Detection of Pathogenic and Non-pathogenic Bacteria in Drinking Water and Associated Biofilms on the Crow Reservation, Montana, USA

Authors: Crystal L. Richards, Susan C. Broadaway, Margaret J. Eggers, John Doyle, Barry H. Pyle, Anne K. Camper, and Timothy E. Ford

The final publication is available at Springer via http://dx.doi.org/10.1007/s00248-015-0595-6.

Richards, Crystal L. , Susan C. Broadaway, Margaret J. Eggers, John Doyle, Barry H. Pyle, Anne K. Camper, and Timothy E. Ford. "Detection of Pathogenic and Non-pathogenic Bacteria in Drinking Water and Associated Biofilms on the Crow Reservation, Montana, USA." Microbial Ecology 76, no. 1 (July 2018): 52-63. DOI:10.1007/s00248-015-0595-6.

Detection of *Mycobacteria*, *Legionella*, and *Helicobacter* in Drinking Water and Associated Biofilms on the Crow Reservation, Montana, USA

Crystal L. Richards[1], Susan C. Broadaway[1], Margaret J. Eggers[1,2], Emily Colgate[3], John Doyle[4,5,6], Barry H. Pyle[1], Anne K. Camper[7*], and Timothy E. Ford[8]

[1]Department of Microbiology and Center for Biofilm Engineering
Montana State University
Bozeman, MT 59717

[2]Little Big Horn College
Crow Agency, MT 59022

[3]University of Vermont
College of Medicine
Burlington, VT 05405

[4]Apsaalooke Water and Wastewater Authority
Hardin, Montana 59034

[5]Crow Tribal Member
Crow Agency, MT 59022

[6]Big Horn County Commissioner
Hardin, Montana 59034

[7]Department of Civil Engineering and Center for Biofilm Engineering
Montana State University
Bozeman, MT 59717

[8]Department of Research and Graduate Studies
University of New England
Biddeford, ME 04005

Journal: Applied and Environmental Microbiology
Journal Section: Public Health

(*)Corresponding author. Mailing address:
Center for Biofilm Engineering,
Montana State University, EPS 366, Bozeman, MT 59717.
Phone: (406) 994-4906. Fax: (406) 994-6098. E-mail: anne_c@biofilm.montana.edu

ABSTRACT

Public health has been shown to be directly related to water quality, and although drinking water quality has improved in much of the United States, rural areas typically have underserved water systems. Private residences in rural areas with water systems that are not adequately regulated, monitored, and updated could have drinking water that poses a health risk. To investigate water quality on the Crow Reservation in Montana, water and biofilm samples were collected from 57 public buildings and private residences served by both treated municipal and individual groundwater well systems. Three bacterial genera, with members that are potential drinking water pathogens, were chosen for investigation. *Mycobacteria*, *Legionella*, and *Helicobacter* were detected by PCR and/or standard culture techniques. Free and total chlorine, temperature, and pH were recorded at the time of sampling. Fecal coliform bacteria and heterotrophic plate count (HPC) bacteria were enumerated using m-Coliblue24® and R2A agar, respectively. All three target genera were detected in drinking water systems on the Crow Reservation. Species detected included the opportunistic and frank pathogens *Mycobacterium avium*, *M. gordonae*, *M. flavescens*, *Legionella pneumophila*, and *H. pylori*. There was no correlation between the presence of any genera and chlorine (free and total), temperature, pH or fecal coliforms. However, there was an association between HPC bacteria and the presence of *Mycobacteria* and *Legionella* but not the presence of *Helicobacter*. This research has shown that groundwater and municipal drinking water systems and associated biofilms may be reservoirs for *Mycobacteria*, *Legionella*, and *Helicobacter*.

INTRODUCTION

In the United States over 15 million households rely on private ground water wells for their primary drinking water source (82), and in many rural areas private and community groundwater wells provide a major source of drinking water (12). Generally, most water obtained from private groundwater systems is considered safe to drink (23). However, in the United States from 1999-2002, 22% of water-borne illnesses were attributed to individual water systems and 36% were attributed to community systems (24). Private water systems are not routinely monitored for bacteriological water quality, thus little is known about the presence of bacterial pathogens in these systems. Information regarding water quality on Indian Reservations in the United States is equally scant. However, it is known that American Indian populations have disproportionately high disease burdens compared to the overall population of the United States (59). This is due to many factors which include economics, geographic isolation, cultural barriers, and inadequate sewage disposal (5).

The United States Centers for Disease Control report that chronic lower respiratory disease, influenza and pneumonia are among the top ten causes of death among American Indian and Alaska Native populations (82). In Montana, cancer is included as a major cause of death for American Indian populations (21). Although it has been observed that the disease burden of these populations is greater than the overall population of the United States (59), very little research has been done to identify causes and potential routes of exposure to infectious agents and environmental carcinogens. In the present study, three bacterial genera with members that are potential drinking water pathogens, *Mycobacteria*, *Legionella*, and *Helicobacter*, were chosen for investigation due to concerns expressed by Crow Tribal community members about

46 poor drinking water quality and the relationship of these organisms to respiratory disease and
47 stomach cancer (28).
48 *Mycobacteria* are common inhabitants of drinking water systems and are known to
49 survive and proliferate in biofilms (29, 49). Several species of this genus cause respiratory
50 disease in mainly immunocompromised humans. Species include members of the
51 *Mycobacterium avium* complex, *M. gordonae*, *M. flavescens*, and others (18, 36, 54, 55).
52 *Legionella* are ubiquitous throughout aquatic environments including ground and surface water,
53 and manmade water reservoirs such as potable water systems and cooling towers. (20, 53, 81).
54 *Legionella pneumophila* is the main causative agent for respiratory disease in that genus, causing
55 Legionellosis in the form of Legionnaire's disease and Pontiac fever (30). Legionellosis is
56 thought to occur when *Legionella* are aerosolized and inhaled (62). However, it has been
57 suggested that transmission of the different forms of Legionellosis, and the resultant severity of
58 disease, may be related to an association with biofilms (42). *Helicobacter* are pathogens of the
59 gastrointestinal tract of mammals but have been found in many environments such as well, river,
60 and pond water, in addition to house flies, and cattle feces (68). *Helicobacter pylori* are the
61 primary bacterial cause of gastritis, as well as peptic and duodenal ulcers in people around the
62 world (63). Infection is known to increase the risk of the development of gastric mucosa-
63 associated lymphoma and adenocarcinoma (62). Water is a short term reservoir, with the
64 pathogen often occurring sporadically in drinking water supplies that have been exposed to
65 sewage, or have been contaminated by infected animals (10).
66 Drinking water samples and their associated biofilms were tested for heterotrophic and
67 coliform bacteria by traditional culture methods. *Mycobacteria, Legionella,* and *Helicobacter*
68 species were detected by culture and PCR. The aim of this study was to investigate whether

these organisms are common inhabitants of drinking water systems on the Crow Reservation in southeast Montana.

MATERIALS AND METHODS

Study Area. The Crow Indian Reservation, Montana, USA was the primary location for sample collection and analysis. Fifty-seven locations were sampled across the Crow Reservation (41 private residences and 14 public buildings) from March 2007 through July 2009. The Crow Reservation, the largest reservation in Montana, is rural with an average population density of 1.9 individuals per square mile (82). The Crow tribe has an enrolled membership of 11,357 and approximately 72% of members live on or near the Reservation (65). This Reservation has a diverse landscape spanning the Wolf, Big Horn and Pryor Mountain ranges, as well as the Big Horn and Little Big Horn River valleys. Land use is typical of rural areas in Montana with approximately 68% grazing rangeland, 12% dry cropland, 3% irrigated cropland, 15% forested areas, 1% wild land, and 1% developed areas (4). The Crow Reservation area receives approximately 12-18 total inches annual precipitation (4). The surface water in the area is dependent on precipitation, snowpack and groundwater for recharge while the aquifers on the reservation rely on infiltration from rivers, streams, precipitation, stock ponds and reservoirs (38). The major township on the reservation, Crow Agency, has drinking water provided by treated surface water, while other townships utilize community and private groundwater wells and springs (33). The Crow Agency treatment facility performs reliably and adequately; however the distribution system in Crow Agency is nearly 100 years old and is vulnerable to cracks and leaks (27). Most of the residents outside of designated townships have privately maintained groundwater wells, often only drilled to first water.

92 **Sample Collection and Processing.** Samples were primarily collected from kitchen sinks in private
93 residences and kitchen or restroom sinks in public buildings. Biofilm samples were collected first,
94 before any flushing or sterilization of the tap. Biofilm samples were collected by systematically
95 wiping the inside of the drinking water faucet with a sterile cotton swab before any flushing or
96 sterilization of the tap. Three swabs were collected from one faucet at each residence or building
97 and were placed in individual tubes containing sterile water for transport. To calculate surface area
98 of the biofilm, the faucet dimensions (depth and width) were measured and recorded. After biofilm
99 collection, the faucet was wiped with 95% ethanol to sanitize it before bulk water collection. After
100 sanitization of the tap, one liter of water was collected without flushing and is denoted as "first
101 flush". First flush sample collection was added in 2008 (n=20) and thus this fraction was only
102 analyzed for groundwater wells. After first flush collection, the water was run from the tap for two
103 minutes minimum or until water temperature stabilized prior to parameter measurement. Physical
104 and chemical characteristics were measured using standard methodologies. The presence and
105 quantity of free and total chlorine was measured using a colorimetric method (Hach kit model CN-
106 70 chlorine test kit, Hach Co., Loveland, CO). The temperature and pH were measured using a
107 multi-parameter probe (Oakton, Vernon Hills, IL). After a minimum of two minutes of flushing,
108 "post flush" water was collected in three separate one liter sterile plastic bottles. All samples were
109 placed on ice and transported to the laboratory and processed within 24 h. In the laboratory, tubes
110 containing biofilm samples were vortexed for one minute and the cell suspensions from each swab
111 were pooled and mixed. Pooled cell suspensions from the same source were used for all biofilm
112 analysis. To concentrate water samples, 900 ml from each liter sample was filtered through a 0.45
113 µm 25mm diameter mixed cellulose ester filter (Pall Corp., Ann Arbor, MI). The filter was vortexed
114 in 1.5 ml of PBS at maximum speed for one minute, and then removed and the cell suspension

centrifuged at 13,000 x g for 10 minutes. The supernatant was removed and the cell pellet was resuspended in 100 µl of sterile water. Pooled biofilm and both concentrated and un-concentrated water samples were used in DNA extractions. Pooled biofilm and un-concentrated water samples were used for genus specific culture methods.

