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#165

SUSCEPTIBILITY OF LA CROSSE MUNICIPAL WELLS TO ENTERIC VIRUS CONTAMINATION FROM SURFACE WATER CONTRIBUTIONS

A Final Report prepared for the

WISCONSIN DEPARTMENT OF NATURAL RESOURCES

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EXECUTIVE SUMMARY

Human enteric viruses have been identified in municipal drinking water wells, but few studies have examined the routes responsible for virus contamination. In this study we monitored enteric viruses in La Crosse, Wisconsin municipal wells and surrounding river water, and determined if surface water infiltration related to virus occurrence. From March 2001-February 2002, one river water site and four municipal pumping locations predicted to have variable degrees of surface water infiltration were sampled monthly for enteric viruses, indicators of fecal contamination, and oxygen ($^{18}O/^{16}O$) and hydrogen ($^{2}H/^{1}H$) isotopes. Surface water was identified in two of the four municipal pumping locations using water isotopes. Analyses of 48 water samples from 6 municipal wells resulted in 24 samples (50%) testing positive for enteric viruses including enteroviruses, rotavirus, hepatitis A virus, and Norwalk-like virus. Of 12 river water samples, 10 (83 %) were virus positive. Virus occurrence cannot be related to infectiousness as the method used in this work identifies virus RNA but cannot ascertain if the virus is active or inactive. None of the well water samples were positive for indicators of sanitary quality, though all river samples were positive for one or more indicators. This result suggests that indicators are not surrogates for virus occurrence in the La Crosse hydrologic system. There was an appreciable incidence of enteric viruses in each La Crosse municipal well sampled, including those without measurable surface water infiltration as indicated by water isotopes. This suggests an unidentified source, in addition to surface water, was responsible for the virus occurrence.

INTRODUCTION

Human gastrointestinal viruses occur in groundwater (Borchardt et al. 2003; Abbaszedegan et al. 1999) and are thought to be responsible for a significant proportion of infectious gastroenteritis related to groundwater consumption. Between 1971 and 1996, there were 642 outbreaks of waterborne disease outbreaks in the United States of which 371 (58%) were associated with groundwater sources; in 9% of the outbreaks, the etiologic agent was identified as a virus (EPA 2000). In the most recent compilation of waterborne disease outbreak data for the years 1999 and 2000, 26 of 37 infectious disease outbreaks were attributed to groundwater, and a virus was identified in four of these (Lee et al. 2002). The etiologic agent could not be identified in roughly half of groundwater-related outbreaks, but these outbreaks were presumably viral in origin because of the absence of bacterial and protozoan pathogens (EPA 2000; Craun et al. 1997).

Few studies have systematically monitored groundwater sources of drinking water for the occurrence of gastrointestinal viruses. In a nation-wide study, 539 samples from 448 groundwater sites in 35 states were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) for enteroviruses, rotavirus, hepatitis A virus (HAV), and human caliciviruses (Abbaszedegan 2002). Among all samples, 141 (31.5%) were positive for at least one virus; 68 (15.2%), 62 (13.8%), 31 (6.9%), and three (0.9%) were positive for enteroviruses, rotavirus, HAV, and caliciviruses, respectively (Abbaszedegan 2002). In another study using similar techniques, Borchardt, et al. (2003) tested 50 private household wells located throughout Wisconsin for gastrointestinal viruses. Four wells (8%) were positive. Three wells were positive for HAV; another well was positive for rotavirus and calicivirus in one sample and an enterovirus in another sample. Other unpublished studies report virus contamination in community and non-community wells (EPA 2000). Past studies have primarily focused on estimating the prevalence of virus contamination. An ideal next step would be to model groundwater flow near potential fecal sources and develop the ability to estimate the likelihood of virus contamination in a particular well. In order to predict virus contamination, it is necessary to consider virus surface-to-groundwater transport.

Numerous factors affect viral transport in the subsurface environment: organic matter content, mineral content, soil particle size, salt species and concentrations, water infiltration, and virus type (Woessner et al. 2001; Yates and Yates 1988; Gerba and Bitton 1984; Goyal and Gerba 1979). Virus adsorption to sediment particles via electrostatic attractions is the primary virus removal mechanism; sediment pH and virus isoelectric point dictate the degree of adsorption (Chattopadhyay and Puls 2000; Dowd et al. 1998). However, these attractive interactions can be disrupted when there is a large influx of water, for example after a heavy

rainfall, leading to virus desorption (Yates and Yates 1990; Gerba and Bitton 1984). Similarly, it is possible that viruses are desorbed and readily transported when fecally contaminated surface water from a nearby lake or river rapidly infiltrates underlying groundwater.

Stable hydrogen and oxygen isotope ratios can be valuable tools for investigating surface water infiltration into groundwater systems (Mazor 1997; Clark and Fritz 1997). These isotopes are ideal conservative tracers of water sources because they are part of the water molecule itself. Stable isotope ratios of water are conserved in aquifers at low temperature, but the water becomes isotopically fractionated on the surface at less than 100% humidity (Gat 1970). Because the vapor pressure of $H_2{}^{16}O$ is greater than $H_2{}^{18}O$, the residual liquid is characterized by a higher $H_2{}^{18}O$ content after evaporation. Protium (^1H) and deuterium (^2H) fractionate to a greater extent than oxygen due to their larger percent relative mass difference. Thus, characteristic $^{18}O/^{16}O$ and $^2H/^1H$ ratios can provide fingerprints for water sources. These approaches have only recently been applied to drinking water supplies

Many communities in the U.S. rely on groundwater that is under the influence of surface water. The City of La Crosse, Wisconsin, is located adjacent to the Mississippi River, but its drinking water source is the alluvial sand-gravel aquifer beneath the city. The United States Geologic Survey and the Wisconsin Geological and Natural History Survey recently conducted a Source Water Area Protection project for La Crosse County to identify quantitatively the zone of contribution for each municipal groundwater supply in the county, from which a 3D model was completed (Hunt et al. 2003). Simulations using the model suggested that some wells in the City of La Crosse receive large amounts of surface water, some receive only small amounts, and others appear not to receive any. The Mississippi River likely contains gastrointestinal viruses;

therefore, we conjectured that virus occurrence in the La Crosse wells may be related to surface water contributions.

The objectives of this study were to test for gastrointestinal viruses in wells that had predicted varying amounts of surface water, quantify the amount of surface water in the discharge of these wells using water isotope analyses, and then relate the amount of surface water to the presence and frequency of virus detection. This understanding could be transferable to other communities with similar hydrogeologic characteristics, thus enabling identification of wells vulnerable to virus contamination without costly virus sampling of all the wells in a pumping system. However, instead of finding virus contamination limited to the subset of wells with the most surface water contributions, all the wells (even those without measurable surface water) were contaminated with viruses, suggesting that there was another fecal source besides the Mississippi River that was reaching the aquifer.

