling period than the first, third, and fourth sampling periods ($p=0.0469$, $p=0.0059$, $p=<0.0001$, respectively). Enterococci contamination of the susceptible wells in the fourth sampling period was also significantly greater than the third sampling period ($p=0.0249$). The fourth sampling period had greater average enterococci values as compared to the first sampling period, but 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: the *E. coli* contamination in the fourth sampling period was significantly greater than the contamination of the first and second sampling periods ($p=0.0164$, $p=0.002$, respectively).

In combination, the seasonality results of the bacteria parameters indicate that bacteria levels were greatest during the spring thaw compared to summer and fall. The fourth sampling period had the most bacterial contamination, the third sampling period had the second-greatest amount of bacterial contamination, and the second sampling period had the least amount of bacterial contamination. As stated earlier, the presence of enterococci and/or *E. coli* in a groundwater well indicates that the well was contaminated with some type of human or animal waste. Due to the nature of *E. coli* and enterococci, both of which are found in the intestines of warm-blooded animals, our results suggest as many as 46% of our wells were contaminated with animal or human waste in the fourth sampling period.

When the dataset was analyzed as a whole, a significant difference was found among the four sampling periods for nitrate ($p=0.0151$). The Tukey adjustment indicated that this was due to a significant difference between the first and fourth sampling periods ($p=0.0086$). When the control and susceptible wells were

analyzed separately, it was found that the control wells did not differ significantly among the four sampling periods (p=0.6543). Thus, the difference between sampling periods was due to a difference in contamination of the susceptible wells, which had significantly greater nitrate contamination in the first sampling period as compared to the fourth (p=0.0081).

Unlike bacteria and nitrate results, EEqs were significantly lower in the fourth sampling period vs. 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 the greatest average EEqs and the most estrogenicity detections (p=0.6995). Sampling period three also had significantly less contamination than sampling 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 sampling period two and three (p=0.25).

Overall, fewer estrogenicity detections were found in the groundwater wells as compared to bacteria and nitrate detections in all the sampling periods. This could be due to several reasons. Firstly, estrogen contamination may simply occur less frequently in our subject wells than bacteria and nitrate contamination events. Perhaps there are fewer sources of estrogen contamination in our study area than bacteria or nitrate sources. Secondly, some samples may have had estrogenic activity that measured below the LOQ of our assay, preventing it from being detected. Thirdly, 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 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 either reducing EEqs or pushing values below the LOQ and preventing detection all together. Toxicity occurred very frequently in our assays, especially during the third and fourth sampling period. As such, it is possible that the estrogenicity of the groundwater samples may be greater than our results indicate, particularly during the third and fourth sampling period, since cell death due to the presence of toxic chemicals in the sample prevents or lowers EEq detection by the E-screen. Thus, it is possible that wells with apparent toxicity that registered below detection in the E-screen may have contained estrogenic chemicals, but the dose-dependent response of the cells was masked by the toxic components of the sample. These limitations of bioassay 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 gas chromatography-mass spectrometry (GC-MS; Drewes et al. 2005; Chen et al. 2006; Soliman et al. 2007).

Objective 4: To discern whether levels of groundwater contamination by EDCs correlate with other water quality indicators (nitrates, fecal coliform, E. coli levels)

Our study did not find strong correlations between estrogenicity and the other water quality parameters. We did not find any strong correlations between our E-screen data and the other water quality parameters, though one significant, 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 a conclusion. As discussed above, the weak relationship between *E. coli* and estrogenicity in the fourth sampling period was driven by two samples, F05-4 and F07-4. Both of these samples tested positive for estrogenicity, *E. coli*, coliform, and enterococci.

Several possible explanations exist for the lack of correlation between our water quality parameters. For example, toxicity of groundwater samples during the fourth sampling period – which may have led to low or undetectable EEqs - may have prevented the detection of a correlation of bacteria and estrogenicity data. Also, sources of contamination are plentiful, and estrogenic activity may be coming from a source other than that which causes bacterial contamination (e.g., estrogenic pesticides; pharmaceuticals from leaky underground septic tank).

Objective 5: To measure estradiol levels in water samples through use of an enzyme-linked immunosorbent assay (ELISA)

Attempts were made to measure concentrations of 17β-estradiol in the groundwater sample extracts using enyzme-linked immunosorbent assay (ELISA) kits obtained from Oxford Biomedical Research, Inc.