Quantification of Fecal Indicator Bacteria and Heterotrophic Bacteria. Fecal indicator bacteria were quantified for first flush and post flush water samples using standard methodologies. The presence of fecal contamination was determined by growth on the selective and differential m-Coliblue24® broth (Hach Co., Loveland, CO). This medium was used to culture coliform bacteria and differentiates *Escherichia coli* by using an enzymatic indicator. One hundred ml of each water sample and appropriate dilutions were filtered and the filter placed on a pad soaked with 2 ml of the Coliblue24® broth. The filters were incubated at 37˚C and growth was observed at 24 h. Post-flush water samples were collected in triplicate and each replicate was analyzed separately. Sterile water was filtered as a negative control, and sterile water was inoculated with environmental isolates of *Escherichia coli* and *Klebsiella pneumoniae* obtained from drinking water samples from New Haven, CT as a positive control. Heterotrophic bacteria were enumerated for biofilm, first flush and post flush samples. Each sample was diluted and plated on R2A agar followed by incubation at 30ºC for 2 weeks.

Control Bacterial Strains and Growth Conditions. Representative species from each genus of interest were kept as frozen stocks at -70˚C and used as positive controls in both PCR and culture methods. The *M. avium* W2001 strain used in this study was originally isolated from drinking water in the Boston area and has been classified as *M. avium* subsp. *hominisuis* based on the *hsp65* gene (80). The *M. avium* strain was cultured on Middlebrook 7H10 (Difco) and incubated at 37˚C for 10 days. *L. pneumophila* strain 33153 was obtained from the American Type Culture Collection,

cultured on *Legionella* agar (Difco) enriched with 0.7% L-cysteine and 0.3% ferric pyrophosphate (Difco) and incubated at 37˚C for 7 days. The *H. pylori* strain 43504 was obtained from the American Type Culture Collection and was cultured on *H. pylori* specific HP medium (25). The *Helicobacter* cultures were placed in a BBL anaerobe jar with a BBL CampyPak Plus™ sachet, which creates a microaerophilic atmosphere of 5-10% oxygen and 10% carbon dioxide, and incubated for one week at 37˚C. All control strains were grown and sequentially transferred twice prior to use as positive controls.

Culture of Drinking Water and Biofilm Samples. The drinking water and biofilm samples were analyzed for the presence of *Mycobacteria*, *Legionella*, and *Helicobacter*, by organism appropriate culture techniques. Due to overgrowth by other micoorganisms, specific selection methods were employed to target the organisms of interest. To select for members of the genus *Mycobacteria*, two hundred microliters of each unconcentrated water sample as well as the pooled biofilm suspension were treated with a final concentration of 0.005% cetyl pyridinium chloride (CPC) (Sigma, St. Louis, MO) for 30 minutes as previously described (70). Sterile tap water was inoculated with *M. avium* W2001 and treated with CPC as a positive control. The CPC treated cells were washed with phosphate buffered saline twice by centrifuging at 10,000 x g for 5 minutes. Subsequently, one hundred microliters were plated onto M7H10 agar (Difco), two replicates were plated for each sample and were incubated at 37˚C for up to three weeks. To select for *Legionella* species, each sample was heated to 50˚C for 30 minutes in a water bath (9). Subsequently, one hundred microliters were plated onto enriched *Legionella* agar (Difco), two replicates were plated for each sample and incubated at 37˚C for one week. Sterile tap water was inoculated with *L. pneumophila* ATCC 33153 and treated with heat as a positive control. The samples were also cultured on *H. pylori* specific HP medium (25). *H. pylori* ATCC 43504 was inoculated into sterile water and

161 plated as a positive control. The plates were placed in a BBL anaerobe jar with a BBL CampyPak
162 Plus™ sachet, and incubated for one week at 37˚C. All presumptive isolates were subcultured and
163 subsequently identified by PCR and phylogenetic analysis.
164 **DNA Extraction from Biofilm and Water Samples.** Nucleic acids were extracted from the pooled
165 biofilm suspensions and from concentrated and unconcentrated water samples. DNA was extracted
166 within 48 h of sampling and the extracts were immediately frozen at -20˚C. Two ml of each biofilm
167 sample was centrifuged at 12,000 x g for 15 minutes. Subsequently, all but 100µl of the supernatant
168 was removed, the pellet was mixed thoroughly into the liquid and the suspension was added to 2 ml
169 plastic screw cap tubes with o-rings (Fisher) containing 0.4g of 0.1 mm sterile glass beads.
170 Similarly, 200µl of each concentrated and unconcentrated water sample was added to individual
171 sterile bead tubes for DNA extraction. Two hundred microliters of lysis buffer consisting of 20 mM
172 sodium acetate (Fisher Scientific, Fair Lawn, New Jersey), 0.5% sodium dodecyl sulfate (Fisher),
173 and 1mM ethylenediamine-tetraacetic acid (Fisher) and 500 µl phenol (pH 8.1) (Fisher) was also
174 added to each 2 ml tube and the mixture was homogenized in a Fastprep® FP120 cell disrupter at
175 speed 5.0 for 40 seconds. After homogenization, samples were placed on ice and allowed to rest for
176 10 minutes. The samples were then centrifuged at 12,000 x g for 10 minutes. The DNA was
177 precipitated by transferring the supernatant to a fresh 2 ml tube containing an equal volume of
178 chloroform: isoamylalcohol (24:1). The samples were vortexed for 30 seconds and then centrifuged
179 at 12,000 x g for 5 minutes. The supernatant was transferred to another fresh tube containing an
180 equal volume of isopropanol and 1/10 volume of 3M sodium acetate and held at -20˚C for 24 h. The
181 nucleic acids were subsequently pelleted by centrifugation, washed once with 70% ethanol, air-
182 dried, and finally resuspended in 100µl of Tris-EDTA buffer (TE) consisting of 10mM Tris and
183 1mM EDTA (Fisher).

184 **PCR Amplification, Sequencing, and Phylogenetic Analysis.** The detection limit of each primer
185 set was determined by amplification of a 10-fold dilution series of purified genomic DNA (10ng-
186 0.0001pg). The target genes, sequences, product sizes, and PCR conditions are listed in Table 1. To
187 ensure that the PCR reaction was not inhibited by environmental contaminants, amplification of each
188 sample was performed using eubacterial 16S rRNA primers as described by Voytek et al. (84).
189 Amplification of the PCR products was done in 25µl PCR mixture containing 1x PCR buffer II, 50-
190 200ng template DNA, 200µM (each) deoxynucleoside triphosphates (Takara Bio Inc., Japan), 0.1
191 µM (each) of primer (Integrated DNA Technologies, Coralville, IA), and 1U LA Taq polymerase
192 (Takara). Aliquots of each PCR product were separated by electrophoresis in a 0.8% (w/v) agarose
193 gel (Fisher) in TBE buffer consisting of 90mM Tris-HCl (Fisher), 80mM boric acid (Fisher), 2.5mM
194 EDTA (Fisher) and stained with ethidium bromide (0.5µg/ml). PCR products were purified using
195 the Qiaquick PCR purification kit (Qiagen, Valencia, CA) according to the manufacturer's
196 instructions. Automated sequencing from both strands of PCR products of positive samples was
197 performed by the Molecular Research Core Facility at Idaho State University. DNA sequences were
198 assessed for their similarity to published DNA sequences using the BLAST database
199 (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were aligned with ClustalW (77). Phylogenetic
200 trees were constructed with the neighbor-joining method (67) and the Jukes-Cantor distance model
201 (41) with bootstrap values of 1,000 replicates within MEGA v4.0 (75). All sequences were deposited
202 in GenBank, accession numbers HQ018935-HQ018989.
203 **Statistical Analysis**. All data were compiled and for all instances where plate count values had a
204 value of zero indicating none detected, a substitution rule was used (83). An arbitrary value (0.25)
205 was chosen to replace all zero plate counts so that log transformations could be performed. Multiple
206 linear regression and logistic regression tests were performed in Minitab® to determine correlations

between pH, temperature, drinking water and biofilm heterotrophic bacteria, total coliform bacteria, *Helicobacter*, *Legionella* and, *Mycobacteria* (44). Additionally, paired and Welch two sample t-tests were performed on heterotrophic and total coliform bacteria to determine if there were significant differences between first flush, post flush, and biofilm samples (44). Fisher's exact tests were performed to determine if the presence of *Mycobacteria*, *Legionella*, and/or *Helicobacter* had a relationship with each other (19). Fisher's exact tests were also done to determine if there was a relationship between *Mycobacteria*, *Legionella*, and *Helicobacter* and the source of the drinking water (treated municipal or groundwater well) (19). A Benjamini-Hochberg correction (10%) was applied to all analyses to minimize false discovery due to multiple comparisons (11).

RESULTS

Physical Characteristics of Sampled Drinking Water. A total of 57 sites were sampled during this study. Sixteen samples were collected from public buildings and private residences that had drinking water supplied by treated municipal systems, while the 41 remaining systems were community and private groundwater wells. Total and free chlorine were quantified in drinking water sampled from municipal systems and ranged from none detected - 2.5 mg/L and none detected - 1.3 mg/L with means of 0.34 mg/L and 0.27 mg/L, respectively. The pH of the drinking water ranged from 5.82-9.56 with a mean of 7.42. The temperature of the bulk water was recorded after flushing the tap and ranged from 8 - 33ºC with a mean of 15.7ºC and one outlier at 46ºC. Treated municipal systems had a mean temperature of 21.5ºC, while groundwater well systems had a mean temperature of 14.2ºC. Simple linear regression and logistic regression were used to analyze relationships between the measured physical characteristics and HPC bacteria, total coliforms, *Legionella*,

Mycobacteria, and *Helicobacter*. No significant statistical correlation was detected between the measured physical characteristics and any of the bacteria identified.