MATERIALS AND METHODS

Sampling design. Four drinking water wells and one Mississippi River site in La Crosse, Wisconsin, were sampled monthly for one year from March 2001 to February 2002 (Fig. 1). The wells selected for sampling included one well predicted to have high surface water contributions (Well 24), one well predicted to have low surface water contributions (Well 14), and two wells with intermediate contributions (Wells 10 and 25) (Fig. 1). Due to maintenance issues Wells 10 and 14 were shut down during one of water sanitary quality, and the $^{18}O/^{16}O$ ratio as a measure of surface water contribution. All samples from municipal wells were taken at the wellhead, prior to chlorination, from a standard tapered coliform bacteria-sampling tap. River water samples were taken from a levee using an immersible sump pump submerged 0.5 to

1.0 m. In December 2001, two samples were taken from two piezometers using a battery powered peristaltic pump. One piezometer was located between the river and Well 24 and the other was located between the river and Well 10 (Fig. 1).

Analysis of indicators of fecal contamination. Indicator samples were collected before the virus filtration apparatus was attached to the tap; gloves were worn and the tap was disinfected with 70% EtOH. Four liters of well water were collected into a sterile polypropylene container and transported to the laboratory on ice on the day of collection. All indicator analyses were set-up that same day or the next day, in which case the samples were stored overnight at 4°C in a refrigerator.

Somatic and male-specific coliphages were enumerated using the single agar layer procedure as described in EPA method 1602 (EPA 2001). The analysis volume for each coliphage type was 100 ml. Samples were analyzed as a batch (i.e., 1 river and 4 well samples each analysis period). Each batch included a positive and negative control to test for method efficacy, and each sample included matrix controls to test for interferences. Matrix controls were performed by seeding 80-plaque forming units (PFU) of somatic coliphage phiX-174 (ATCC 13706-B1) and 200 PFU of male-specific by $R = 100\ (N_{sp}\text{-}N)/T$, where R = percent recovery, N_{sp} = number PFU in spiked sample, N_{usp} = number PFU in unspiked sample, and T = number coliphage spiked. In addition, the recovery efficiency of the method was measured with each sample batch by testing two 100-ml volumes of sterile reagent grade water, one that had been seeded with 80 PFU of phiX-174 and the other seeded with 200 PFU of MS2.

Total coliforms and *Escherichia coli* bacteria were detected using the chromogenic substrate assays Colilert and Colisure (both from IDEXX Laboratories, Westbrook, ME). Both assays were performed in parallel for each sample, and if either assay was positive, a sample was

classified as indicator positive. Fecal enterococci were tested using the chromogenic substrate assay Enterolert (IDEXX Laboratories). The test volume for all bacterial indicators was 100 ml. Results were scored as indicators present/absent.

Water isotope analysis. Water isotope samples were collected unfiltered in clean, dry, 20-ml glass vials and sealed with a polyseal cap. Paraffin™ film was used to secure the cap after sample collection to prevent evaporation that can alter $^{18}O/^{16}O$ and $^{2}H/^{1}H$ ratios. Two samples were taken from each well during each sampling period; one was analyzed and one was held as a duplicate sample in case of breakage during shipping. Water isotopes were analyzed at the United States Geologic Survey Stable Isotope Laboratory, Reston, VA. Oxygen isotope ratios were measured using CO_2-H_2O equilibration (Epstein and Mayeda 1953). Stable hydrogen isotope ratios were determined by H_2-H_2O equilibration (Coplen et al. 1991). Oxygen and hydrogen isotopic results are reported in per mil relative to Vienna Standard Mean Ocean Water (VSMOW) and normalized on scales such that the oxygen and hydrogen isotopic values of Standard Light Antarctic Precipitation (SLAP) were -55.5 per mil and -428 per mil, respectively (Coplen 1994). Analytical error (2σ) is estimated at ±0.2 per mil and ±2.0 per mil for $\delta^{18}O$ and δD, respectively.

Virus sampling. Viruses were sampled by filtering a target water volume of 1,500 L through a 1-MDS filter (CUNO, Meriden, CT) according to the American Public Health Association (1995). A sediment prefilter (McMaster and Carr, Los Angeles, CA) was used for the river site. All samples were collected aseptically; filter housings were autoclaved; tubing, gauges, sump pump, and other non-autoclavable components of the virus filtration apparatus were disinfected with 10% bleach for 30 min followed by 2.5% sodium thiosulfate for 30 min and two rinses with deionized H_2O (Fout et al. 2001). Water temperature and pH were measured

prior to virus sampling. Filters were transported to the laboratory on ice and processed within two days after collection.

Virus elution and flocculation. Viruses were eluted by slowly passing 1 L of 1.5% beef extract (wt/vol) with 0.05 M glycine (pH 9.5) backwards through the filter twice. The prefilter from the river sample was eluted in series with the 1-MDS filter. For the first 45 samples (March 2001 to November 2001), the eluate was flocculated by acidification where the beef extract was adjusted to pH 3.5, stirred for 3 min at room temperature, and centrifuged at 2,500 x g for 15 min at 4°C. The pellet was resuspended in 30 ml of sterile 0.15 M Na_2HPO_4 (pH 9.25), centrifuged again at 6,000 x g for 10 min at 4°C, and the supernatent was adjusted to pH 7.25 +/- 0.25. For the last 17 samples (December 2001 to February 2002), the eluate was flocculated using polyethylene glycol (PEG). The reason for switching flocculation methods was that during the sample collection period we validated in our laboratory the reports of other investigators (Guyader et al. 1994; Green and Lewis 1995; Schwab et al. 1996) -- that compared to acidification, the PEG method yielded higher virus recovery efficiency in a smaller final concentrated sample volume (see Chapter 3). Flocculation using PEG was accomplished by adjusting the beef extract pH to 7.5 +/- 0.25 with 1 N HCl, adding NaCl (0.2 M final concentration) and 8% PEG 8,000 (wt/vol) (Sigma Chemical Co., St. Louis, Mo.), stirring this mixture for 16 h at 4°C, and centrifuging at 4,200 x g for 45 min at 4°C. The pellet was resuspended in 2 ml of sterile 0.15 M Na_2HPO_4 (pH 9.25), aliquoted, and frozen immediately at -80°C. The final concentrated samples that had been flocculated by acidification (March 2001 to November 2001) were subsequently flocculated again by the PEG method so that (to the extent possible) samples collected from different periods were concentrated to a similar degree. To accomplish this, 24 ml of final concentrated sample from acidification was treated with PEG as

described previously and with the amounts of reagents adjusted accordingly. The remaining portion (approximately 6 ml) of the acidified final concentrated sample volume (FCSV) was archived. All final concentrated samples were stored at -80°C.

