Science of the Total Environment 618 (2018) 165–173

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Exploring the links between groundwater quality and bacterial communities near oil and gas extraction activities

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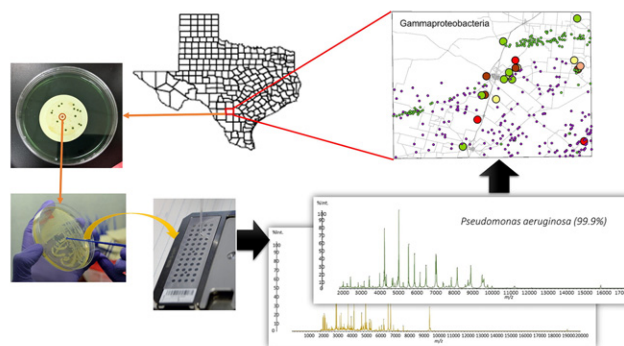
HIGHLIGHTS

- Stressful environments change bacterial communities.
- Groundwater samples located near agricultural and UD activities were collected.
- The bacteria present in contaminated groundwater were identified using MALDI-TOF MS.
- Mainly bacteria from the Phylum *Proteobacteria* were isolated.
- The bacterial communities varied significantly with the compositional differences.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 8 August 2017
Received in revised form 22 October 2017
Accepted 23 October 2017
Available online 9 November 2017

Editor: D. Barcelo

Keywords:
MALDI-TOF MS
Unconventional drilling
Groundwater microbiome
Hydraulic fracturing
Biogeochemical cycles
Unconventional oil and gas

ABSTRACT

Bacterial communities in groundwater are very important as they maintain a balanced biogeochemical environment. When subjected to stressful environments, for example, due to anthropogenic contamination, bacterial communities and their dynamics change. Studying the responses of the groundwater microbiome in the face of environmental changes can add to our growing knowledge of microbial ecology, which can be utilized for the development of novel bioremediation strategies. High-throughput and simpler techniques that allow the real-time study of different microbiomes and their dynamics are necessary, especially when examining larger data sets. Matrix-assisted laser desorption-ionization (MALDI) time-of-flight mass spectrometry (TOF-MS) is a workhorse for the high-throughput identification of bacteria. In this work, groundwater samples were collected from a rural area in southern Texas, where agricultural activities and unconventional oil and gas development are the most prevalent anthropogenic activities. Bacterial communities were assessed using MALDI-TOF MS, with bacterial diversity and abundance being analyzed with the contexts of numerous organic and inorganic groundwater constituents. Mainly denitrifying and heterotrophic bacteria from the Phylum *Proteobacteria* were isolated. These microorganisms are able to either transform nitrate into gaseous forms of nitrogen or degrade organic compounds such as hydrocarbons. Overall, the bacterial communities varied significantly with respect to the compositional differences that were observed from the collected groundwater samples. Collectively, these data

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https://doi.org/10.1016/j.scitotenv.2017.10.264
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provide a baseline measurement of bacterial diversity in groundwater located near anthropogenic surface and subsurface activities.

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1. Introduction

Microorganisms such as bacteria, viruses, and fungi are ubiquitous on earth (Horner-Devine et al., 2004). They are present in humans and animals, food, and the environment. These cells do not exist as individuals but interact and communicate with other cells and therefore act as a dynamically changing microbial community. Consequently, changes in their environment will eventually change their interactions and community (Blaser et al., 2016). According to Baas Becking's hypothesis, "everything is everywhere but the environment selects" (Fondi et al., 2016). Only specifically-adapted organisms will survive and proliferate in a particular environment. Therefore, understanding the factors that modulate diversity within a microbial ecosystem, such as physical (e.g. temperature) and chemical (e.g. nutrients) factors, is essential from a microbiological and ecological point of view (Blaser et al., 2016). Studying the responses of the microbiome to environmental changes can provide important knowledge for the development of new microbiological applications such as remediation of contaminated soil and water, and the search of novel biochemicals (Horner-Devine et al., 2004).