Detection of Heterotrophic Bacteria and Fecal Indicator Bacteria. Heterotrophic bacteria were enumerated to assess whether these organisms were associated with the presence of potential pathogens. Table 2 shows the range and arithmetic mean of HPC bacteria in first flush, biofilm and post flush drinking water samples. The significance from the statistical analyses of the interactions between HPC bacteria counts and response variables are shown in Tables 3 and 4. Differences in the mean HPC populations between the first flush and post flush fractions collected were evaluated using Minitab® (Table 3). There was a significant difference in mean HPC bacteria when first flush and bulk water samples were compared ($P = 0.025$, significant with Benjamini-Hochberg correction) with first flush samples having higher numbers of HPC bacteria on average. Differences in HPC bacteria between treated municipal and groundwater wells were also evaluated (Table 5). There was a difference between the mean HPC bacteria in biofilm samples and the drinking water source ($P = 0.049$), however after applying the Benjamini-Hochberg correction this relationship was not significant. Overall, biofilm samples collected from groundwater wells had higher HPC bacteria counts than biofilm samples collected from treated municipal systems. There was not a significant difference in HPC bacteria numbers between the source water types for the post flush water samples.

Total coliform bacteria were enumerated in first flush and post flush drinking water samples. Coliform bacteria were found in both treated municipal (37.5% or 6/16) and untreated groundwater wells (40% or 16/40) in post flush water samples. *Escherichia coli* was not observed in treated municipal samples but was found in 10% or 4/40 of post flush groundwater well samples. Table 2 shows the range and arithmetic mean of coliform bacteria and *E. coli* in drinking water samples. Although there was a significant difference in HPC bacteria in first flush and post flush fractions,

252 there was not a significant difference between mean coliform counts in first flush and post flush
253 fractions ($P > 0.13$). *E. coli* had a positive association with post flush HPC bacteria ($P = 0.026$,
254 significant with correction) (Table 3).
255 **Presence of *Mycobacteria*, *Legionella*, and *Helicobacter*.** *Mycobacterium* species were detected in
256 35.1% or 20/57 of the locations sampled, with 15 found in the biofilm fraction, and 8 in the drinking
257 water fraction. Three of these occurrences of *Mycobacteria* were found in both the drinking water
258 and biofilm fractions. From the biofilm fractions, 7 of the 15 positive samples were identified by
259 PCR alone and 5 were identified by culture alone while 3 were identified by both PCR and culture.
260 From the drinking water fractions, 2 were identified by both PCR and culture while the remaining 6
261 were identified by PCR only. Fig. 1 shows the phylogenetic relatedness of the PCR and culture
262 isolates. The *Mycobacterium* species sequences detected were closely related to known species
263 including *M. gilvum*, *M. mucogenicum*, *M. murale*, *M. flavescens*, *M. gordonae*, *M. manitobense* and
264 members of the *Mycobacterium avium* complex (MAC) (>95% similarity).
265 To assess whether total coliforms or HPC bacteria influence the likelihood that *Mycobacteria*
266 may be present, logistic regression was applied in Minitab®. Identical analyses were performed for
267 all three genera tested in this study. The analysis showed a relationship between *Mycobacteria* and
268 both post flush ($P = 0.044$, significant after correcting for multiple comparisons) and biofilm HPC
269 bacteria ($P = 0.01$, not significant after corrections) (Tables 3 and 4 respectively). This showed that,
270 in general, as HPC bacteria increased, the odds of encountering *Mycobacteria* increased as well.
271 *Mycobacteria* were detected when Coliforms were present in 50% or 10/20 of the locations sampled.
272 Of the 20 locations that tested positive for *Mycobacteria*, 8 were treated municipal systems and 12
273 were groundwater well systems. There were no significant relationships between the presence of

274 *Mycobacteria* and total coliforms (logistic regression) or the source type of the drinking water
275 system (Fisher's exact test).

276 *Legionella* species were detected in 21% or 12 of the 57 locations sampled with 5 of those in
277 the biofilm fraction, 8 in the drinking water fraction and only one occurrence of *Legionella* in both
278 the biofilm and drinking water. Of the 5 positive biofilm samples, 3 were identified by PCR alone, 1
279 was identified by culture alone, and 1 was identified by both PCR and culture. From the 8 drinking
280 water samples, 2 were identified by both PCR and culture while the remaining 6 were identified by
281 PCR alone. Fig. 2 shows the phylogenetic relatedness of *Legionella* detected by PCR directly and
282 from culture isolates. The *Legionella* species detected include uncultured *Legionella* sp., *L.*
283 *pneumophila*, *Legionella* sp., *L. fairfieldensis*, and *L. dresdeniensis* (sequence similarity >95%).

284 The results of the logistic regression showed a positive relationship between post flush HPC
285 bacteria counts and *Legionella* in the system ($P = 0.003$, significant after correcting for multiple
286 comparisons) (Table 3). In general, as post flush HPC bacteria increased, the odds of encountering
287 *Legionella* increase as well. The greatest interaction occurred between *Legionella* detected in the
288 biofilm fraction and post flush HPC bacteria ($P = 0.001$). *Legionella* detected in the drinking water
289 fraction did not have a significant interaction ($P = 0.068$) with post flush fractions (Table 3). There
290 was no significant relationship between the presence of *Legionella* (in either the biofilm or drinking
291 water fractions) and biofilm HPC bacteria (Table 4). Coliforms were present in 6 of the 12 samples
292 where *Legionella* were detected, but there was no significant relationship between the presence of
293 *Legionella* and total coliforms ($P = 0.679$). However, there was a significant association between
294 the presence of *Legionella* and *E. coli* ($P = 0.018$). Of the 12 samples positive for *Legionella,* 8
295 were at treated municipal sites and 4 were in groundwater. Unlike *Mycobacteria*, the source type of

296 the drinking water system did have a relationship with the presence of *Legionella* ($P = 0.002$) (Table
297 5).

298 *Helicobacter* species were detected in 7% or 4/57 of locations sampled, with 2 of those in the
299 biofilm and 2 in the drinking water. There were no occurrences of *Helicobacter* in the drinking
300 water and biofilm concurrently. All of the positive samples were identified by PCR alone. Fig. 3
301 shows the phylogenetic relatedness of the *Helicobacter* sequences detected by PCR directly. The
302 only *Helicobacter* species detected was *H. pylori*. Coliforms were found in 2 of the 4 samples where
303 *Helicobacter* were detected. Logistic regression did not demonstrate any significant correlation
304 between the presence of *Helicobacter* and any of the biological or physical parameters collected or
305 the source type of the drinking water.

306 **Interactions Between Potentially Pathogenic Genera**. To determine whether there was a
307 relationship between the presence of the three genera of interest a Fisher's exact test was performed.
308 There was no statistically significant relationship between the three genera. Interestingly, 50% or
309 6/12 of locations positive for *Legionella* were also positive for *Mycobacteria* while only one location
310 had both *Legionella* and *Helicobacter*. Conversely, there were 20 occurrences of *Mycobacteria* with
311 six of these samples also positive for *Legionella* (28.5%) and two samples positive for *Helicobacter*
312 (9.5%). *Helicobacter* was found alone in one location (25% or 1/4).

313

314 DISCUSSION

315 The results of our study show that *Mycobacteria*, *Legionella*, and *Helicobacter* can be found
316 in drinking water and associated biofilms on the Crow Reservation, in both treated municipal water
317 and untreated well water. The data also indicated that the number of HPC bacteria correlated with
318 the presence of *Mycobacteria* or *Legionella*.

Fecal Coliforms and HPC Bacteria in Drinking Water. Although the presence of coliform bacteria in drinking water is a potential indicator of fecal contamination and may indicate the possible presence of harmful pathogens in drinking water (48), members of the coliform group are also common inhabitants of rural drinking water systems (45). This study found that 40% of community and private groundwater wells contained coliform bacteria while 37.5% of treated municipal samples were positive. This is in agreement with an Iowa statewide rural well water survey that found that 44% of private groundwater systems were contaminated with coliforms (37). In our study area, surface water municipal and groundwater systems are vulnerable to contamination, particularly during wet seasons that result in flooding events. These flooding events can drastically increase the turbidity of surface waters and hinder water treatment, potentially allowing coliform contamination of finished water. During March 2007, the largest treatment facility on the Reservation was required to shut down due to mud and debris that clogged the intake pipe after a flood (16). This particular event accounts for all of the coliform positive municipal system samples except one, which occurred shortly after this flood. The temporary closure of the treatment facility required a town-wide boil order and resulted in turbid water at the tap. Groundwater wells in rural areas are vulnerable to flooding, but are also susceptible to contamination from septic systems and inappropriate disposal of sewage effluents and sludges (12). During sample collection, we occasionally observed instances where well heads were completely inundated after precipitation, and water at the tap was turbid and/or odiferous.

Coliform detection has inherent limitations and high levels of background bacteria can interfere with the assays (17, 32, 48). Coliform bacteria often do not adequately predict the presence of pathogens, as has been demonstrated in waterborne outbreaks of *Cryptosporidia*,

342 *Giardia*, and *Salmonella* (43). The lack of concurrence between the detection of *Mycobacteria*,
343 *Legionella*, and *Helicobacter* and coliforms indicates that fecal indicator bacteria have limited
344 use in predicting the presence of these environmental pathogens. Our finding agrees with that of
345 others who found no correlation between these organisms and fecal coliform bacteria (61, 72, 84,
346 86).

347 Heterotrophic plate count bacteria are the normal flora of drinking water and include a
348 wide range of organisms including *Acinetobacter*, *Aeromonas*, *Bacillus*, *Corynebacterium*,
349 *Pseudomonas*, *Mycobacteria*, and *Legionella* (2). *Helicobacter* also utilize organic nutrients for
350 growth and thus fit the general definition of a heterotroph, but their microaerophilic lifestyle
351 make them less suited for growth in the drinking water environment (1, 35). This study has
352 shown that HPC bacteria can occur in numbers $>10^6$ CFU/ml and that water that was stagnant in
353 plumbing (first flush) had significantly greater numbers of HPC bacteria than water that has been
354 collected after flushing ($P = 0.025$). Water stagnation in drinking water pipes promotes bacterial
355 accumulation and may compromise microbiological quality of drinking water when those
356 organisms are flushed out (52). In this study, groundwater wells generally had higher levels of
357 HPC bacteria than treated municipal water, which can be at least partially explained by the
358 presence of chlorine residuals in municipal systems.