(Product Number EA 70). Specific 17β-estradiol concentrations in samples were not measurable with the ELISA, due to cross-reactivity and/or matrix effects.

Conclusions and Recommendations

Results from the study indicate that groundwater contamination with EDCs, bacteria and nitrate is a common problem in karst areas of northeast Wisconsin. EDC contamination was greatest during the months of August and November, times at which land application of manure is frequent. Potential sources of EDC contamination within our study area (e.g., pharmaceuticals from leaky septic systems, land-applied manure, estrogenic pesticides) remain speculative based on the information provided in this study, and 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 quick transport of surface runoff to the groundwater. The impact of individual well characteristics (well depth, depth to bedrock, age, and soil type) on water quality parameters, likewise, is worthy of study. Finally, the specific contaminants exerting estrogenic activity within the water samples should be analyzed with a more reliable method of detection than the ELISA, such as liquid chromatography-mass spectrometry.

References

Brown County Land Conservation Department. 2007. Groundwater and well information. http://www.co.brown.wi.us/Land_Conservation/GroundwaterWell2.htm.

Calumet County, 2006. Calumet County Land and Water Resource Management Plan 2007-2011. (www.co.calumet.wi.us/uploads/document/CalumetLWRMP2007_2011.pdf).

Celico, F., Varcamonti, M., Guida, M., and Naclerio, G. (2004) *Influence of Precipitation and Soil on Transport of Fecal Enterococci in Fracture Limestone Aquifers.* Applied and Environmental Microbiology. Vol. 70, No. 5: 2843-2847.

Cody, R. P. and Smith, J. K. (2006) *Applied Statistics and the SAS Programming Language.* 5th Edition. Pearson Education, Inc. Upper Saddle River, NJ 07458

Cohn, B.A., Wolff, M.S., Cirillo, P.M. and Sholtz, R.I. (2007). DDT and breast cancer in young women: New data on the significance of age at exposure. Environmental Health Perspectives 115(10): 1406-1414.

Colburn, T., Dumanoski, D. and Myers, J.P. (1996). Our Stolen Future: Are We Threatening Our Fertility, Intelligence and Survival? A Scientific Detective Story. New York, Dutton.

Dorgan, J.F., Longcope, C., Stephenson, H.E., Falk, R.T., Miller, R., Franz, C., Kahle, L., Campbell, W.S., Tangrea, J.A., and Schatzkin, A. (1996). Relation of prediagnostic serum estrogen and androgen levels to breast cancer risk. Cancer Epidemiol Biomark Prev 5:533-539.

Fisher, J.S., Turner, K.J., Brown, D. and Sharpe, R.M. (1999). Effect of Neonatal Exposure to Estrogenic Compounds on Development of the Excurrent Ducts of the Rat Testis through Puberty to Adulthood**.** Environmental Health Perspectives 107:397-405.

Fleming, L.E., Bean, J.A., Rudolph, M. and Hamilton, K. (1999). Mortality in a cohort of licenced pesticide applicators in Florida. Occupational and Environmental Medicine 56:14-21.

Gray, L.E., Ostby, J., Monosson, E., and Kelce, W.R. (1999). Environmental antiandrogens: low doses of the fungicide vinclozolin alter sexual differentiation of the male rat. Toxicology and Industrial Health 15: 48-64.

Hanselman, T. A., Graetz, D. A., and Wilkie, A. C. (2003) *Manure-Borne Estrogens as Potential Environmental Contaminants: A Review.* American Chemical Society. Vol. 37, No. 24: 5471-5478.

Hanselman, T.A., Graetz, D.A., Wilkie, A.C., Szabo, N.J. and Diaz, C.S. (2006). Determination of steroidal estrogens in flushed dairy manure wastewater by gas chromatography-mass spectrometry. J. Environ. Qual. 35:695-700.

Harper, S. and Sinha, P. (2006) *Tackling Emerging Contaminants at POTWS.* Pollution Engineering. Nov. 06: 22-25. www.pollutionengineering.com

Hemming, J., Shafer, M., Barry, T. and Schauer, J. (2007). Assessing occurrence, persistence and biological effects of hormones released from livestock waste. Presented at the *Fate and Effects of Hormones in Waste from Concentrated Animal Feeding Organizations (CAFOs)* Workshop sponsored by the EPA. (http://es.epa.gov/ncer/publications/workshop/08_20_07_cafo.html).