Several environmental studies have postulated that unconventional oil and gas development processes (UD), including hydraulic fracturing, may change the chemical composition of groundwater overlying hydrocarbon-rich petroliferous strata (Fontenot et al., 2013; Hildenbrand et al., 2017, 2016, 2015) and may affect the microbial communities that they support. Previous investigations have examined the impacts of hydraulic fracturing on surrounding environmental microbiomes in headwater stream ecosystems and surface waters (Fahrenfeld et al., 2016; Trexler et al., 2014). In both studies, the authors revealed that the microbial communities changed in response to altered conditions due to UD activities. In these works, DNA sequencing was used to characterize the water microbiome. High-throughput and simpler techniques that allow the real-time study of different microbiomes and their dynamics are necessary, especially when examining larger data sets and accounting for the costs and limited scope that are associated with more traditional techniques.

Mass spectrometry (MS), particularly matrix assisted laser desorption ionization - time of flight (MALDI-TOF), has already proved to be a workhorse for the identification of microorganisms (Basile and Mignon, 2016) and its application to environmental microbiology has increased significantly (Santos et al., 2016). MALDI-TOF MS allows the analysis of large biomolecules such as proteins by soft ionization meaning no fragmentation is induced. A matrix is overlaid on top of the sample to promote desorption and ionization of the analytes followed by acceleration in a vacuum through the application of an electric potential. The mass-to-charge ratio (m/z) will determine the time necessary to travel the flight tube and reach the detector (Dingle and Butler-Wu, 2013). A single colony can be used to obtain a protein profile that is unique for each microorganism thereby allowing its identification and/or differentiation (Freiwald and Sauer, 2009; Ghyselinck et al., 2011). In previous work, we demonstrated the ability of this technique to characterize the microbial ecology of groundwater located near UD activities. It was shown that the presence of high concentrations of hydrocarbon contaminants promotes the presence of pathogenic bacteria such as *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Stenotrophomonas maltophilia*, and *Bacillus cereus* (Martin et al., 2017; Santos et al., 2017).

In the work presented here, the connection between groundwater constituents and specific bacteria was evaluated using MALDI-TOF MS to further elucidate how environmental factors modulate the survival and proliferation of microorganisms in highly variable ecosystems. These data are some of the first to comprehensively characterize microbial communities in groundwater overlying oil and gas development, in a rural region engaged in various agricultural activities.

2. Materials and methods

2.1. Sample collection and analysis

Groundwater samples were collected from 19 water wells throughout Frio County, in southern Texas, overlying the Eagle Ford Shale. In situ measurements were performed using a YSI Professional Plus multiparametric probe and are presented in Table S1. To assess their degree of impairment, the chemical composition of groundwater samples was assessed by measuring volatile organic compounds (VOCs) using gas chromatography – mass spectrometry as described previously (Hildenbrand et al., 2016, 2015). Pertinent metal ions and anions were determined by inductively coupled plasma - mass spectrometry (ICP-MS) and ion chromatography (IC), as per EPA methods 200.7, 245.1, and 300A. Samples for the microbial analyses were collected in duplicate in 500 mL HDPE sterile sample bottles (Thermo Scientific™ Nalgene™) by filling bottles completely and leaving minimal headspace. Sterile deionized water was used as a transport blank to ensure no contamination, either chemical or biological, due to transportation of samples. Samples were stored on ice in coolers until they were processed.

2.2. Microbial analysis

Groundwater samples were filtered within 24 h of collection using the membrane filter technique. Volumes of 100 mL were filtered through sterile filter units coupled with sterile membrane filters of 0.22 μm pore size (EMD Millipore). All filtration was performed under aseptic techniques and in triplicate. Membranes were plated onto Nutrient agar (NA), m-Endo Agar LES, and Aeromonas Isolation Agar (Sigma-Aldrich, St. Louis, MO, USA). The spread plate technique was also performed and 0.1 mL of each water sample was spread in the agar plates. All plates were incubated at 25 °C for 24–48 h, except for the m-Endo agar media which was incubated at 37 °C. Bacteria quantification in water samples was performed according to Standard Methods for Water and Wastewater Analysis (APHA-AWWA-WPCF) (APHA et al., 1999) and results were expressed as colony forming units (CFU)/100 mL. The colonies were isolated in NA and incubated at 37 °C for 24 h. Pure cultures were preserved at −80 °C in Nutrient Broth (NB, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 15% (v/v) sterile glycerol (Amresco, Ohio, USA).