359 Heterotrophic plate count bacteria in biofilm and post flush drinking water fractions had
360 significant relationships with the presence of both *Mycobacteria* and *Legionella*. Logistic
361 regression showed that as the number of HPC bacteria increase, the odds of encountering
362 *Mycobacteria* or *Legionella* increase as well. The presence of *Mycobacteria* in the system had a
363 stronger relationship with HPC bacteria in post flush drinking water fractions (odds ratio 1.68, P
364 = 0.044) than in biofilm fractions (odds ratio 0.63, $P = 0.010$). The data showed a relationship

between *Mycobacteria* identified in different fractions (biofilm and drinking water) and the number of HPC bacteria in the different fractions. The most significant relationship was the interaction between the presence of *Mycobacteria* in the drinking water and elevated HPC bacteria in post flush water (odds ratio 2.05, $P = 0.030$). September et al. (72) found that water quality parameters do not provide any indication of the possible presence of *Mycobacteria* in drinking water biofilms, while another group found a relationship between elevated HPC counts and *Mycobacteria* in surface waters (39). The relationship between *Mycobacteria* and HPC in drinking water systems remains unclear and more research is required to elucidate all of the factors involved. The presence of *Legionella* in the system had a stronger relationship with HPC bacteria in post flush drinking water (odds ratio 2.75, $P = 0.003$) than in biofilm samples (odds ratio 0.77, $P = 0.185$). The relationship between *Legionella* identified in biofilm and drinking water and the number of HPC bacteria in the corresponding fractions was analyzed. Although the minority of *Legionella* sequences were found in biofilm samples (41.6%), they accounted for the significant interaction with elevated HPC bacteria in post flush water. Finding elevated levels of HPC bacteria in post flush samples significantly increased the odds of encountering *Legionella* in a biofilm (odds ratio 31.66, $P = 0.001$). There is very little data regarding the usefulness of HPC counts for predicting the presence of *Legionella*. It has been shown that certain common HPC bacteria inhibit the growth of *Legionella* while others stimulate it (79). Our data is in agreement with LeChevallier et al. (47) who concluded that HPC bacteria were useful for predicting the presence of opportunistic pathogens and provide insight into the overall quality of drinking water. There was no relationship between HPC bacteria and the presence of *Helicobacter* in any fraction.

Sampling Strategy Influences Detection of *Mycobacteria*, *Legionella*, and *Helicobacter*. This research has attempted to identify the presence of potential pathogens and identify factors that may play a role in where and when these organisms may be present. Because the residents on the Crow Reservation were concerned with overall drinking water quality, samples of drinking water and associated biofilms were taken at public buildings and private residences. This resulted in samples being collected from treated municipal and untreated groundwater systems. In this study, biofilm samples were collected in addition to drinking water samples according to the recommendations for *Legionella* (VAMC; Pittsburgh, Pa., CDC; Atlanta, Ga.). Although this recommendation specifically addresses *Legionella* detection, it is in agreement with many other findings that *Mycobacteria*, *Legionella*, and *Helicobacter* can be harbored and detected in drinking water biofilms (35, 69, 74). Finally, two methods for detecting the organisms of interest, PCR and culturing, were chosen. It is well documented that traditional culturing techniques underestimate the quantity and diversity of microorganisms in the environment (60). However, when looking at issues of public health it is also important to identify whether these organisms are viable and perhaps capable of infection. By combining molecular detection with traditional culturing methods, it is possible to increase the likelihood of detecting an organism of interest.

Mycobacteria, *Legionella*, and *Helicobacter* were found in both treated municipal water and untreated well water systems. *Mycobacteria* were found more often in groundwater systems than in treated municipal systems (61.9% and 38.1% of the 20 samples positive for *Mycobacteria*, respectively). Reports of the detection of *Mycobacteria* in groundwater have been sporadic, but generally have shown relative frequencies from not detected to up to 68% of locations testing positive (46, 71). *Mycobacteria* have been detected in treated systems with

410 varying results as well (22, 46, 78). Overall, our results are consistent with other reports of
411 *Mycobacteria* in treated municipal and groundwater systems. Unlike *Mycobacteria*, *Legionella*
412 had a statistically significant relationship with the source of the drinking water. *Legionella* were
413 found more often in treated municipal systems (66.7%) than in groundwater systems (33.3%). In
414 other studies, *Legionella* has been frequently found in municipal water systems and sporadically
415 in groundwater (14, 20, 53, 87). It has been shown that the presence and diversity of *Legionella*
416 varies spatially in drinking water distribution systems and in groundwater (87). It is possible that
417 premise plumbing in buildings with light or sporadic use could promote the planktonic and/or
418 necrotrophic growth of *Legionella* as described by others (51, 76). It is also likely that the
419 overall warmer temperature of the treated municipal system is supportive for *Legionella* survival
420 and growth. *Helicobacter* were detected in 4 locations of our study area with 50% in treated
421 municipal systems and 50% in untreated groundwater systems. One of the instances of
422 *Helicobacter* occurred during the flood event that closed the water treatment facility for a short
423 period of time. Reports of *Helicobacter* detection in drinking water have been intermittent with
424 most reports finding infrequent positive samples (15, 40, 85). Although the environmental
425 reservoir of *Helicobacter* is unknown, it is possible that water distribution systems may be
426 vulnerable to contamination through breaks or leaks in distribution pipes. Groundwater systems
427 that are too shallow may be under the influence of surface water which could be contaminated by
428 agricultural practices and inadequate sewage disposal (12).
429 *Mycobacteria*, *Legionella*, and *Helicobacter* were detected in both biofilm and drinking
430 water samples. *Mycobacterium gilvum* and *M. avium* complex were most frequently identified
431 (>95% sequence similarity) and were both found in biofilms more often than drinking water.
432 *Legionella pneumophila* occurred more often in biofilm samples while sequences identified as

433 *Legionella* sp. were more often identified in drinking water samples. Interestingly, both
434 *Mycobacteria* and *Legionella* had greater rates of culture positive tests in biofilm samples. This
435 could indicate that tap water biofilms are protective and supportive for these organisms. *H. pylori*
436 sequences occurred in two drinking water samples and two biofilm samples. Although *H. pylori*
437 were not detected in a large number of locations, biofilm sampling doubled the detection of this
438 organism. These data are consistent with reports of others that indicate that all three of these
439 genera can be found in both drinking water and associated biofilm samples (50, 53).

440 Consistent with other reports, molecular detection of all three genera was more successful
441 than traditional culture methods (3, 49, 73, 85). The majority of detections were achieved by PCR,
442 with only a small fraction of the samples positive for *Mycobacteria* and *Legionella* culture isolates.
443 While it is known that molecular techniques are important for detecting organisms that are injured,
444 or viable but not culturable (1, 7), culture techniques provide valuable information as well. In this
445 study, both *Mycobacteria* and *Legionella* were detected by culture methods and PCR. However, in a
446 minority of cases, culture positive locations could not be identified by PCR performed directly on
447 the samples. This has been documented by others as well and could be due to PCR inhibitors, such
448 as heavy metals, intrinsic to the drinking water system (31, 66).

449 **Health Consequences of *Mycobacteria*, *Legionella*, and *Helicobacter* in Drinking Water.** All of
450 the *Mycobacteria* sequences detected in this study were of the nontuberculous Mycobacteria group
451 (NTM). One important detected NTM are the slow-growing opportunistic pathogens in the *M.*
452 *avium* complex (MAC), which includes the *M. avium* subsp. *avium, M. avium* subsp. *intracellulare,*
453 *M. avium* subsp. *hominisuis,* and *M. avium* subsp. *paratuberculosis* (64). MAC accounts for over 70
454 percent of nontuberculous mycobacterial disease in the United States and for more than 95 percent
455 of nontuberculous disease among persons infected with human immunodeficiency virus (HIV) (64).

456 It has been shown that MAC isolates recovered from hospital water had a close relationship (large-
457 restriction-fragment pattern analysis) with clinical isolates recovered from patients indicating that
458 water could be the reservoir for infection (6). Other sequences identified in this study were of >95%
459 similarity to *M. gordonae*, *M. flavescens,* and *M. mucogenicum*. These species are all known to be
460 inhabitants of drinking water systems and have been implicated in adverse health consequences (36,
461 46, 54, 55). One interesting fast-growing *Mycobacterium* species that we encountered fairly
462 frequently was *M. gilvum*. This bacterium is an environmental mycobacterium that has been isolated
463 from soils in Montana (57), which is known to degrade polyaromatic hydrocarbons and has not been
464 implicated in any health effects.
465 The *Legionella* species sequences detected in this study were mainly *L. pneumophila* and
466 *Legionella* sp. but also included sequences similar to *L. fairfieldensis*, *L. dresdeniensis*, and *L.*
467 *birminghamiensis*. *L. pneumophila* is a well-documented opportunistic pathogen that has a low
468 infection rate (1-6%), but a mortality rate of 10-15%, and accounts for 1-4% of all pneumonia cases
469 in the United States' general population (62). *L. pneumophila* is the primary disease causing species
470 of this genus but there have been occasional cases of disease caused by other *Legionella* sp. (58).
471 Other *Legionella* species such as *L. fairfieldensis* and *L. birminghamiensis* have been documented in
472 drinking water systems (26), but are not implicated in health effects.
473 This study detected *H. pylori* sequences at four of the locations sampled. Recent research
474 using the ^{13}C urea breath test has shown that the prevalence of *H. pylori* in one rural community of
475 Montana is greater than 50% (56). Their research also indicated that the presence of *H. pylori*
476 infection was associated with regular consumption of city water as indicated by questionnaire results
477 (Unpublished data, USEPA) (56). Untreated well water has also been implicated in clinical

infections in the United States (8, 10). Areas with poor water quality may be more likely to have higher rates of water-borne transmission of disease, especially in children (15, 85).

In conclusion, microbes such as *M. avium*, *L. pneumophila*, and *H. pylori* can be found in drinking water systems in rural underserved areas. Coliforms were shown to be inadequate indicators for all of these organisms, while HPC bacterial levels did have a relationship with the presence of *Mycobacteria* and *Legionella*. Both treated municipal water and groundwater fed systems can harbor these organisms, which can be found in both bulk water and associated biofilms. Consequently, it is impossible to rule out drinking water as a route of infection for pathogenic bacteria such as *M. avium*, *L. pneumophila*, and *H. pylori*. To address health disparities in underserved communities such as American Indian reservations it is important to determine potential reservoirs of infection. These results are pertinent to water utility managers, regulatory agencies, as well as epidemiologists interested in identifying disease causing agents in rural drinking water systems.

ACKNOWLEDGEMENTS

The authors would like to thank the entire Crow Environmental Health Steering Committee, Ada Bends, Urban Bear Don't Walk, John Doyle, Brandon Goodluck, Vernon Hill, Larry Kindness, Myra Lefthand, Henry Pretty On Top, Ronald Stewart, and Sara Young, for providing insight into research in reservation communities and community support for this work. Thanks to Crow community coordinators, Crescentia Cummins and Gail Whiteman, without whom sample collection would not have been possible. Additional thanks to Al Parker for statistical support.