A method recovery control was performed approximately every three months during the study period according to the quality control procedure for virus-monitoring that is described in the Information Collection Rule (Fout et al. 1996). Two-hundred PFU of poliovirus (Sabin 3) were seeded into 40 L dechlorinated tap water, which was then filtered through a sterile 1-MDS filter, eluted into 1 L beef extract as described above, and flocculated by either acidification (3 times) or PEG (1 time). The seeded virus captured in the FCSV was cultured on buffalo green monkey kidney (BGMK) cells, and the quantity of virus was determined by the most probable number assay (Fout et al. 1996). The mean recovery for flocculation by acidification was 79% (n = 3, range 33 to 130%). Recovery was 137% for the single control when PEG was used as the flocculation agent.

Viral RNA extraction and concentration. Prior to RNA extraction, all work surfaces and pipettors were scrubbed with RNase ZAP (Sigma), 10% bleach, and 70% ethanol to remove nucleases and contaminants. The work surfaces and pipettors in the areas used for master mix preparation and template addition were similarly cleaned each time before use. Viral RNA was extracted by adding 500 µl guanidine solution (4 M guanidine thiocyanate and 20 mM sodium acetate, pH 5.2) to 500 µl final concentrated sample, vortexing for 3 min, and then adding 1 ml buffered saturated acidic phenol-chloroform (5:1, pH 4.7). After vortexing again for 3 min, the solution was centrifuged at 12,000 x g for 20 min at room temperature, and 800 µl of the aqueous phase was transferred to 800 µl of chloroform/isoamyl alcohol (24:1). Vortexing and centrifugation steps were repeated. Sephadex G-100 columns were prepared in 5-ml syringe

barrels plugged with silane/DEPC-treated glass wool by adding a sufficient volume of reconstituted Sephadex G-100 (Sigma) to produce a 3-ml resin bed. After rinsing three times with 1 ml Tris-EDTA buffer (TE, pH 7.6), 750-µl of the aqueous phase of the sample/chloroform/isoamyl alcohol mixture was added to the top of the column. The first column eluate (fraction 1) was discarded; three consecutive 750-µl volumes of TE were then added, displacing three 750-µl eluate volumes (fractions 2, 3, and 4). These fractions were placed into separate RNase-free microcentrifuge tubes containing 50 µl 20% Chelex resin (Sigma). Fractions 2 and 4 were stored at -80°C. Fraction 3, which had been found previously to contain the highest concentration of viral RNA (Borchardt et al. 2003), was concentrated using a sterile, nuclease-free Centricon YM-100 ultrafilter (Millipore, Bedford, MA). The ultrafilter was sterilized with 70% EtOH and treated with 0.2% DEPC-treated water at 37°C for 2 h prior to use. The entire fraction 3 was placed on the filter and centrifuged at 2,000 x g for 12 min at 4°C. RNA Storage Solution (300 µl) (Ambion, Inc., Austin, TX) was pipetted onto the filter, which was then inverted and placed on a new, autoclave-sterilized cupule and centrifuged (1,000 x g for 2 min at 4°C). The fraction 3 RNA concentrate was stored at -80°C.

Reverse transcription-polymerase chain reaction. A two-step RT-PCR reaction was performed to detect five groups of viruses: enteroviruses, rotavirus, hepatitis A virus (HAV), and Norwalk-like viruses genogroups 1 and 2 (NLV G1 and G2). The RT master mix was prepared as follows (with final concentrations reported for one reaction): 74.5 µl nuclease-free water (Fisher Scientific, Fair Lawn, NJ), *Taq* buffer (50 mM KCl, 10 mM Tris-HCl, [Applied Biosystems, Branchburg, NJ]), 3.0 mM $MgCl_2$ (Applied Biosystems), 10 mM dithiothreitol (Invitrogen, Carlsbad, CA), 70 µM each deoxynucleotide triphosphate, (Applied Biosystems), 200 U RNase inhibitor (Promega, Madison, WI), and 500 U Superscript II Molony Murine

Leukemia Virus reverse transcriptase (Invitrogen). The PCR master mix contained 0.4 µM of each upstream and downstream primer (Integrated DNA Technologies, Coralville, IA), 10 U *Taq* polymerase (Applied Biosystems), and 4.0 µl nuclease-free water. Final concentrations of the primers were 0.43 µM each, except for NLV G1 where the one upstream and three downstream primers had a final concentration of 0.30 µM each. The primers are described in Borchardt et al. (2003). Separate reactions were run for each virus group.

Fifty microliters of fraction 3 RNA concentrate was added to 50 µl nuclease-free water and 2 µg random hexamers (Fisher), heated at 99°C for 4 min, placed on ice, and then the RT master mix was added, for a final RT reaction volume of 290 µl. Temperature conditions for RT were 25° C (15 min), 42° C (60 min), 99° C (5 min), and then 4° C until the PCR master mix was added. The entire RT volume served as the PCR template, at a final total volume of 298.6 µl. The PCR thermocycling conditions for enterovirus, rotavirus, and HAV were an initial denaturation step at 94°C (3 min) followed by 35 cycles at 94°C (75 s), 55°C (75 s), and 72°C (75 s) and ending with a final extension period at 72°C for 7 min. The thermocycling conditions for both of the NLVs were identical to the other viruses except there were 40 cycles and the annealing and extension temperatures were 50°C and 60°C, respectively.

Controls for extraction, reverse transcription-polymerase chain reaction, and inhibition. The extraction negative control consisted of final concentrate from 1 L beef extract that had been flocculated by PEG. An aliquot of this final concentrate (500 µl) was extracted each day a batch of samples was extracted, reserving the control for last in the sequence of extractions so that if contamination occurred it would be more likely to be detected. The RT-PCR negative control consisted of the same reaction components as for the samples except that 50µl water was substituted for the fraction 3 RNA concentrate. The RT-PCR positive controls

consisted of one of the five target viruses seeded into 1 L beef extract and flocculated by PEG. To minimize the possibility of contamination the final concentrates of the virus, positive controls were extracted on different dates than the field samples; moreover, only one positive control was run per set of RT-PCR reactions. All controls were processed and analyzed in the same fashion as the field samples.

Each sample was tested for RT-PCR inhibition by seeding 50 µl of fraction 3 RNA concentrate with a Norwalk virus RNA internal standard (NIS). This standard consisted of a portion of the Norwalk virus RNA sequence encoding the polymerase gene that had been synthetically constructed to include a 123-bp deletion, thus it could be distinguished from wildtype virus amplicon (Schwab et al. 1997). Lyophilized NIS was mixed with 1 ml nuclease-free water, vortexed gently, centrifuged for 1 min at 5,000 x g, aliquoted, and stored at -80°C. The NIS was diluted (10^{-7}) in nuclease-free water, and 50 µl of this dilution was combined with 50 µl of fraction 3 RNA concentrate, random hexamers, and RT and PCR master mixes. Primers NVp35 and NVp36 were used as described by Schwab et al. (1997). Thermocycling conditions were the same as for NLV G1 and G2. The inhibition control was run separately from the samples, instead of internally. A sample was scored as inhibited if the predicted 347-bp NIS amplicon was absent on an agarose gel.