2.3. Microbial identification

2.3.1. MALDI-TOF MS

For the identification of microorganisms, two protein extraction methods were used according to the instrument manufacturer (Shimadzu Corporation, Kyoto, Japan): the Direct Smear *plus* Formic Acid method and the protein extraction method. The microbial colony or the protein extraction solution were placed on Fleximass™ DS disposable MALDI targets (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA). A 40 mg/mL alpha-cyano-4-hydroxycinnamic acid matrix solution (CHCA, Sigma-Aldrich, St. Louis, MO, USA) in 33/33/33 acetonitrile/water/ethanol (J.T. Baker, Phillipsburg NJ, USA; Decon Labs, Inc., King of Prussia PA, USA) with 3% trifluoroacetic acid (Sigma-Aldrich,

St. Louis, MO, USA) was used as matrix. Samples were analyzed in duplicate.

The mass spectra were obtained using the AXIMA-iD Plus MALDI-TOF MS Platform (Kratos Analytical, Manchester, UK) operated in linear mode with a laser power of 60–80 and a detector voltage of 2600 V. Experiments were performed with 100 profiles per spectra and 5 shots per spot. The mass range analyzed was set at a mass-to-charge (m/z) ratio of 2000–20,000 Da. *Escherichia coli* DH5α was used as a calibration. From the spectra, the generated peak lists were automatically exported into the Shimadzu Axima Confidence plus SARAMIS microorganism database (BioMérieux, SA, Marcyl'Etoile, France). A match score was then generated based on peak mass and intensities of sample spectra in comparison to database spectra. A score higher than 90% indicated a high degree of confidence in the identification of a species. A score between 70% and 90% was still accepted, though with a lower degree of confidence whereas any score under 70% meant no significant similarity of the spectrum with any database entry. This provides a level of confidence for the identification of the sample microorganism to the matched database spectra (Santos et al., 2016).

2.3.2. 16S rRNA gene sequence analysis

For bacterial identification confirmation, 16S rRNA gene sequencing was performed by the Genomics Core Facility (Department of Biology, UT Arlington, Arlington, TX). Polymerase chain reaction (PCR) amplification was performed on samples using the primers 27F and 534R (Yarza et al., 2014), which are specific for the 16S ribosomal RNA gene. These primers flank the V1–V3 variable regions, located between base pairs (bp) 69–497 ("16S ribosomal DNA | Lutzoni Lab,", n.d.) numbering system as per *Escherichia coli*. Sequence quality was evaluated, and trimmed as needed to remove low quality chromatogram reads. A minimum of two independent PCRs were sequenced for each sample.

The partial 16S rRNA gene sequences of the isolated strains were submitted to the GenBank database under accession numbers: MF464657, *Brevundimonas* sp. strain FC2; MF464658, *Bacillus* sp. FC3; MF464659, Arthrobacter sp. strain FC4; MF464660, *Achromobacter* sp. strain FC14; MF464661, *Alcaligenes faecalis* strain FC; MF464865, *Cupriavidus* sp. strain FC; MF464866, *Serratia marscescens* strain FC; MF464867, *Sphingobacterium* sp. strain FC; and MF470199, *Microbacterium* sp. Strain FC4.

3. Results and discussion

3.1. Impact of anthropogenic activities on groundwater microbiome

The primary aim of this study was to investigate the influence that anthropogenic activities can have on the groundwater microbiome using MALDI-TOF MS. The rural study area facilitates a wide range of conventional and unconventional oil and gas activities (production wells and waste disposal wells), in addition to agricultural activities. Table 1 shows a summary of groundwater constituents in relation to detected bacterial communities.