Herschler, R.C., Olmsted, A.W., Edwards, A.J., Hale, R.L., Montgomery, T., Preston, R.L., Bartle, S.J. and Sheldon, J.J. (1995). Production responses to various doses and rations of estradiol benzoate and trenbolone acetate implants in steers and heifers. J Anim Sci 73:2873-2882.
Ho, S.-M., Tang, W.-Y., Belmonte de Frausto, J. and Prins, G.S. (2006). Developmental Exposure to Estradiol and Bisphenol A Increases Susceptibility to Prostate Carcinogenesis and Epigenetically Regulates Phosphodiesterase Type 4 Variant 4. Cancer Research 66: 5624-5632.
Irwin, L.K., Gray, S., Oberdorster, E. (2001). Vitellogenin induction in painted turtle, *Chrysemys picta*, as a biomarker of exposure to environmental levels of estradiol. Aquat Toxicol 55:49-60.
Murray,T.J., Maffini, M.V., Ucci, A.A., Sonnenschein, C. and Soto, A.M. (2006). Induction of mammary gland ductal hyperplasias and carcinoma in situ following fetal bisphenol A exposure. Reproductive Toxicology 23: 383-390.
National Oceanic and Atmospheric Adminstration's National Weather Service (Accessed March 2009). *National Weather Service Forecast Office: Green Bay, WI.* Green Bay Weather Forecast Office. Green Bay, WI. *http://www.weather.gov/climate/xmacis.php?wfo=grb*
National Research Council (1999). Hormonally Active Agents in the Environment. National Academy Press, Washington, D.C.
Northeast Wisconsin Karst Task Force Executive Summary (February 9, 2007). Accessed at https://mywebspace.wisc.edu/kaerb/web/karst%20task%20force%20EXECUTIVE%20SUMMARY.pdf.
Panter, G.H., Thompson, R.S., and Sumpter, J.S. (1998). Adverse reproductive effects in male fathead minnows (*Pimephales promelas*) exposed to environmentally relevant concentrations of the natural oestrogens, oestradiol and oestrone. Aquat Toxicol 42:243-253.
Peterson, F. W., Davis, R. K., and Orndorff, H. A. (2000) *17β-Estradiol as an Indicator of Animal Waste Contamination in Mantled Karst Aquifers.* Journal of Environmental Quality. 29:826-834. Peterson, F.W., Davis, R.K. and Orndorff, H.A. (2000). 17 b-estradiol as an indicator of animal waste contamination in mantled karst aquifers. J. Environ. Qual. 29:826-834.
Sakaue, M., Ohsako, S., Ishimura, R., Kurosawa, S., Kurohmaru, M., Hayashi, Y., Aoki, Y., Yonemoto, J. and Tohyama, C. (2001). Bisphenol-A Affects Spermatogenesis in the Adult Rat Even at a Low Dose. Journal of Occupational Health 43:185 -190.
Shappell, N. W. (2006) *Estrogenic Activity in the Environment: Municipal Wastewater Effluent, River, Ponds, and Wetlands.* Journal of Environmental Quality. 35:122-132.
Shappell, N. W., Billey, L.O., Forbes, D., Matheny, T.A., Poach, M.E., Reddy, G. B., and Hunt, P.G. (2007) *Estrogenic Activity and Steroid Hormones in Swine Wastewater through a Lagoon Constructed-Wetland System.* Environmental Science and Technology. 41:444-450.
Skakkebæk, N.E., Rajpert-De Meyts, E. and Main, K.M. (2001). Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects**.** Human Reproduction 16:972-978
Susiarjo, M, TJ Hassold, E Freeman and PA Hunt. 2007. Bisphenol A Exposure ***In Utero*** Disrupts Early Oogenesis in the Mouse**.** *PLoS Genetics, 3(1): e5. doi:10.1371/journal.pgen.0030005*.
Toppari, J., Haavisto, A.-M. and Alanen, M. (2002). Changes in male reproductive health and effects of endocrine disruptors in Scandanavian Countries. Cad Saude Publica, Rio de Janeiro 18(2):413-420.
Toppari, J., Larsen, J., Christiansen, P., Giwercman, A., Grandjean, P., Guillette, L.J. Jr., Jegou, B., Jensen, T.K., Jouannet, P., Keiding, N., Leffers, H., McLachlan, J.A., Meyer, O., Muller, J., Rajpert-De Meyts, E., Thomas Scheike, Sharpe, R., Sumpter, J. and N.E. Skakkebaek, N.E. (1996). Male Reproductive Health and Environmental Xenoestrogens. Environmental Health Perspectives 104 (Suppl4):741-803.
Toniolo, P.G., Levitz, M., Zeleniuch-Jacquotte, A., Banerjee, S., Koenig, K.L., Shore, R.E., Strax, P. and Pasternack, B.S. (1995). A prospective study of endogenous estrogens and breast cancer in postmenopausal women. J Natl Cancer Inst 87:190-197.
Weir, H.K., Marrett, L.D., Kreiger, N., Darlington, G.A. and Sugar, L. (2000). Pre-natal and peri-natal exposures and risk of testicular germ cell cancers. International Journal of Cancer 87:438-443.