Agarose gel electrophoresis. The RT-PCR amplicons were resolved by electrophoresis in 1.6% agarose gels containing ethidium bromide followed by UV light illumination (Gel-Doc System; Bio-Rad Laboratories, Hercules, CA). The gel was observed for amplicons that had sizes corresponding to enteroviruses (196-bp), rotavirus (211-bp), HAV (192-bp), and the Norwalk-like viruses (123-bp).

Oligoprobe hybridization. Gels were depurinated in 0.4 M HCl for 15 min with rocking, rinsed twice with DEPC-treated water, and then denatured in 0.4 M NaOH for 15 min. Amplicons were transferred from the gel to a nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ) using a vacuum blotter (Bio-Rad™ model 785). Transfer proceeded in 10X saline sodium citrate (SSC, 1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with a vacuum pressure of 130 mm Hg for 90 min. After blotting, membranes were soaked in 0.4 M NaOH for 1 min followed by 1.0 M Tris-HCl (pH 7.5) and 5X SSC for 1 min. The membrane-bound DNA was immobilized with a Stratalinker™ UV-Crosslinker at 1.2 kJ m^{-2}. Oligoprobes (Integrated DNA Technologies) specific to each of the five virus types (Borchardt et al. 2003) were labeled with digoxygenin (DIG) using the DIG oligonucleotide 3'-end labeling kit (Boerhinger Mannheim, Mannheim, Germany) following manufacturer instructions and performing the kit controls. Membranes were prehybridized with ExpressHyb hybridization buffer (20 ml/100 cm^2 membrane) (Clontech Laboratories, Inc., Palo Alto, CA) at 42°C for 60 min in a rotisserie hybridization oven. The hybridization buffer containing labeled oligoprobe was boiled for 10 min, briefly cooled, and added to the membrane after removing the pre-hybridization buffer. The hybridization incubation period was 6 h at 42°C. Afterwards, the membrane was washed following the instructions of the hybridization buffer manufacturer (two washes at room temperature with 50 to 100 ml 2X SSC-0.1% sodium dodecyl sulfate [SDS] followed by two additional washes at 42°C with 0.1X SSC-0.1% SDS). Successful hybridization was visualized using an enzyme-linked immunoassay DIG detection kit based on colorimetric substrates (Boehringer Mannheim); the color reaction was stopped after 16 h.

A sample was scored as virus-positive when the oligoprobe hybridized to the membrane at the location of the amplicon specific to the virus group tested. If the gel was positive for the amplicon but it did not hybridize, the sample was scored as virus negative.

Reverse transcription-polymerase chain reaction method detection limit. To provide a basic estimate for the limit of the RT-PCR method for detecting low levels of virus present in groundwater samples, we seeded approximately 250 plaque forming units (PFU) of poliovirus (Sabin 3) into 1 L beef extract and proceeded with the method procedure as described above. The poliovirus was enumerated at the time it was seeded by the standard agar overlay plaque assay using BGMK cells. The plaque assay was performed in duplicate (i.e., two independent serial dilutions of the polio stock) using both 1/10 and 1/2 serial dilutions within a replicate to ensure that more than one dilution yielded countable plaques. The method detection limit was estimated on two separate occasions, once where the beef extract was flocculated by acidification and once by the PEG method. After flocculation, the final concentrated samples were diluted serially two-fold (1/2 to 1/64) with unseeded final concentrated sample as the diluent, and then each dilution was carried through the remainder of the procedure until amplicon visualization after agarose gel electrophoresis. Hybridization of the gel products was not completed due to time constraints.

RESULTS

Characteristics of the study wells and water samples. The 6 municipal wells sampled in the present study were conventionally drilled wells in active service and were opened to the shallow sand and gravel aquifer. Well ages ranged from 17 to 65 y (Table 1). Well depths and casing depths were at least 30 m and 15 m, respectively, with static water levels between 3 and

14 m. During the study period, the pumping rate from the wells was on the order of hundreds of gallons per min, and the total volume pumped during the 12-month period was on the order of hundreds of millions of gallons per well (Table 1).

Well water pH was generally between 7 and 8 (Table 2), except for seven samples from Well 24 that had a pH ≥ 8.00. These samples were adjusted to pH 7.00 to 7.50 with 0.1 N HCl during sample filtration to facilitate virus adsorption. River water pH was similar to that of the well water (Table 2), and only one river sample had a pH greater than 8.0. Well water temperature ranged from 9.8 to 15.0°C, whereas river water temperatures ranged from 2.0 to 26.1°C (Table 2).

All the well water samples for viruses met or exceeded the 1,500-L target sample volume, but the river samples clogged the pre-filter after several hundred liters (Table 2). The fraction of the sample volume analyzed by RT-PCR for viruses, after accounting for the 500-µl aliquot of the final concentrated sample and the 50-µl aliquot of the fraction 3 RNA concentrate, averaged 2.6% (median 1.7%, range 0.4 to 5.0%). These fractions corresponded to an average sample volume analyzed of 36.0 L (median = 25.2 L, range 1.0 to 137.6 L).

Virus occurrence as determined by reverse transcription-polymerase chain reaction. Of the 62 water samples collected during the present study, 36 (58%) contained at least one enteric virus as detected by RT-PCR and confirmed by Southern hybridization (Table 3). Fifty percent (24/48) of the well water samples were positive for at least one virus group; 11 out of the 48 well water samples (23%) were positive for two or more virus groups. Viruses detected in the wells included enteroviruses (21 samples, 43%), rotavirus (11 samples, 23%), HAV (4 samples, 8%) and Norwalk-like viruses genogroup 1 (3 samples, 6%). Among the river samples, 83% (10/12) were positive for at least one virus including enteroviruses, rotavirus, and Norwalk-like virus

genogroup 1 (Table 2). Hepatitis A virus was not detected in the river, and Norwalk-like virus genogroup 2 was not detected in any of the samples. Both piezometer samples were virus positive, one for enteroviruses and the other for rotavirus.

Reverse transcription-polymerase chain reaction inhibition. Reverse transcription-polymerase chain reaction was inhibited in 10 samples, as indicated by the absence of amplicon from the NIS (Table 3). River samples were inhibited at the highest frequency. Inhibition did not appear to be seasonally related. River samples were inhibited in April, June, August, September, October, and December. The well samples were inhibited in May, July, January, and March. Inhibition was alleviated in all 10 samples by diluting the fraction 3 RNA concentrate by 1:10 in nuclease-free water. Of the 10 diluted samples, 5 were subsequently determined to be rotavirus positive, and the remaining 5 samples were negative for all viruses tested.