This work was supported by the National Institute for Health, Center for Native Health Partnerships, (M201-10-W2724). Dr. Ford is supported in part by a grant from the U.S.

Environmental Protection Agency's Science to Achieve Results (US-EPA STAR) program and Crystal Richards is supported by a US-EPA STAR Fellowship. Although the research described in the article has been funded in part by the U.S. Environmental Protection Agency's STAR program through grant numbers RD833706 and FP916936, it has not been subjected to any EPA review and therefore does not necessarily reflect the views of the Agency, and no official endorsement should be inferred.

REFERENCES

1. **Adams, B. L., T. C. Bates, and J. D. Oliver.** 2003. Survival of *Helicobacter pylori* in a natural freshwater environment. Appl. Environ. Microbiol. **69:**7462-7466.

2. **Allen, M. J., S. C. Edberg, and D. J. Reasoner.** 2004. Heterotrophic plate count bacteria - what is their significance in drinking water? Int. J. Food Microbiol. **92:**265-274.

3. **Angenent, L. T., S. T. Kelley, A. St Amand, N. R. Pace, and M. T. Hernandez.** 2005. Molecular identification of potential pathogens in water and air of a hospital therapy pool. Proc. Natl. Acad. Sci. U.S.A. **102:**4860-4865.

4. **Anonymous.** 2002. Crow Indian Reservation Natural, Socio-Economic, and Cultural Resources Assessment and Conditions Report, Crow Tribe of Indians, Montana.

5. **Anonymous.** 1999. The Health Care Challenge: Acknowledging Disparity, Confronting Discrimintaion, and Ensuring Equality. United States National Archives and Records Administration, Washington D.C. 20402.

6. **Aronson, T., A. Holtzman, N. Glover, M. Boian, S. Froman, O. G. W. Berlin, H. Hill, and G. Stelma.** 1999. Comparison of large restriction fragments of *Mycobacterium*

avium isolates recovered from AIDS and non-AIDS patients with those of isolates from potable water. J. Clin. Microbiol. **37:**1008-1012.

7. **Atlas, R. M.** 1999. *Legionella*: from environmental habitats to disease pathology, detection and control. Environ. Microbiol. **1:**283-293.

8. **Baker, K. H., and J. P. Hegarty.** 2001. Presence of *Helicobacter pylori* in drinking water is associated with clinical infection. Scand. J. Infect. Dis. **33:**744-746.

9. **Bartie, C., S. N. Venter, and L. H. Nel.** 2003. Identification methods for *Legionella* from environmental samples. Water Res. **37:**1362-1370.

10. **Bellack, N. R., M. W. Koehoorn, Y. C. MacNab, and M. G. Morshed.** 2006. A conceptual model of water's role as a reservoir in *Helicobacter pylori* transmission: a review of the evidence. Epidemiol. Infect. **134:**439-449.

11. **Benjamini, Y., and Y. Hochberg.** 1995. Controlling the false discovery rate - a practical and powerful approach to multiple testing. J. R. Stat. Soc. Series B Stat. Methodol. **57:**289-300.

12. **Bitton, G., and C. P. Gerba.** 1984. Groundwater Pollution Microbiology: the Emerging Issue, p. 1-7. In G. Bitton and C. Gerba (ed.), Groundwater pollution microbiology. John Wiley & Sons, New York.

13. **Boddinghaus, B., T. Rogall, T. Flohr, H. Blocker, and E. C. Bottger.** 1990. Detection and Identification of Mycobacteria by Amplification of RNA. J. Clin. Microbiol. **28:**1751-1759.

14. **Brooks, T., R. A. Osicki, V. S. Springthorpe, S. A. Sattar, L. Filion, D. Abrial, and S. Riffard.** 2004. Detection and identification of *Legionella* species from groundwaters. J. Toxicol. Environ. Health. **67:**1845-1859.

15. **Bunn, J. E. G., W. G. MacKay, J. E. Thomas, D. C. Reid, and L. T. Weaver.** 2002. Detection of *Helicobacter pylori* DNA in drinking water biofilms: implications for transmission in early life. Lett. Appl. Microbiol. **34:**450-454.

16. **Burkland, B.** 2010. Personal Communication regarding treatment facility at Crow Agency, US Environmental Protection Agency. Helena, MT.

17. **Burlingame, G. A., J. McElhaney, M. Bennett, and W. O. Pipes.** 1984. Bacterial interference with coliform colony sheen production on membrane filters. Appl. Environ. Microbiol. **47:**56-60.

18. **Cassidy, P. M., K. Hedberg, A. Saulson, E. McNelly, and K. L. Winthrop.** 2009. Nontuberculous Mycobacterial Disease Prevalence and Risk Factors: A Changing Epidemiology. Clin. Infect. Dis. **49:**E124-E129.

19. **Conover, W. J.** 1999. Practical nonparametric statistics, Third ed. Elm Street Publishing Services, Inc., New York.

20. **Costa, J., I. Tiago, M. S. da Costa, and A. Verissimo.** 2005. Presence and persistence of *Legionella* spp. in groundwater. Appl. Environ. Microbiol. **71:**663-671.

21. **Covers Up, T., R. Turnsplenty, D. Wetzel, and J. Giroux.** 2005. 1990-2002 Montana and Wyoming American Indian Top Ten Causes of Death. Rocky Mountain Tribal Epidemiology Center.

22. **Covert, T. C., M. R. Rodgers, A. L. Reyes, and G. N. Stelma.** 1999. Occurrence of nontuberculous mycobacteria in environmental samples. Appl. Environ. Microbiol. **65:**2492-2496.

23. **Craun, G. F., R. L. Calderon, and T. J. Wade.** 2006. Assessing waterborne risks: an introduction. J. Water Health **04. Suppl 2:**3-18.

24. **Craun, M. F., G. F. Craun, R. L. Calderon, and M. J. Beach.** 2006. Waterborne outbreaks reported in the United States. J. Water Health **04. Suppl 2:**19-30.

25. **Degnan, A. J., W. C. Sonzogni, and J. H. Standridge.** 2003. Development of a plating medium for selection of *Helicobacter pylori* from water samples. Appl. Environ. Microbiol. **69:**2914-2918.

26. **Diederen, B. M. W., C. M. A. de Jong, I. Aarts, M. F. Peeters, and A. van der Zee.** 2007. Molecular evidence for the ubiquitous presence of *Legionella* species in Dutch tap water installations. J. Water Health **5:**375-383.

27. **Doyle, J.** 2010. Personal communication regarding municipal distribution system at Crow Agency, Crow Environmental Health Steering Committee. Crow Agency, MT.

28. **Doyle, J., L. Kindness, U. Bear Don't Walk, S. Young, and M. Lefthand.** 2006. Personal communication regarding water quality on Crow Reservation, Crow Environmental Health Steering Committee. Crow Agency, MT.

29. **Falkinham, J. O., C. D. Norton, and M. W. LeChevallier.** 2001. Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*, and other mycobacteria in drinking water distribution systems. Appl. Environ. Microbiol. **67:**1225-1231.

30. **Fields, B. S., R. F. Benson, and R. E. Besser.** 2002. *Legionella* and Legionnaires' disease: 25 years of investigation. Clin. Microbiol. Rev. **15:**506-526.

31. **Fiume, L., M. A. Bucca Sabattini, and G. Poda.** 2005. Detection of *Legionella pneumophila* in water samples by species-specific real-time and nested PCR assays. Lett. Appl. Microbiol. **41:**470-475.

32. **Franzblau, S. G., B. J. Hinnebusch, L. M. Kelley, and N. A. Sinclair.** 1984. Effect on noncoliforms on coliform detection in potable groundwater - improved recovery with an anaerobic membrane-filter technique. Appl. Environ. Microbiol. **48:**142-148.

33. **Geach, J.** 2007. Source Water Delineation and Assessment Report, p. 1-34. United States Environmental Protection Agency, Helena, MT.

34. **Germani, Y., C. Dauga, P. Duval, M. Huerre, M. Levy, G. Pialoux, P. Sansonetti, and P. A. D. Grimont.** 1997. Strategy for the detection of *Helicobacter* species by amplification of 16S rRNA genes and identification of *H. felis* in a human gastric biopsy. Res. Microbiol. **148:**315-326.

35. **Giao, M. S., N. F. Azevedo, S. A. Wilks, M. J. Vieira, and C. W. Keevil.** 2008. Persistence of *Helicobacter pylori* in heterotrophic drinking-water biofilms. Appl. Environ. Microbiol. **74:**5898-5904.

36. **Guillen, S. M., J. S. Hospital, E. G. Mampaso, A. G. Espejo, C. E. Baquedano, and A. O. Calderon.** 1986. Gluteal abscess caused by *Mycobacterium flavescens*. Tubercle **67:**151-153.

37. **Hallberg, G. R., and B. C. Kross.** 1990. Iowa Statewide Rural Well Water Survey - Summary of Results. Iowa Department of Natural Resources Geologocal Survey Bureau and University of Iowa Center for Health Effects of Environmental Contamination, Iowa City, IA 52242.

38. **Heath, R. C.** 1984. Ground-Water Regions of the United States, p. 1-78. U.S. Geological Survey Supply Paper 2242.

39. **Iivanainen, E. K., P. J. Martikainen, P. K. Vaananen, and M. L. Katila.** 1993. Environmental factors affecting the occurrence of mycobacteria in brook waters. Appl. Environ. Microbiol. **59:**398-404.

40. **Janzon, A., A. Sjoling, A. Lothigius, D. Ahmed, F. Qadri, and A. M. Svennerholm.** 2009. Failure To Detect *Helicobacter pylori* DNA in Drinking and Environmental Water in Dhaka, Bangladesh, Using Highly Sensitive Real-Time PCR Assays. Appl. Environ. Microbiol. **75:**3039-3044.

41. **Jukes, T. H., and C. R. Cantor.** 1969. Evolution of protein molecules, p. 21-132. *In* H. N. Munro (ed.), Mammalian protein metabolism. Academic Press, New York.

42. **Kramer, M. H., and T. E. Ford.** 1994. Legionellosis: ecological factors of an environmentally 'new' disease. Zentralbl Hyg Umweltmed **195:**470-82.