Appendix A: Publications and Presentations

Wingert, S.E.*;* Bauer-Dantoin, A. Fermanich, K.J.; Zorn, M.E. Assessing Levels and Potential Health Effects of Endocrine Disrupting Chemicals in Groundwater Associated with Karst Areas in Northeast Wisconsin. The 33rd Annual Meeting of the American Water Resources Association (AWRA) Wisconsin Section. March 5-6, 2009. Stevens Point, Wisconsin.

Wingert, S.E. Assessing Levels of Endocrine Disrupting Chemicals in Groundwater Associated with Karst Areas in Northeast Wisconsin. Master's Thesis in Environmental Science and Policy, University of Wisconsin – Green Bay, December, 2010.

Appendix B: Water quality indicators for individual wells during each sampling period

Table 1: Results from the first sampling period (August 11-12, 2008).

County	Well ID	Group	Sample Date	Nitrate + Nitrate (mg/L N)	Conductivity (mS/cm)	Coliform (MPN/100 mL)	Enterococci (MPN/100mL)	*E. coli* (MPN/100 mL)	Escreen (EEq in pM)*, Above LOD	All Escreen Detects (Eeq in pM)
Brown	B02	Susceptible	8/12/2008	15.60	1.107	5.2	0.0	0.0	unknown	unknown
Brown	B04	Susceptible	8/12/2008	11.60	0.848	19.9	0.0	0.0	unknown	unknown
Brown	B05	Susceptible	8/12/2008	23.60	1.228	1.0	0.0	0.0	BD	0.086
Brown	B06	Susceptible	8/12/2008	19.80	1.141	0.0	0.0	0.0	BD	0.068
Brown	B07	Control	8/12/2008	<0.1	0.953	90.4	20.1	0.0	0.040	0.040
Brown	B08	Susceptible	8/12/2008	22.00	1.036	4.1	0.0	0.0	0.184	0.184
Brown	B10	Control	8/12/2008	<0.1	1.600	0.0	0.0	0.0	0.279	0.279
Brown	B11	Susceptible	8/12/2008	21.90	1.390	0.0	0.0	0.0	0.120	0.120
Brown	B12	Susceptible	8/12/2008	9.27	0.899	6.3	0.0	0.0	BD	0.049
Brown	B14	Susceptible	8/11/2008	18.90	0.932	1.0	0.0	0.0	0.163	0.163
Calumet	C01	Susceptible	8/11/2008	13.20	0.867	0.0	0.0	0.0	0.250	0.250
Calumet	C02	Susceptible	8/11/2008	14.50	0.979	6.3	0.0	0.0	0.374	0.374
Calumet	C03	Control	8/11/2008	<0.1	0.274	18.7	0.0	0.0	0.461	0.461