Reverse transcription-polymerase chain reaction method detection limit. If we assume that all viruses captured by the 1-MDS filter are eluted and recovered in the beef extract and further assuming a water sample volume of 1,500 L, then the method detection limit estimated using the acidification flocculation method was 0.6 PFU/L. The PEG flocculation method yielded a method detection limit of 1.1 PFU/L. Southern hybridization can detect amplicon bands not readily visible on agarose gels. Thus, it is likely that the sensitivity of the procedure would increase and the method detection limit would improve if Southern hybridization was performed in addition to agarose gel electrophoresis.

Surface water infiltration into municipal wells. Only Wells 10 and 24 contained appreciable amounts of surface water, as indicated by water isotopes $^{18}O/^{16}O$ and $^{2}H/^{1}H$ that were substantially on the evaporative side (right side) of the local meteoric water line (LMWL) (Fig. 2). Other wells had only one or two samples slightly on the evaporative side of the LMWL.

These wells are not considered to be in the same category as Wells 10 and 24 because the LMWL is considered more of a flattened ellipse as opposed to a discrete, sharp line (Hunt et al. 1998), thus samples near the LMWL may be within the natural variability of unevaporated sources. Moreover, the majority of the samples collected from these other wells showed no influence of surface water, whereas the majority of samples from Well 10 and 24 did show surface water contributions.

The piezometer samples were also influenced by surface water and had a pronounced seasonal signature similar to that observed in the surface water (Hunt et al. in review, included in appendix). This observation reflects the municipal well capturing both terrestrial and surface-water derived water; in contrast the piezometers (located along a flowpath from the surface water to the well) were more characteristic of the surface water supplying the well. Because these wells were located closer to the surface water source than the associated municipal well, the travel times from the source to the piezometer were shorter.

Chapel et al. (2003) constructed numerical ground-water flow models of non-flood, average conditions in the vicinity of the municipal wells sampled during this study. These models were used to obtain the time for surface water to reach the well and are shown here to provide insight into virus viability (Table 3). In addition to non-flood conditions, Hunt et al. (included in appendix) used a water isotope time-series from Well 24 to show that flooding short-circuits the groundwater system and decreases the expected time of travel to around two months. For Well 24, travel times from the surface water source to the municipal well was within the one-year limit where viruses are thought to remain infectious in the subsurface. Well 10, in contrast, had travel times from the surface water source greater than 1 year.

Association of surface water infiltration with virus occurrence. The relationship between virus occurrence and the amount of surface water infiltrating a well was confounded because nearly all wells were virus positive regardless of surface water amount. Wells 14 and 25, which had no detectable surface water, were positive for viruses in 5 of 11 and 6 of 12 samples, respectively. Well 24, which had an appreciable amount of surface water with a travel time of only two months from the river, was virus positive in 4 of 12 samples.

Virus occurrence compared to clinical seasonality. The correspondence between virus occurrence in the wells and the seasonal pattern of clinical infections typically observed in Wisconsin was equivocal (Table 4). Hepatitis A virus and rotavirus were found in the wells at approximately the same time the viruses are found in patients, the winter months (Hollinger and Ticehurst 1996), although rotavirus was also found in the wells in the autumn before the usual clinical season. Enteroviruses, whose infections usually occur in the late summer and autumn (Melnick 1996; Centers for Disease Control and Prevention 2000), were found in the La Crosse wells in nearly every monthly sample. In the past, the administration of the Sabin live-attenuated poliovirus vaccine resulted in the presence of the shed virus in environmental waters. The recent change from administering the live-attenuated virus to the Saulk-killed vaccine may prevent this occurrence in the future. Caliciviruses occur clinically year round (Kapikian et al. 1996), but they were in the wells only one month. The seasonal correspondence was not any clearer when the wells were sub-grouped into high, intermediate, and low surface water contributions.

Indicators of water sanitary quality. The coliphage matrix controls did not show any evidence of interferences from the river or well water samples. The mean matrix recovery of male-specific coliphage was 95% (n = 62, median = 94%, range 74 to 153%), whereas the matrix recovery of somatic coliphage had a mean of 96% (n = 62, median = 95%, range 49 to 174%).

The lower end of the recovery range for both phage types falls within the quality control acceptance criteria of EPA Method 1602 (EPA 2001). The mean method recovery control for male-specific coliphage was 90% (n = 13, median = 90%, range 78 to 104%), and the mean method recovery for the somatic coliphage was 95% (n = 13, median = 95%, range 74 to 118%).

All 12 river samples were positive for total coliforms and somatic coliphages, and 11 river samples were positive for *E. coli* and fecal enterococci (Table 5). The mean concentration of somatic coliphages in the river samples was 71 PFU/100 ml (n = 12, median = 64 PFU/100 ml, range 4 to 141 PFU/100ml). Male-specific coliphages were identified in only four out of 12 river samples with a mean concentration of 2 PFU/100ml (n = 12, median = 0, range 0 to 17). None of the 50 well and piezometer samples were positive for any of the indicators (Table 5). In contrast, 26 of the 50 samples were positive for enteric viruses (Table 2). Thus, in the well water and groundwater, enteric virus occurrence was not associated with indicators of water sanitary quality.

DISCUSSION

Detection of enteric viruses in surface water and municipal well water. Our study was unique in attempting to identify the source of groundwater contamination with enteric viruses. By relating enteric virus presence to the proportion of surface water in groundwater, our aim was to determine the likelihood of Mississippi River water as a source for enteric virus contamination of La Crosse, Wisconsin, municipal wells. Monthly sampling of each water source proceeded for one year, allowing for analysis of viral contamination throughout the seasonal variation experienced in a community with a climate such as in La Crosse. Previously, the highest reported enteric virus contamination rate in groundwater was 31.5% as detected by RT-PCR, with individual positive detection rates for enteroviruses, rotavirus, HAV, and Norwalk

viruses at 15.2%, 13.8%, 6.9%, and 0.9%, respectively (Abbaszadegan 2002). Our study detected enteric viruses in 50% of groundwater samples, with contamination rates for enteroviruses, rotavirus, HAV, and the combined G1 and G2 genogroups of the NLVs at 41.7%, 20.8%, 8.3%, and 8.3%, respectively. In addition, we detected enteric viruses in 36 of 62 (58.1%) river and groundwater samples combined. These results indicate that enteric viruses were present in both La Crosse municipal well water and in surrounding Mississippi River water, with viral incidence exceeding past studies (Abbaszadegan 2002; Borchardt et al. 2003).