43. **Kramer, M. H., B. L. Herwaldt, R. L. Calderon, and D. D. Juranek.** 1996. Surveillance for Waterborne-Disease Outbreaks -- United States, 1993-1994, p. 1-33, vol. 45. Centers for Disease Control, Washington D.C.

44. **Kutner, M., C. Nachtsheim, J. Neter, and W. Li.** 2004. Applied linear statistical models, Fifth ed. McGraw-Hill/Irwin, New York.

45. **Lamka, K. G., M. W. LeChevallier, and R. J. Seidler.** 1980. Bacterial contamination of drinking water supplies in a modern rural neighborhood. Appl. Environ. Microbiol. **39:**734-738.

46. **Le Dantec, C., J. P. Duguet, A. Montiel, N. Dumoutier, S. Dubrou, and V. Vincent.** 2002. Occurrence of mycobacteria in water treatment lines and in water distribution systems. Appl. Environ. Microbiol. **68:**5318-5325.

47. **LeChevallier, M. W., R. J. Seidler, and T. M. Evans.** 1980. Enumeration and characterization of standard plate-count bacteria in chlorinated and raw water supplies. Appl. Environ. Microbiol. **40:**922-930.

48. **Leclerc, H., D. A. A. Mossel, S. C. Edberg, and C. B. Struijk.** 2001. Advances in the bacteriology of the coliform group: their suitability as markers of microbial water safety. Annu. Rev. Microbiol. **55:**201-234.

49. **Lehtola, M. J., E. Torvinen, J. Kusnetsov, T. Pitkanen, L. Maunula, C. H. von Bonsdorff, P. J. Martikainen, S. A. Wilks, C. W. Keevil, and I. T. Miettinen.** 2007. Survival of *Mycobacterium avium*, *Legionella pneumophila*, *Escherichia coli*, and caliciviruses in drinking water-associated biofilms grown under high-shear turbulent flow. Appl. Environ. Microbiol. **73:**2854-2859.

50. **Mackay, W. G., L. T. Gribbon, M. R. Barer, and D. C. Reid.** 1998. Biofilms in drinking water systems - A possible reservoir for *Helicobacter pylori*. Water Sci. Technol. **38:**181-185.

51. **Mampel, J., T. Spirig, S. S. Weber, J. A. J. Haagensen, S. Molin, and H. Hilbi.** 2006. Planktonic replication is essential for biofilm formation by *Legionella pneumophila* in a complex medium under static and dynamic flow conditions. Appl. Environ. Microbiol. **72:**2885-2895.

52. **Manuel, C. M., O. C. Nunes, and L. F. Melo.** 2010. Unsteady state flow and stagnation in distribution systems affect the biological stability of drinking water. Biofouling **26:**129-139.

53. **Marciano-Cabral, F., M. Jamerson, and E. S. Kaneshiro.** 2010. Free-living amoebae, *Legionella* and *Mycobacterium* in tap water supplied by a municipal drinking water facility. J. Water Health **08:**71-82.

54. **Marshall, C., J. Samuel, A. Galloway, and S. Pedler.** 2008. *Mycobacterium mucogenicum* from the Hickman line of an immunocompromised patient. J. Clin. Pathol. **61:**140-141.

55. **Mazumder, S. A., A. Hicks, and J. Norwood.** 2010. *Mycobacterium gordonae* pulmonary infection in an immunoocompetent adult. N. A. J. Med. Sci. **2:**205-207.

56. **Melius, E., R. Wierzba, S. Davis, J. Sobel, B. Gold, A. Henderson, and J. Cheek.** 2005. Risk factors for *Helicobacter pylori* in a rural community.

57. **Miller, C. D., R. Child, J. E. Hughes, M. Benscai, J. P. Der, R. C. Sims, and A. J. Anderson.** 2007. Diversity of soil mycobacterium isolates from three sites that degrade polycyclic aromatic hydrocarbons. J. Appl. Microbiol. **102:**1612-1624.

58. **Muder, R. R., and V. L. Yu.** 2002. Infection due to *Legionella* species other than *L. pneumophila*. Clin. Infect. Dis. **35:**990-998.

59. **National Center for Health Statistics.** 2010. Health, Unites States, 2009: With Special Feature on Medical Technology., Hyattsville, MD.

60. **Pace, N. R.** 1997. A molecular view of microbial diversity and the biosphere. Science **276:**734-740.

61. **Palmer, C. J., G. F. Bonilla, B. Roll, C. Paszkokolva, L. R. Sangermano, and R. S. Fujioka.** 1995. Detection of *Legionella* species in reclaimed water and air with the ENVIROAMP *Legionella* PCR kit and direct fluorescent-antibody staining. Appl. Environ. Microbiol. **61:**407-412.

62. **Percival, S., R. Chalmers, M. Embrey, P. Hunter, J. Sellwood, and P. Wyn-Jones.** 2004. Microbiology of Waterborne Diseases. Elsevier Academic Press, California.

63. **Perez-Perez, G. I., D. Rothenbacher, and H. Brenner.** 2004. Epidemiology of *Helicobacter pylori* infection. Helicobacter **9:**1-6.

64. **Reed, C., C. F. von Reyn, S. Chamblee, T. V. Ellerbrock, J. W. Johnson, B. J. Marsh, L. S. Johnson, R. J. Trenschel, and C. R. Horsburgh.** 2006. Environmental risk factors for infection with *Mycobacterium avium* complex. Am. J. Epidemiol. **164:**32-40.

65. **Research and Analysis Bureau.** 2008. Demographic and Economic Information for the Crow Reservation, p. 1-8. Montana Department of Labor and Industry, Helena, MT 59624.

66. **Riffard, S., S. Douglass, T. Brooks, S. Springthorpe, L. G. Filion, and S. A. Sattar.** 2001. Occurrence of *Legionella* in groundwater: an ecological study. Water Sci. Technol. **43:**99-102.

67. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method - a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406-425.

68. **Sasaki, K., Y. Tajiri, M. Sata, Y. Fujii, F. Matsubara, M. G. Zhao, S. Shimizu, A. Toyonaga, and K. Tanikawa.** 1999. *Helicobacter pylori* in the natural environment. Scand. J. Infect. Dis. **31:**275-280.

69. **Schulze-Robbecke, R., B. Janning, and R. Fischeder.** 1992. Occurrence of mycobacteria in biofilm samples. Tuber. Lung Dis. **73:**141-144.

70. **Schulze-Robbecke, R., A. Weber, and R. Fischeder.** 1991. Comparison of decontamination methods for the isolation of mycobacteria from drinking water samples. J. Microbiol. Methods **14:**177-183.

71. **Schwartz, T., S. Kalmbach, S. Hoffmann, U. Szewzyk, and U. Obst.** 1998. PCR-based detection of mycobacteria in biofilms from a drinking water distribution system. J. Microbiol. Methods **34:**113-123.

72. **September, S. M., V. S. Brozel, and S. N. Venter.** 2004. Diversity of nontuberculoid *Mycobacterium* species in biofilms of urban and semiurban drinking water distribution systems. Appl. Environ. Microbiol. **70:**7571-7573.

73. **Springer, B., L. Stockman, K. Teschner, G. D. Roberts, and E. C. Bottger.** 1996. Two-laboratory collaborative study on identification of mycobacteria: Molecular versus phenotypic methods. J. Clin. Microbiol. **34:**296-303.

74. **Storey, M. V., J. Langmark, N. J. Ashbolt, and T. A. Stenstrom.** 2004. The fate of legionellae within distribution pipe biofilms: measurement of their persistence, inactivation and detachment. Water Sci. Technol. **49:**269-275.

75. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24:**1596-1599.

76. **Temmerman, R., H. Vervaeren, B. Noseda, N. Boon, and W. Verstraete.** 2006. Necrotrophic growth of *Legionella pneumophila*. Appl. Environ. Microbiol. **72:**4323-4328.

77. **Thompson, J. D., T. J. Gibson, and D. G. Higgins.** 2002. Multiple sequence alignment using ClustalW and ClustalX. Curr Protoc Bioinformatics **Chapter 2:**Unit 2.3.

78. **Torvinen, E., S. Suomalainen, M. J. Lehtola, I. T. Miettinen, O. Zacheus, L. Paulin, M. L. Katila, and P. J. Martikainen.** 2004. Mycobacteria in water and loose deposits of drinking water distribution systems in Finland. Appl. Environ. Microbiol. **70:**1973-1981.

79. **Toze, S., L. I. Sly, I. C. Macrae, and J. A. Fuerst.** 1990. Inhibition of growth of *Legionella* species by heterotrophic plate-count bacteria isolated from chlorinated drinking water. Curr. Microbiol. **21:**139-143.

80. **Turenne, C. Y., M. Semret, D. V. Cousins, D. M. Collins, and M. A. Behr.** 2006. Sequencing of hsp65 distinguishes among subsets of the Mycobacterium avium complex. J. Clin. Microbiol. **44:**433-440.

81. **Turetgen, I., E. I. Sungur, and A. Cotuk.** 2005. Enumeration of *Legionella pneumophila* in cooling tower water systems. Environ. Monit. Assess. **100:**53-58.

82. **United States Census Bureau.** 2007. Current Housing Reports, Series H150/07, p. 1-642, American Housing Survey for the United States: 2007. U.S. Government Printing Office, Washington D.C.

83. **United States Environmental Protection Agency.** 1998. Guidance for data quality assessment-Practical methods for data analysis. Office of Research and Development, Washington, D.C.

84. **Voytek, M. A., J. B. Ashen, L. R. Fogerty, J. D. Kirshtein, and E. R. Landa.** 2005. Detection of *Helicobacter pylori* and fecal indicator bacteria in five North American rivers. J. Water Health **03:**405-422.

85. **Watson, C. L., R. J. Owen, B. Said, S. Lai, J. V. Lee, S. Surman-Lee, and G. Nichols.** 2004. Detection of *Helicobacter pylori* by PCR but not culture in water and biofilm

744 samples from drinking water distribution systems in England. J. Appl. Microbiol.
745 **97:**690-698.
746 86. **Whan, L., H. J. Ball, I. R. Grant, and M. T. Rowe.** 2005. Occurrence of
747 *Mycobacterium avium* subsp *paratuberculosis* in untreated water in Northern Ireland.
748 Appl. Environ. Microbiol. **71:**7107-7112.
749 87. **Wullings, B. A., and D. van der Kooij.** 2006. Occurrence and genetic diversity of
750 uncultured *Legionella* spp. in drinking water treated at temperatures below 15 degrees C.
751 Appl. Environ. Microbiol. **72:**157-166.