Calumet	C04	Susceptible	8/11/2008	18.90	0.790	108.1	8.5	0.0	0.383	0.383
Calumet	C05	Control	8/11/2008	1.24	1.011	0.0	0.0	0.0	unknown	0.236
Calumet	C06	Susceptible	8/11/2008	14.40	0.825	1732.9	156.5	1.0	unknown	unknown
Calumet	C10	Susceptible	8/11/2008	7.12	0.930	195.6	13.5	6.3	0.040	0.040
Calumet	C11	Susceptible	8/11/2008	6.15	0.850	2.0	0.0	0.0	0.028	0.028
Calumet	C12	Susceptible	8/11/2008	6.74	0.787	>2419.6	275.5	0.0	0.011	0.011
Calumet	C15	Susceptible	8/11/2008	24.00	1.021	0.0	0.0	0.0	0.042	0.042
Fond du Lac	F01	Susceptible	8/11/2008	3.39	1.023	1046.2	13.5	25.6	unknown	unknown
Fond du Lac	F02	Susceptible	8/11/2008	6.70	0.895	0.0	0.0	0.0	BD	0.031
Fond du Lac	F03	Control	8/11/2008	4.11	0.860	0.0	0.0	0.0	0.172	0.172
Fond du Lac	F04	Susceptible	8/11/2008	15.70	1.106	2.0	0.0	0.0	BD	0.072
Fond du Lac	F05	Susceptible	8/11/2008	1.78	0.932	78.5	0.0	0.0	BD	BD
Fond du Lac	F06	Control	8/11/2008	0.26	0.602	0.0	0.0	0.0	0.299	0.299
Fond du Lac	F07	Susceptible	8/11/2008	15.00	0.769	59.4	5.2	1.0	BD	BD
Fond du Lac	F08	Susceptible	8/11/2008	12.30	0.884	0.0	0.0	0.0	0.094	0.094
Dodge	F09	Susceptible	8/11/2008	6.31	1.065	0.0	0.0	0.0	BD	0.017
Dodge	F10	Susceptible	8/12/2008	17.50	0.921	88.2	1.0	1.0	unknown	unknown
Kewaunee	K01	Susceptible	8/12/2008	24.30	0.862	0.0	0.0	0.0	BD	0.036
Kewaunee	K02	Susceptible	8/12/2008	20.20	0.777	0.0	0.0	0.0	0.146	0.146
Kewaunee	K03	Susceptible	8/12/2008	11.00	0.878	2.0	0.0	0.0	0.040	0.040
Kewaunee	K04	Control	8/12/2008	4.10	0.906	187.2	6.3	0.0	BD	BD
Kewaunee	K05	Susceptible	8/12/2008	5.13	0.773	6.3	0.0	0.0	BD	BD
Kewaunee	K06	Susceptible	8/12/2008	19.90	0.821	0.0	0.0	0.0	0.102	0.102
Kewaunee	K07	Susceptible	8/12/2008	13.00	0.886	10.9	4.1	0.0	BD	BD
Kewaunee	K08	Susceptible	8/12/2008	15.10	0.952	4.1	0.0	0.0	0.067	0.067
Kewaunee	K09	Susceptible	8/12/2008	8.51	0.680	0.0	0.0	0.0	BD	BD
Kewaunee	K13	Control	8/12/2008	0.82	1.047	1119.9	129.6	0.0	BD	0.069