Relationship between surface water infiltration and enteric virus occurrence. We are unable to associate surface water infiltration to enteric virus contamination in La Crosse, Wisconsin, municipal wells based on the presence of enteric viruses in each of the sampled municipal wells regardless of surface water presence as determined by water isotopes. Wells that received detectable levels of surface water (Wells 10 and 24) were expected to be most frequently contaminated with enteric viruses due to transport from virally contaminated river water. Instead, our data indicated that all wells, including those without appreciable surface water content (Wells 14 and 25), also contained enteric viruses. Surface water was not determined to be the sole source for enteric virus occurrence in municipal well water in this study, so at least one other route of virus contamination must be present. One likely source for contamination in the municipal well water assayed here was municipal sewer lines. With the exception of Well 24, each of the wells sampled were located within residential neighborhoods containing high-density sewer lines. Considering the likelihood that old/leaky sewage lines may be present in these areas, we propose that sanitary sewer lines near the wellhead are a potential source for virus contamination. Although surface water can be a source of viruses to drinking water (as evidenced by Well 24, a well without sanitary sewer lines), it appears that surface

water is not the only source (as evidenced by virus detection in wells without appreciable surface water contributions).

Virus transport to La Crosse municipal wells. Wells 10 and 24 contained both surface water and enteric viruses. Travel times are sufficiently short to expect virus survival at Well 24 (Hunt et al., included in appendix); however, travel times from the surface water to Well 10 are greater than 12 months, so virus survival may be questionable. Numerous factors have been identified as influences on viral viability and transport with water through the subsurface (Bitton and Harvey 1992; Jansons et al. 1989; Yates et al. 1985). The duration that a specific virus can remain infectious in the subsurface depends on both the characteristics of the individual virus and the conditions encountered in the subsurface. Generally, viral viability increases as environmental temperature decreases and moisture content increases, with most viral species experiencing permanent inactivation in both dry and warm sediments (Yeager and O'Brien 1979; Sobsey et al. 1986; Ramia 1985). Virus survival also increases with association with aqueous suspended solids; one study indicated the infectious duration of poliovirus and rotavirus to increase from nine to 19 days when compared between "free" and sediment-associated waters (Rao et al. 1984). Sandy soil, as found in the aquifer underlying the La Crosse municipal wells, has also been associated with transport of certain viruses and other contaminants in soil (Woessner et al. 2001). Sandy soils have less surface area for sorption than finer textured clay soils, thus decreasing virus to soil surface interactions, resulting in considerable passage of poliovirus through the subsurface (Gerba and Bitton 1984; Duboise et al. 1976). Although factors including soil pH and salt concentration, organic matter content, mineral colloid content, relative subsurface temperature, and moisture content were not addressed in our study, they have been shown to have an effect on virus viability and mobility in the subsurface (Jin et al. 2000;

Redman et al. 1999; Yates et al. 1985; Yates and Yates 1988; Dowd et al. 1998). Briefly, saturated soil with a pH less than 5.0 is generally a good adsorber of enteric viruses due to viral isoelectric points resulting in negatively charged viral capsids, which in turn interact with positively charged soil particles (Goyal and Gerba, 1979; Redman et al. 1997).

Based on the environmental factors involved in viral transport stated here, we would expect some degree of viral transport through the subsurface in La Crosse. The combination of La Crosse's alluvial sand aquifer, relative water pH of approximately 7.0 to 8.0, and low organic matter content in the groundwater help to support the hypothesis that viral contamination of groundwater may be due in part to co-transport of viruses with infiltrated surface water.

Indicator reliability as a predictor of enteric viruses. The promise of microbial water quality indicators as inexpensive and less tedious predictors of enteric virus occurrence has been proposed in numerous studies, although their reliability has been questioned (Abbaszadegan et al. 1996; Gratacap-Cavallier et al. 2000; Leclerc et al. 2000; Wait and Sobsey 2001; Nasser and Oman 1999). Enteric viruses were identified in all sites sampled in this study: four municipal wells under varying degrees of surface water infiltration and one Mississippi River surface water site. Our study detected multiple microbial indicators in every river water sample (12 of 12), with male-specific coliphages, a promising surrogate for enteric virus contamination, present in 33.3% (4 of 12) of these samples. Ten of the 12 river water samples (83.3%) were positive for at least one enteric virus. Although indicators were not detected in any of the municipal water samples, 50% contained at least one enteric virus. The disconnect between the presence of indicators in surface and groundwater samples and the enteric virus presence in each source suggests that total coliforms, fecal enterococci, *E. coli*, and coliphages (somatic and male-specific) are unreliable indicators of enteric viruses (enteroviruses, rotavirus, HAV, and the G1

and G2 genogroups of the NLVs) in this study. This finding may be the result of the inherent differences between the structures and sizes of bacterial indicator species and enteric viruses. Coliphages, which are viruses, have not been demonstrated to be any more effective than the bacterial species in this study. Further experimentation will be required to improve reliability of surrogate microorganisms as indicators of virus contamination in environmental water samples.

Study limitations. This study used molecular techniques for detecting enteric virus RNA. The greatest limitation of this study relates to our ability to only detect viral nucleic acid in water samples without determining any epidemiologic ramifications of our findings (Sobsey 1994). Alhough this study has identified enteric viruses in a high proportion of municipal well water samples (50%) in La Crosse, Wisconsin, we cannot deduce the infectious nature of the virus-positive samples. The public health ramifications of this study will remain in question unless a comprehensive epidemiologic study relating ingestion of enteric virus contaminated water to infectious risk of consuming such water is completed.

Reverse transcription-polymerase chain reaction may provide greater sensitivity in detecting enteric viruses in environmental samples compared to plaque assays; presently, cell culturing is the sole method for determining the proportion of infectious virions in a sample. Many enteric viruses, including HAV and the NLVs are either poorly culturable or non-culturable using current techniques. It has been estimated that the ratio of infectious to non-infectious viruses in stool and environmental samples can range from 1:1,000 to 1:100,000, thereby limiting the ability to determine infectious risk from RT-PCR virus-positive samples alone (Sobsey 1994). Further study may provide for risk assessment analysis of individuals ingesting water from the sites involved in this study. An additional area of interest involves our municipal water samples, which were collected at the wellhead prior to chlorination. Dose-

response analysis of the effects of residual chlorine (0.4 mg/L for the La Crosse Water Department) on the inactivation rates for the viruses could prove valuable in assessing infectious risk.

Another concern about this study involves assay sensitivity in relationship to inhibitors of molecular detection techniques. Organic compounds such as humic and fulvic acids are known to inhibit RT-PCR in environmental samples (Schwab et al. 1995; Shieh et al. 1995). Ten La Crosse water samples contained RT-PCR inhibitors (six river water, two from Well 14, one from Well 10, and one from Well 25) as indicated by the absence of amplification of the NIS. After successful removal of the inhibitory compounds with dilution (1/10), 50% (two river water, two from Well 14, and one from Well 25) of those initially inhibited samples were negative for enteric viruses after RT-PCR. We cannot determine if the absence of viruses in these samples resulted from their being virus negative or simply being negative due to the viral RNA dilution with the inhibitory compounds. Unfortunately, current sample concentration methods may prevent the complete removal of inhibitors without potential loss of viruses from the samples, especially in samples consisting of complex matrices.