Table 1. Primer Sequences, References and PCR Conditions.

Target (Reference)	Sequence	Product size	PCR Conditions
16S RNA gene *Legionella* spp. (87)	LEG-225 5'AAGATTAGCCTGCG TCCGAT; LEG-858 5'GTCAACT TATCGCGTTTGCT	656 bp	94˚C 2 min (1 cycle); 94˚C 20 sec, 60˚C 30 sec, 72˚C 40 sec (40 cycles); 72˚C 5 min (1 cycle)
16S RNA gene *Mycobacterium* spp. (13)	MycgenF 5' AGAGTTTGATCCT GGCTCAG; MycgenR 5' TGCAC ACAGGCCACAAGGGA	1,030 bp	95˚C 2 min (1 cycle); 93˚C 1 min, 60˚C 1 min, 72˚C 1 min (35 cycles); 72˚C 5 min (1 cycle)
16S RNA gene *Helicobacter* spp. (34)	HS1 5' AACGATGAAGCTTCT AGCTTGCTAG; HS2 5' GTGCT TATTCGTTAGATACCGTCAT	400 bp	94˚C 5 min (1 cycle); 94˚C 1 min, 65˚C 1 min, 72˚C 1 min (35 cycles); 72˚C 5 min (1 cycle)
16S RNA gene Eubacteria (84)	46f 5' GCYTAACACATGCA AGTCGA; 519r 5' GTATTACCG CGGCKGCTG	490 bp	95˚C 5 min (1 cycle); 94˚C 0.5 min, 56˚C 0.5 min, 72˚C 1.5 min (30 cycles); 72˚C 7 min (1 cycle)

Table 2. Range and Arithmetic Mean of HPC bacteria, total coliforms, and *E. coli*

Bacteria	Measurement	Source	
		Treated Municipal (n=16)	Groundwater Well (n=41)
Heterotrophic plate counts			
In first flush (CFU/ml)	Range	*	1.5×10^0 - 5.12×10^7
	Arithmetic Mean	*	2.81×10^6
In water (CFU/ml)	Range	3.57×10^2 - 5.15×10^5	2.0×10^0 - 9.23×10^5
	Arithmetic Mean	9.02×10^4	5.7×10^4
In biofilm (CFU/mm^2)	Range	<1 - 1.24×10^5	<1 - 3.22×10^5
	Arithmetic Mean	1.79×10^4	4.29×10^4
Total Coliforms			
In first flush (CFU/100ml)	Range	*	<1 - 1.19×10^3
	Arithmetic Mean	*	1.17×10^2
In water (CFU/100ml)	Range	<1 - 2.63×10^1	<1 - 2.96×10^3
	Arithmetic Mean	2.71×10^0	1.07×10^2
Escherichia coli			
In first flush (CFU/100ml)	Range	*	<1
	Arithmetic Mean	*	<1
In water (CFU/100ml)	Range	<1	<1 - 2.22×10^2
	Arithmetic Mean	<1	5.68×10^0

* No samples were taken in this category.

Table 3. Statistical Analysis of the Interactions between HPC Bacteria in Drinking Water and Response Variables

		Significance and FDR	
Response Variable	Model	*P*	$P_{(12)}$
Helicobacter (BF)	Binary Logistic Regression	0.95	0.100
Mycobacteria (BF)	Binary Logistic Regression	0.778	0.092
Helicobacter (System)	Binary Logistic Regression	0.628	0.083
Total coliforms (DW)	Simple Linear Regression	0.463	0.075
Helicobacter (DW)	Binary Logistic Regression	0.457	0.067
Legionella (DW)	Binary Logistic Regression	0.068	0.058
Mycobacteria (System)	Binary Logistic Regression	0.044*	0.050
Mycobacteria (DW)	Binary Logistic Regression	0.03*	0.042
Escherichia coli (DW)	Simple Linear Regression	0.026*	0.033
HPC bacteria (FF)	Paired t-test	0.025*	0.023
Legionella (System)	Binary Logistic Regression	0.003*	0.017
Legionella (BF)	Binary Logistic Regression	0.001*	0.008

* Denotes P-value with statistical significance.
(FDR) false discovery rate, (FF) first flush, (BF) biofilm, (DW) drinking water, (System) combines all sample fractions.

Table 4. Statistical Analysis of the Interactions between HPC Bacteria in Biofilms and Response Variables

		Significance and FDR	
Response Variable	Model	*P*	$P_{(10)}$
Helicobacter (BF)	Binary Logistic Regression	0.872	0.100
Helicobacter (DW)	Binary Logistic Regression	0.746	0.082
Helicobacter (System)	Binary Logistic Regression	0.726	0.073
Total coliforms (DW)	Simple Linear Regression	0.606	0.064
Mycobacteria (DW)	Binary Logistic Regression	0.509	0.055
Legionella (BF)	Binary Logistic Regression	0.204	0.045
Legionella (DW)	Binary Logistic Regression	0.208	0.036
Legionella (System)	Binary Logistic Regression	0.185	0.027
Mycobacteria (BF)	Binary Logistic Regression	0.051	0.018
Mycobacteria (System)	Binary Logistic Regression	0.01	0.009

(FDR) false discovery rate, (BF) biofilm, (DW) drinking water, (System) combines all sample fractions.

Table 5. Statistical Analysis of the Interactions between Drinking Water Source (Treated Municipal or Groundwater Well) and Response Variables

		Significance and FDR	
Variable	Model	*P*	$P_{(7)}$
Escherichia coli (DW)	Two sample t-test	0.418	0.100
Helicobacter (System)	Fisher's exact test	0.393	0.086
Mycobacteria (System)	Fisher's exact test	0.216	0.071
HPC bacteria (DW)	Two sample t-test	0.13	0.057
Total coliforms (DW)	Two sample t-test	0.128	0.043
HPC bacteria (BF)	Two sample t-test	0.049	0.029
Legionella (System)	Fisher's exact test	0.003*	0.014

* Denotes P-value with statistical significance.
(FDR) false discovery rate, (BF) biofilm, (DW) drinking water, (System) combines all sample fractions.

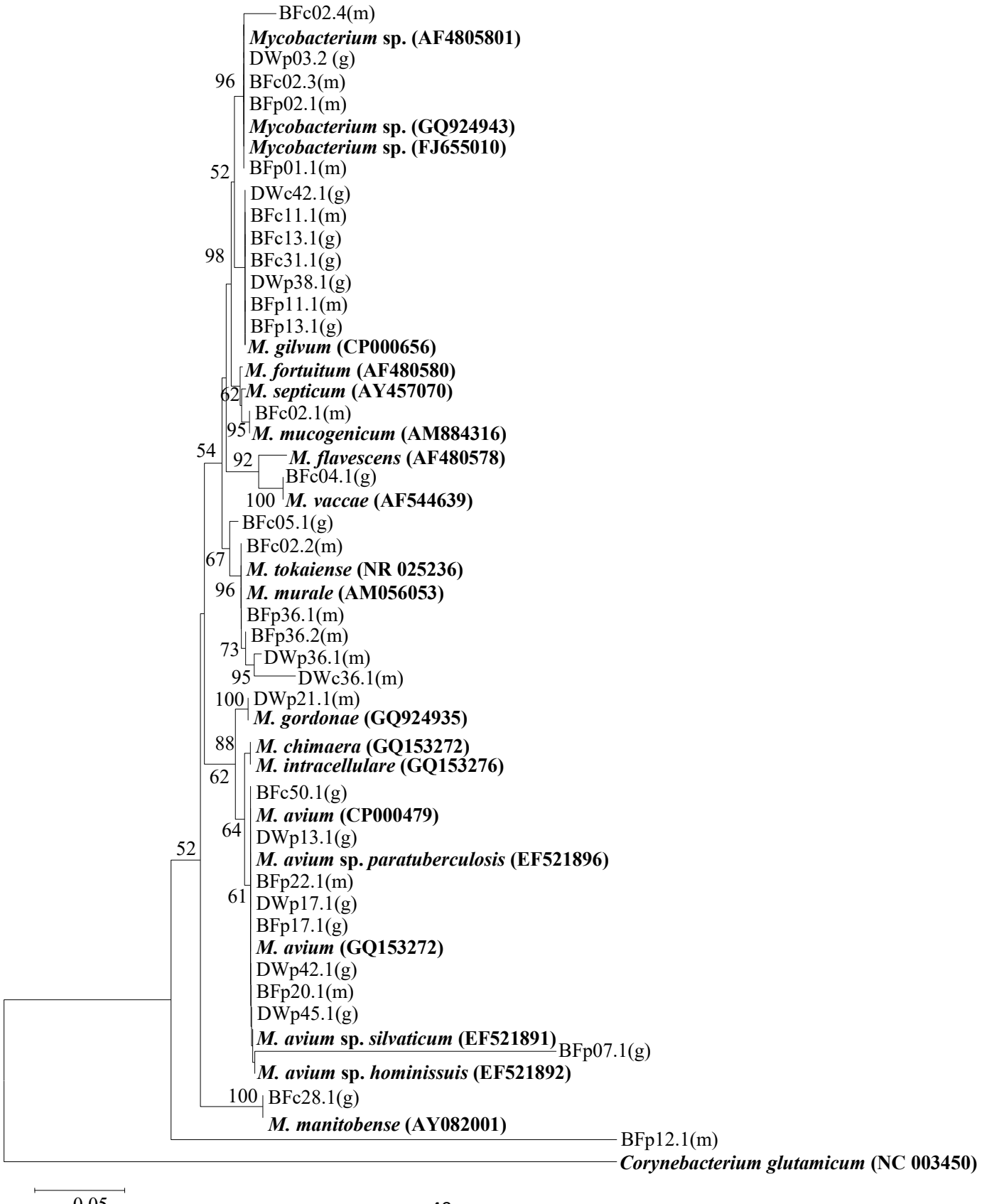
BFc02.4(m)
Mycobacterium sp. (AF4805801)
DWp03.2 (g)
96
BFc02.3(m)
BFp02.1(m)
Mycobacterium sp. (GQ924943)
Mycobacterium sp. (FJ655010)
52
BFp01.1(m)
DWc42.1(g)
BFc11.1(m)
BFc13.1(g)
98
BFc31.1(g)
DWp38.1(g)
BFp11.1(m)
BFp13.1(g)
M. gilvum (CP000656)
M. fortuitum (AF480580)
62
M. septicum (AY457070)
BFc02.1(m)
95
M. mucogenicum (AM884316)
54
92
M. flavescens (AF480578)
BFc04.1(g)
100
M. vaccae (AF544639)
BFc05.1(g)
BFc02.2(m)
67
M. tokaiense (NR 025236)
96
M. murale (AM056053)
BFp36.1(m)
BFp36.2(m)
73
DWp36.1(m)
95
DWc36.1(m)
100
DWp21.1(m)
M. gordonae (GQ924935)
88
M. chimaera (GQ153272)
M. intracellulare (GQ153276)
62
BFc50.1(g)
M. avium (CP000479)
64
DWp13.1(g)
52
M. avium sp. paratuberculosis (EF521896)
BFp22.1(m)
61
DWp17.1(g)
BFp17.1(g)
M. avium (GQ153272)
DWp42.1(g)
BFp20.1(m)
DWp45.1(g)
M. avium sp. silvaticum (EF521891)
BFp07.1(g)
M. avium sp. hominissuis (EF521892)
100
BFc28.1(g)
M. manitobense (AY082001)
BFp12.1(m)
Corynebacterium glutamicum (NC 003450)
0.05

Figure 1. Phylogenetic relationship of 16s rRNA gene amplified with *Mycobacterium* genus-specific primers with *Corynebacterium glutamicum* as the out-group. Reference sequences are in bold with accession numbers in parentheses. Sequences from this study are indicated by code as follows. (DW) drinking water, (BF) biofilm, (p) PCR, (c) culture, (g) groundwater, and (m) municipal.