**Unknown* refers to results that were unquantifiable due to cell death (as a result of groundwater sample toxicity). *BD* refers to results that were below the detection limits of the assay.

Table 2: Results from the second sampling period (November 17-18, 2008).

County	Well ID	Group	Sample Date	Nitrate + Nitrate (mg/L N)*	Conductivity (mS/cm)	Coliform (MPN/100mL)	Enterococci (MPN/100mL)	*E. coli* (MPN/100 mL)	Escreen (EEq in pM)**, Above LOD	All Escreen Detects (Eeq in pM)
Brown	B02	Susceptible	11/17/2008	14.8	1.055	<1	<1	<1	BD	0.048
Brown	B04	Susceptible	11/17/2008	12.5	0.84	2	<1	<1	0.111	0.111
Brown	B05	Susceptible	11/17/2008	19.4	1.095	<1	<1	<1	BD	BD
Brown	B06	Susceptible	11/17/2008	17.4	1.03	<1	<1	<1	0.219	0.219
Brown	B07	Control	11/17/2008	0.0343	0.967	3	<1	<1	BD	0.006
Brown	B08	Susceptible	11/17/2008	15.2	0.973	<1	<1	<1	BD	0.029
Brown	B10	Control	11/17/2008	0.1	1.475	43.5	<1	<1	BD	BD
Brown	B11	Susceptible	11/17/2008	31.1	1.71	<1	<1	<1	0.255	0.255
Brown	B12	Susceptible	11/17/2008	10.8	0.955	<1	<1	<1	12.875	12.875
Brown	B14	Susceptible	11/17/2008	15.5	0.83	5.1	<1	<1	BD	BD
Calumet	C01	Susceptible	11/18/2008	NS	NS	NS	NS	NS	NS	NS
Calumet	C02	Susceptible	11/18/2008	11.5	0.966	<1	<1	<1	BD	BD
Calumet	C03	Control	11/18/2008	0.0144	0.262	9.7	<1	<1	1.066	1.066
Calumet	C04	Susceptible	11/18/2008	15.3	0.751	1	<1	<1	7.190	7.190
Calumet	C05	Control	11/18/2008	1.47	1.009	<1	<1	<1	unknown	BD
Calumet	C06	Susceptible	11/18/2008	NS	NS	NS	NS	NS	NS	NS
Calumet	C10	Susceptible	11/18/2008	7.37	0.818	980.4	<1	13.4	BD	BD
Calumet	C11	Susceptible	11/18/2008	6.71	0.925	1	<1	<1	BD	BD
Calumet	C12	Susceptible	11/18/2008	NS	NS	NS	NS	NS	NS	NS
Calumet	C15	Susceptible	11/18/2008	28	0.994	<1	<1	<1	BD	BD
Fond du Lac	F01	Susceptible	11/18/2008	3.72	1.075	6.3	1	<1	BD	0.009
Fond du Lac	F02	Susceptible	11/18/2008	6.62	0.907	<1	<1	<1	BD	BD
Fond du Lac	F03	Control	11/18/2008	4.51	0.851	<1	<1	<1	0.096	0.096
Fond du Lac	F04	Susceptible	11/18/2008	15.3	1.069	<1	<1	<1	BD	0.016
Fond du Lac	F05	Susceptible	11/18/2008	1.58	0.933	<1	<1	<1	0.600	0.600
Fond du Lac	F06	Control	11/18/2008	0.256	0.582	<1	<1	<1	BD	0.010
Fond du Lac	F07	Susceptible	11/18/2008	6.99	0.819	17.3	1	<1	1.663	1.663
Fond du Lac	F08	Susceptible	11/18/2008	12.8	0.85	<1	<1	<1	unknown	unknown
Dodge	F09	Susceptible	11/18/2008	5.7	1.074	<1	<1	<1	BD	0.044
Dodge	F10	Susceptible	11/18/2008	17.3	0.966	11	<1	<1	BD	0.076
Kewaunee	K01	Susceptible	11/17/2008	16.6	0.979	<1	<1	<1	BD	BD
Kewaunee	K02	Susceptible	11/17/2008	20.1	0.795	<1	<1	<1	unknown	unknown
Kewaunee	K03	Susceptible	11/17/2008	7.5	0.787	<1	<1	<1	BD	BD
Kewaunee	K04	Control	11/17/2008	1.79	0.784	<1	<1	<1	unknown	0.169
Kewaunee	K05	Susceptible	11/17/2008	4.03	0.779	21.6	<1	<1	BD	0.005
Kewaunee	K06	Susceptible	11/17/2008	6.49	1.021	<1	<1	<1	BD	0.010
Kewaunee	K07	Susceptible	11/17/2008	12.5	0.87	61.3	2	<1	unknown	unknown
Kewaunee	K08	Susceptible	11/17/2008	15.1	0.937	52.9	1	<1	BD	BD
Kewaunee	K09	Susceptible	11/17/2008	6.49	0.634	<1	<1	<1	BD	BD
Kewaunee	K13	Control	11/17/2008	0.702	1.012	2	<1	<1	0.117	0.117

**NS* means a well was not sampled during this sampling period.
***Unknown* refers to results that were unquantifiable due to cell death (as a result of groundwater sample toxicity). *BD* refers to results that were below the detection limits of the assay.

Table 3: Results from the third sampling period (February-March 2009).