An additional factor in this study relates to the flood event experienced in La Crosse, Wisconsin, during April 2001. The La Crosse flood resulted in a Mississippi River crest level of 16.41 ft (3rd highest crest on record) and was the most severe flood in the area since 1965 (National Weather Service 2003; USGS 2002). This factor must be considered when determining the source of pathogens involved in this study because large volumes of water may disrupt subsurface sorption of pathogenic microorganisms, thereby enhancing virus transport Conversely, large volumes of flood water may have also diluted the surface water sample sufficiently to prevent virus detection in otherwise contaminated samples in Spring 2001.

Considering this, concessions must be made when extrapolating pathogen occurrence results presented here to non-flood years.

Epidemiologic implications. The health implications for ingesting contaminated groundwater must be addressed. The potential for groundwater contamination with gastroenteric pathogens is real, with contaminating organisms ranging from parasitic protozoa to bacteria and viruses (Robertson and Edberg 1997, Melnick and Gerba 1980). From 1990 to 2000, 66.7% of the 39 total waterborne disease outbreaks from drinking water in the U.S. were attributed to well water (Lee et al. 2002). In addition, viruses and unidentified pathogens were responsible for 10.3% (10 of 39) and 43.6% (17 of 39) of these same outbreaks, respectively (Lee et al. 2002). This corresponds to only 56.4% of waterborne disease outbreaks being traced to a definitive pathogen, a number too small considering the potential serious diseases that can result from waterborne enteric pathogens.

Ideally, municipal well water contamination rates could be compared to clinical records for characteristic illnesses presented in patients living in areas serviced by the contaminated wells. Unfortunately, relatively few enteric viral illnesses are reported due to the usually mild illnesses and generally tolerable symptoms that result. Follow-up studies based on this study may be better suited for determining the infectious risk of ingesting the La Crosse municipal water sampled here.

Conclusion. Results reported in our study represent the highest United States incidence of enteric virus contamination in groundwater to date. Samples containing enteric viruses were collected from La Crosse, Wisconsin, municipal wells that were under varying degrees of surface water infiltration as well as from surrounding river surface water. Although our goal was to associate groundwater contamination with infiltration of virally contaminated surface water into

municipal well groundwater, virus occurrence in wells with little or no surface water infiltration indicates alternative source(s) for contamination. We found no direct association between enteric virus occurrence and commonly proposed waterborne indicators of water sanitary quality in any of the groundwater samples collected.

Our study is unique in its scope in attempting to link virus presence in groundwater to a source. Hopefully, these findings will serve as a base for further research relating to the ability to predict virus contamination of water, leading to preemptive treatment of water sources under the influence of virally contaminated surface water. The geology associated with water sources that are similar to the alluvial sand and gravel aquifers of the La Crosse area is estimated at approximately one-third nationwide (Abbaszadegan 2002). The fact that La Crosse is far from unique in its hydrogeologic characteristics lends to the potential application of our study results toward many communities throughout the U.S.

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Table 1. Construction and pumping data for La Crosse, Wisconsin, municipal well sites

Well no.	Construction date	Well depth (m)	Well casing depth (m)	Static water level (m)	Study period[a] average GPM[b]	Study period[a] total gallons pumped (x1000)
10	01/01/1936	45.7	15.2	11.9	224	117,165
13	01/01/1953	46.0	30.8	13.1	1135	598,046
14	01/01/1952	44.2	29.0	14.0	813	428,780
17	01/01/1955	48.8	33.5	7.8	789	415,803
24	03/27/1980	32.9	21.6	7.5	726	381,687
25	03/30/1984	30.2	19.2	3.0	828	436,542

[a] March 2001 to February 2002
[b] Gallons pumped per min

TABLE 2. Physical characteristics of La Crosse municipal wells and Mississippi River samples

	Wells (n=48)			River (n=12)		
	pH	Temp (°C)	Sample vol (L)	pH	Temp (°C)	Sample vol (L)
Min.	7.3	9.8	1484	7.7	2.0	223
Max.	8.4	15.0	2752	8.1	26.1	681
Mean	7.6	12.7	1611	7.9	12.5	431
Median	7.5	12.8	1526	7.9	11.0	445

TABLE 3. Virus occurrence by site as determined by reverse transcription-polymerase chain reaction and Southern hybridization

Sample site	No. samples	No. samples inhibited[a]	Surface water travel time[b]	No. virus positive samples					
				E[c]	R	H	G1	G2	Any virus
River	12	6	-	5	8	0	1	0	10
Well 10	11	1	>12 months	6	4	2	1	0	8
Well 10 piez.	1	0	~10 to 12 months	1	0	0	0	0	1
Well 13	1	0	No surface water[d]	0	0	0	0	0	0
Well 14	11	2	No surface water	4	1	1	1	0	5
Well 17	1	0	ND[e]	1	0	0	0	0	1
Well 24	12	0	2 months (flood) 6 months (non-flood)	4	3	1	0	0	4
Well 24 piez.	1	0	3 to 4 months (non-flood)	0	1	0	0	0	1
Well 25	12	1	negligable	5	2	0	1	0	6

[a] Determined by Norwalk virus internal standard
[b] Determined by O^{18}/O^{16} ratios and numerical flow modeling of Chapel et al. (2003)
[c] E = enteroviruses, R = rotavirus, H = hepatitis A virus, G1 = Norwalk-like virus genogroup 1, G2 = Norwalk-like virus genogroup 2
[d] Hunt et al. (in review)
[e] Not determined

TABLE 4. Seasonality of virus occurrence[a] in La Crosse wells compared to virus clinical seasonality[b]

Virus	J	F	M	A	M	J	J	A	S	O	N	D
enteroviruses	+	+	-	-	+	+	-	+	+	+	+	+
rotavirus	+	+	-	-	-	-	-	+	+	-	+	+
hepatitis A virus	+	-	-	-	-	-	-	-	-	-	+	+
Norwalk-like virus G1	-	-	-	+	-	-	-	-	-	-	-	-
Norwalk-like virus G2	-	-	-	-	-	-	-	-	-	-	-	-

[a] + = at least one well sample positive for respective virus

[b] Boxes indicate months when respective virus is commonly detected in clinical cases

TABLE 5. Number of samples positive for microbial indicators of water sanitary quality

Sample site	No. samples	Total coliforms	*E. coli*	Fecal enterococci	Somatic coliphage	Male specific coliphage
River	12	12	11	11	12	4
Well 10	11	0	0	0	0	0
Well 10 piezometer	1	0	0	0	0	0
Well 13	1	0	0	0	0	0
Well 14	11	0	0	0	0	0
Well 17	1	0	0	0	0	0
Well 24	12	0	0	0	0	0
Well 24 piezometer	1	0	0	0	0	0
Well 25	12	0	0	0	0	0