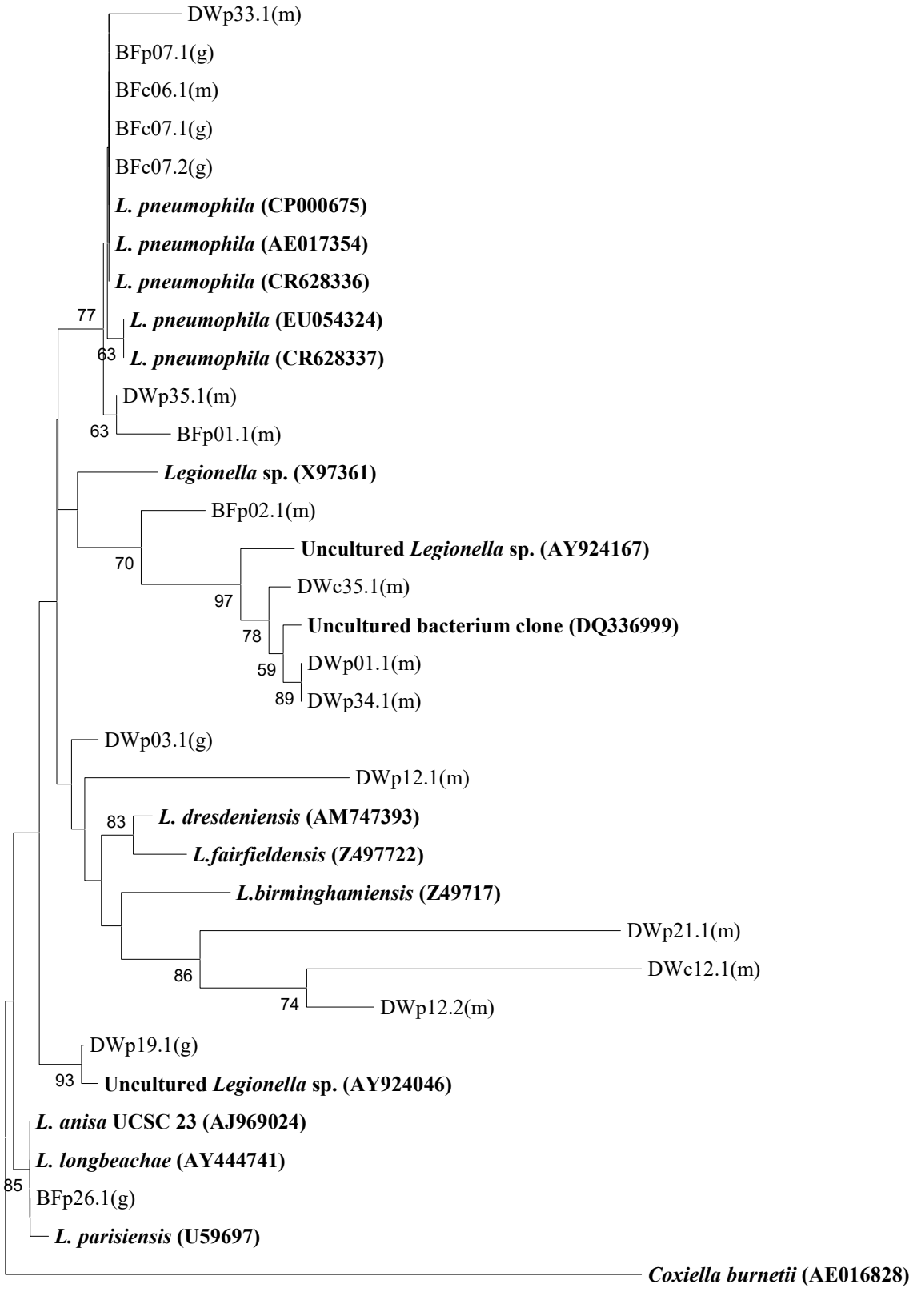
DWp33.1(m)
BFp07.1(g)
BFc06.1(m)
BFc07.1(g)
BFc07.2(g)
L. pneumophila (CP000675)
L. pneumophila (AE017354)
L. pneumophila (CR628336)
77
L. pneumophila (EU054324)
63
L. pneumophila (CR628337)
DWp35.1(m)
63
BFp01.1(m)
Legionella sp. (X97361)
BFp02.1(m)
Uncultured Legionella sp. (AY924167)
70
DWc35.1(m)
97
Uncultured bacterium clone (DQ336999)
78
DWp01.1(m)
59
DWp34.1(m)
89
DWp03.1(g)
DWp12.1(m)
83
L. dresdeniensis (AM747393)
L.fairfieldensis (Z497722)
L.birminghamiensis (Z49717)
DWp21.1(m)
DWc12.1(m)
86
74
DWp12.2(m)
DWp19.1(g)
93
Uncultured Legionella sp. (AY924046)
L. anisa UCSC 23 (AJ969024)
L. longbeachae (AY444741)
85
BFp26.1(g)
L. parisiensis (U59697)
Coxiella burnetii (AE016828)
0.02

Figure 2. Phylogenetic relationship of 16s rRNA gene amplified with *Legionella* genus-specific primers with *Coxiella burnetii* as the out-group. Reference sequences are in bold with accession numbers in parentheses. Sequences from this study are indicated by code as follows. (DW) drinking water, (BF) biofilm, (p) PCR, (c) culture, (g) groundwater, and (m) municipal.

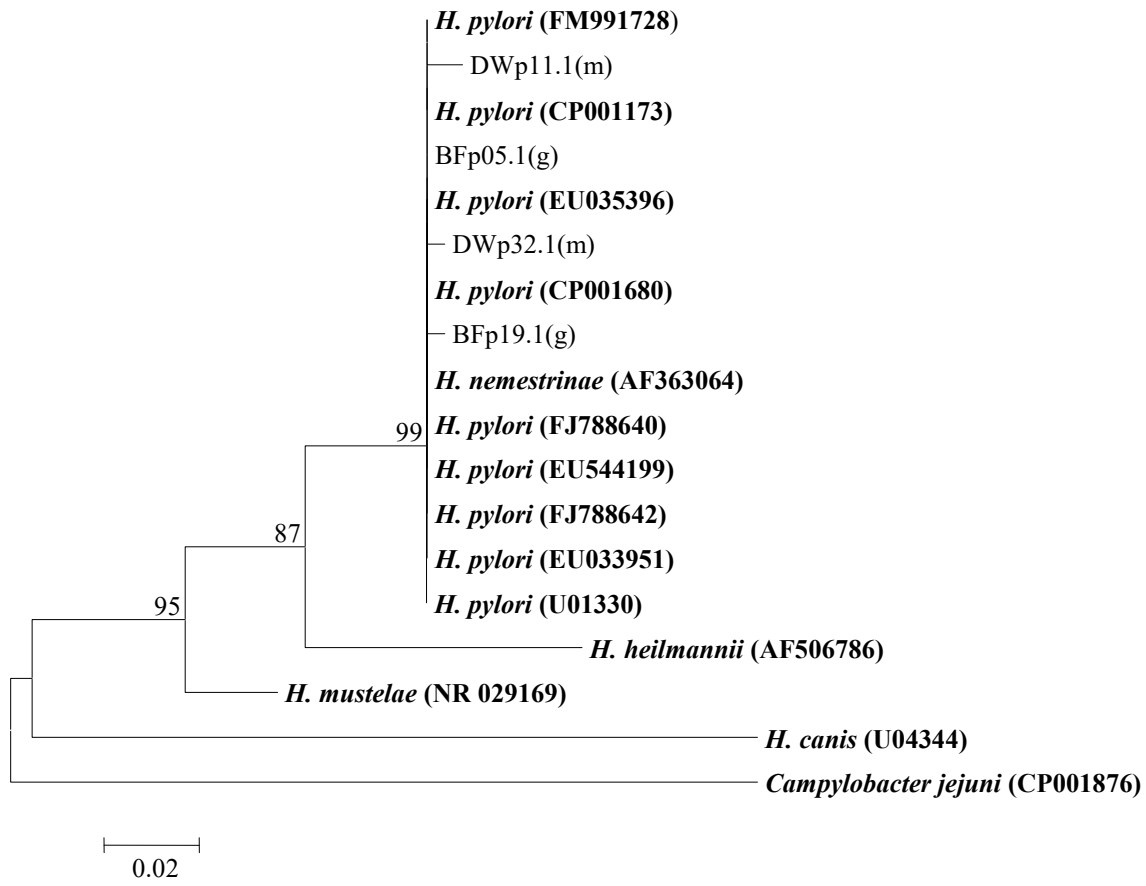

Figure 3. Phylogenetic relationship of 16s rRNA gene amplified with *Helicobacter* genus-specific primers with *Campylobacter jejuni* as the out-group. Reference sequences are in bold with accession numbers in parentheses. Sequences from this study are indicated by code as follows. (DW) drinking water, (BF) biofilm, (p) PCR, (c) culture, (g) groundwater, and (m) municipal.