County	Well ID	Group	Sample Date	Nitrate + Nitrate (mg/L N)*	Conductivity (mS/cm)	Coliform (MPN/100mL)	Enterococci (MPN/100mL)	*E. coli* (MPN/100mL)	Escreen (EEq in pM)**, Above LOD	All Escreen Detects (Eeq in pM)
Brown	B02	Susceptible	2/24/2009	13.6	0.989	387.3	7.3	0	BD	BD
Brown	B04	Susceptible	2/24/2009	9.00	0.723	209.8	1	0	BD	BD
Brown	B05	Susceptible	2/24/2009	19.7	1.095	114.5	<1	0	BD	0.042
Brown	B06	Susceptible	2/24/2009	15.0	1.013	4.1	1	0	unknown	BD
Brown	B07	Control	2/24/2009	0.01	1.031	<1	<1	0	BD	BD
Brown	B08	Susceptible	2/24/2009	23.7	1.161	14.8	10.7	0	BD	0.063
Brown	B10	Control	2/24/2009	0.01	1.530	<1	<1	0	BD	0.071
Brown	B11	Susceptible	2/24/2009	29.5	1.890	<1	<1	0	BD	0.026
Brown	B12	Susceptible	2/24/2009	9.01	0.926	98.7	<1	0	BD	BD
Brown	B14	Susceptible	2/24/2009	13.6	0.836	6.3	<1	0	BD	BD
Calumet	C01	Susceptible	2/24/2009	14.5	0.872	<1	<1	0	BD	0.028
Calumet	C02	Susceptible	2/24/2009	15.1	0.761	<1	<1	0	BD	BD
Calumet	C03	Control	2/24/2009	0.01	0.325	<1	<1	0	unknown	unknown
Calumet	C04	Susceptible	2/24/2009	NS	NS	NS	NS	NS	NS	NS
Calumet	C05	Control	2/24/2009	1.36	1.023	<1	<1	0	BD	0.087
Calumet	C06	Susceptible	2/24/2009	14.7	0.771	<1	<1	0	not run	not run
Calumet	C10	Susceptible	2/24/2009	9.27	1.045	290.9	<1	0	0.125	0.125
Calumet	C11	Susceptible	2/24/2009	8.43	0.833	38.4	<1	0	BD	0.041
Calumet	C12	Susceptible	2/24/2009	10.0	0.776	178.9	<1	0	not run	not run
Calumet	C15	Susceptible	2/24/2009	25.0	1.208	<1	<1	0	BD	BD
Fond du Lac	F01	Susceptible	2/13/2009	3.38	0.958	3	<1	0	BD	BD
Fond du Lac	F02	Susceptible	2/13/2009	5.71	0.857	<1	<1	0	BD	BD
Fond du Lac	F03	Control	2/13/2009	4.46	0.827	<1	<1	0	BD	BD
Fond du Lac	F04	Susceptible	3/2/2009	16.5	NS	4.1		0	BD	BD
Fond du Lac	F05	Susceptible	2/13/2009	1.90	0.956	<1	<1	0	BD	BD
Fond du Lac	F06	Control	2/13/2009	0.231	0.930	<1	<1	0	BD	BD
Fond du Lac	F07	Susceptible	2/13/2009	7.58	0.687	770.1	5.2	344.1	not run	not run
Fond du Lac	F08	Susceptible	2/13/2009	14.3	0.852	1	<1	0	0.104	0.104
Dodge	F09	Susceptible	3/2/2009	5.41	NS	1	NS	0	BD	BD
Dodge	F10	Susceptible	2/13/2009	13.2	0.574	>2419.6	285.1	816.4	0.150	0.150
Kewaunee	K01	Susceptible	2/17/2009	12.1	0.752	2	<1	0	BD	BD
Kewaunee	K02	Susceptible	2/17/2009	18.1	0.797	2	<1	0	0.112	0.112
Kewaunee	K03	Susceptible	2/17/2009	18.9	0.860	101.2	10.4	0	BD	0.025
Kewaunee	K04	Control	2/17/2009	2.73	0.832	2	<1	0	BD	0.019
Kewaunee	K05	Susceptible	2/17/2009	4.93	0.654	81.6	8.2	2	BD	0.020
Kewaunee	K06	Susceptible	2/17/2009	12.2	0.890	10.4	2	0	BD	BD
Kewaunee	K07	Susceptible	2/17/2009	11.5	0.417	274.8	10.4	0	BD	BD
Kewaunee	K08	Susceptible	2/17/2009	13.2	1.007	<1	<1	0	BD	0.035
Kewaunee	K09	Susceptible	2/17/2009	4.92	0.614	<1	<1	0	0.040	0.040
Kewaunee	K13	Control	2/17/2009	0.563	0.973	<1	2	0	BD	0.079

**NS* means a well was not sampled during this sampling period.

** *Unknown* refers to results that were unquantifiable due to cell death (as a result of groundwater sample toxicity). *BD* refers to results that were below the detection limits of the assay. *Not run* means the sample was not run through the E-screen assay.

Table 4: Results from the fourth sampling period (March 18-19, 2009).