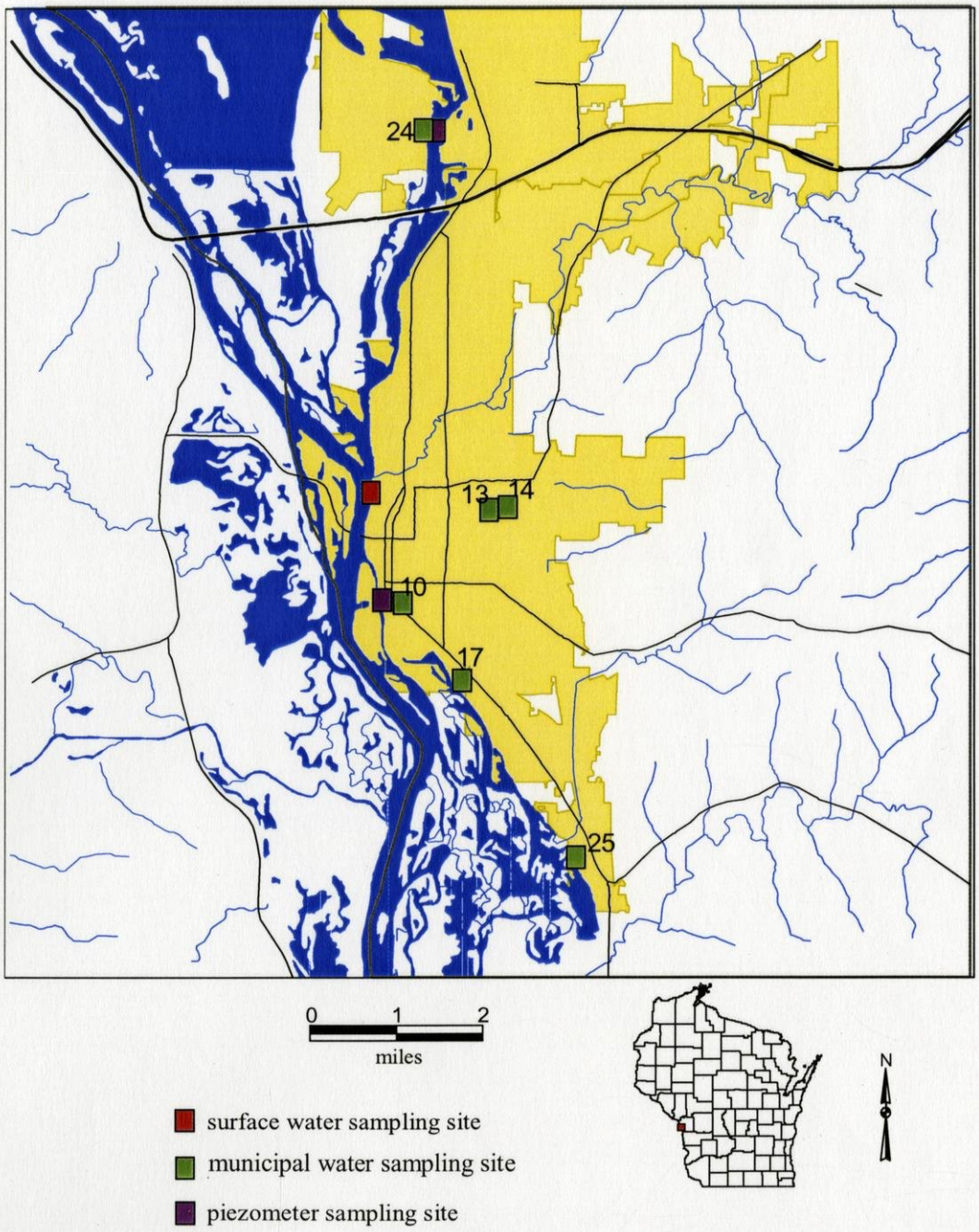

Fig. 1. Map of La Crosse, Wisconsin, indicating surface water, municipal well groundwater, and piezometer groundwater sampling sites.

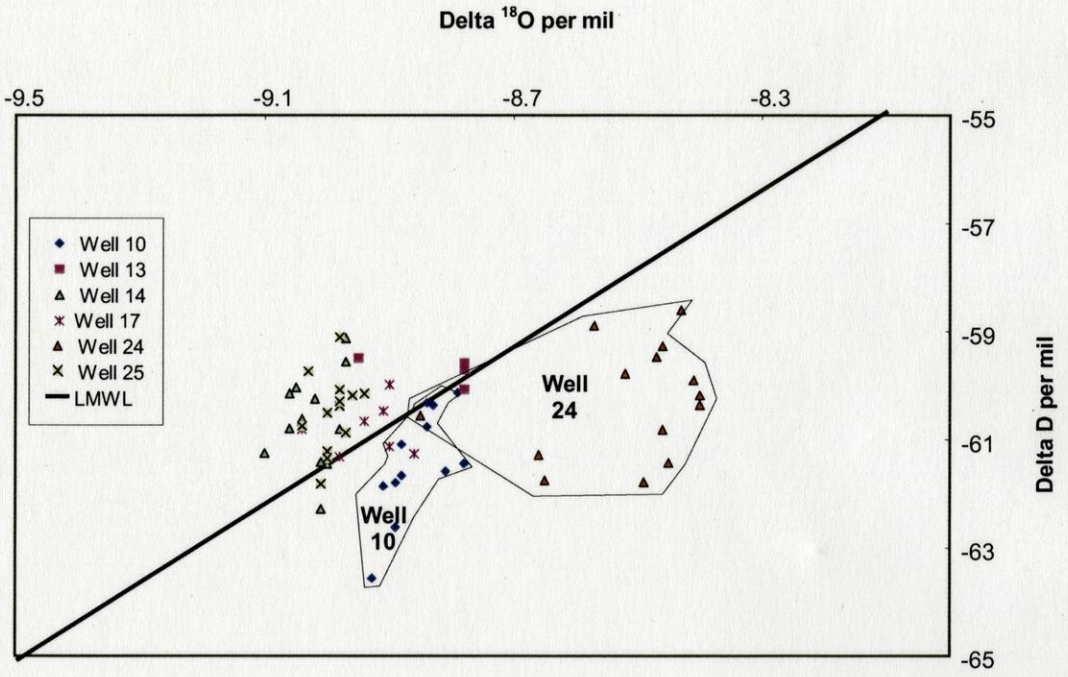

Fig. 2. La Crosse, Wisconsin, municipal well $^{18}O/^{16}O$ and $^{2}H/^{1}H$ isotopes for 2001 to 2002, calculated from data by Hunt et al. (in review).