County	Well ID	Group	Sample Date	Nitrate + Nitrate (mg/L N)*	Conductivity (mS/cm)	Coliform (MPN/100 mL)	Enterococci (MPN/100mL)	*E. coli* (MPN/100mL)	Escreen (EEq in pM)**, Above LOD	All Escreen Detects (Eeq in pM)
Brown	B02	Susceptible	3/19/2009	NS	NS	NS	NS	NS	NS	NS
Brown	B04	Susceptible	3/19/2009	12.6	0.672	727	24.6	3.1	BD	BD
Brown	B05	Susceptible	3/19/2009	17.3	1.006	261.3	7.5	3.1	BD	BD
Brown	B06	Susceptible	3/19/2009	14.9	0.999	17.1	<1	<1	BD	BD
Brown	B07	Control	3/19/2009	0.1	1.031	<1	<1	<1	BD	0.012
Brown	B08	Susceptible	3/19/2009	21.5	1.017	2	<1	<1	BD	0.031
Brown	B10	Control	3/19/2009	0.1	1.550	<1	<1	<1	not run	not run
Brown	B11	Susceptible	3/19/2009	22.5	1.530	<1	<1	<1	BD	BD
Brown	B12	Susceptible	3/19/2009	9.03	0.754	>2419.6	10.8	6.3	BD	BD
Brown	B14	Susceptible	3/19/2009	16.3	0.937	36.9	1	39.7	BD	BD
Calumet	C01	Susceptible	3/19/2009	14.4	0.865	<1	<1	<1	BD	BD
Calumet	C02	Susceptible	3/19/2009	13.2	0.604	25.9	<1	<1	BD	BD
Calumet	C03	Control	3/19/2009	0.1	0.387	<1	<1	<1	BD	0.069
Calumet	C04	Susceptible	3/19/2009	11.8	0.580	195.6	2	<1	BD	0.016
Calumet	C05	Control	3/19/2009	1.40	1.014	<1	<1	<1	BD	BD
Calumet	C06	Susceptible	3/19/2009	14.7	0.775	<1	<1	<1	BD	BD
Calumet	C10	Susceptible	3/19/2009	7.50	0.698	>2419.6	8.5	85.7	BD	BD
Calumet	C11	Susceptible	3/19/2009	7.12	0.753	816.4	<1	<1	BD	BD
Calumet	C12	Susceptible	3/19/2009	8.59	0.632	>2419.6	5.2	<1	BD	BD
Calumet	C15	Susceptible	3/19/2009	NS	NS	NS	NS	NS	NS	NS
Fond du Lac	F01	Susceptible	3/18/2009	2.71	0.884	461.1	4.1	<1	BD	BD
Fond du Lac	F02	Susceptible	3/18/2009	5.50	0.826	<1	<1	<1	BD	BD
Fond du Lac	F03	Control	3/18/2009	4.12	0.881	<1	<1	<1	BD	BD
Fond du Lac	F04	Susceptible	3/18/2009	NS	NS	NS	NS	NS	BD	BD
Fond du Lac	F05	Susceptible	3/18/2009	4.15	0.996	110.6	11.9	27.5	0.118	0.118
Fond du Lac	F06	Control	3/18/2009	0.272	0.589	<1	<1	<1	BD	BD
Fond du Lac	F07	Susceptible	3/18/2009	6.74	0.635	206.4	8.5	3.1	0.113	0.113
Fond du Lac	F08	Susceptible	3/18/2009	14.4	0.855	32.7	3	<1	BD	BD
Dodge	F09	Susceptible	3/18/2009	5.83	1.120	8.5	<1	<1	BD	BD
Dodge	F10	Susceptible	3/18/2009	11.5	0.832	46.4	2	<1	BD	BD
Kewaunee	K01	Susceptible	3/18/2009	9.09	0.735	23.1	<1	<1	BD	BD
Kewaunee	K02	Susceptible	3/18/2009	18.0	0.782	81.6	1	<1	BD	BD
Kewaunee	K03	Susceptible	3/18/2009	13.3	0.937	1046.2	123.6	36.2	BD	BD
Kewaunee	K04	Control	3/18/2009	3.83	0.909	<1	<1	<1	BD	BD
Kewaunee	K05	Susceptible	3/18/2009	2.75	0.392	>2419.6	579.4	27.5	BD	BD
Kewaunee	K06	Susceptible	3/18/2009	11.1	0.711	14.6	1	<1	BD	BD
Kewaunee	K07	Susceptible	3/18/2009	7.92	0.413	1732.9	248.1	1	BD	0.016
Kewaunee	K08	Susceptible	3/18/2009	12.8	1.017	80.9	<1	<1	BD	0.043
Kewaunee	K09	Susceptible	3/18/2009	5.67	0.629	<1	<1	<1	BD	0.017
Kewaunee	K13	Control	3/18/2009	0.730	1.087	<1	<1	<1	BD	BD

**NS* means a well was not sampled during this sampling period.

***BD* refers to results that were below the detection limits of the assay. *Not run* means the sample was not run through the E-screen assay.