Within the collected groundwater samples, microbial abundance and diversity did not exhibit any geospatial patterns (Fig. 1). Each water sample showed a unique microbiome. *Proteobacteria* dominated the communities at all sites. All bacteria isolated, with the exception of *S. multivorum* and *Bacillus* sp., belong to this Phylum (Fig. 2). *Proteobacteria* accounts for mainly gram-negative bacteria and includes five major phylogenetic lines known as *Alpha-proteobacteria*, *Beta-proteobacteria*, *Gamma-proteobacteria*, *Delta-proteobacteria*, and *Epsilon-proteobacteria*. The extreme diversity of energy-generating mechanisms is a unique biochemical characteristic of the *Proteobacteria*: some are chemoorganotrophs (e.g., *Escherichia coli*), others are chemolithotrophs (e.g., the sulfur-oxidizing bacteria such as the thiobacilli) or phototrophs (e.g., the purple colored *Chromatium*) (Kersters et al., 2006).

The *Alpha-proteobacteria* are oligotrophs, which means that the organisms are capable of living in low-nutrient environments such as deep oceanic sediments, glacial ice, or deep undersurface soil. Only one bacteria from this class, *Brevundimonas vesiculares*, was identified in a single water sample (FC19). This suggests that the water environment from where this microorganism was isolated had a low concentration of nutrients, which was verified by the low levels of TN.

The *Beta-proteobacteria* are heterogeneous and highly metabolically diverse containing chemolithoautotrophs, photoautotrophs, and heterotrophs. Nevertheless, the three bacteria isolated from this class are all chemoorganotrophic organisms, *A. faecalis*, *C. pauculus*, and *A. xylosoxidans*, meaning they are able to use a wide variety of carbon sources for growth. Furthermore, *A. faecalis* is a heterotrophic denitrifier, meaning that it breaks down NO_3^-, NO_2^-, and NH_4^+ using organic C as a carbon and energy source. Sample FC1 showed high levels of nitrate which promotes the presence of denitrifying bacteria. Another interesting property of *A. faecalis* is that this microorganism produces biosurfactant compounds (Bharali et al., 2011). These compounds increase the hydrophobicity of the cell surface decreasing surface tension during growth on hydrocarbons. This high surface activity enhances the contact with the hydrocarbons and as a result increases hydrocarbon degradation (Bharali et al., 2011). *A. xylosoxidans* is capable of simultaneous nitrification and denitrification. *Cupriavidus pauculus* is known for its resistance to high levels of metals such as copper.

The *Gamma-proteobacteria* form the largest class with at least 180 genera and 750 species. This class includes 13 major orders and 20 families such as *Enterobacteriaceae*, *Moraxellaceae*, *Aeromonadaceae*, and *Pseudomonadaceae*. Most *Pseudomonads* are versatile and can grow on a variety of organic compounds, including aromatic hydrocarbons. In fact, *Pseudomonas* was the most predominant microorganism as it rapidly adapts to different environmental conditions and are able to colonize a wide range of niches where aliphatic and aromatic hydrocarbons are present (Das and Chandran, 2011). The detected *Pseudomonas* species (*P. aeruginosa*, *P.* stutzeri, and *P. putida*) may be involved in the oxidation of simple or complex organic carbon coupled to nitrate reduction. Although nitrogen was present in all samples, nitrate levels were extremely elevated (148.0 mg/L) in only one sample (FC1). *C. amalonaticus* are chemoheterotrophs and use citrate as their sole carbon source. *C. amalonaticus* also has the ability to accumulate metals from its environment by combining them with phosphates (Macaskie et al., 1992).

Interestingly, *E. coli* and *C. amalonaticus* were found in water samples FC1 and FC2. Both bacteria can originate from the intestines of animals and humans. The sub-surface location of the FC1 water well pump was located near a septic tank that is most likely contaminating the water well. In fact, the presence of high levels of nitrate can also originate from septic tanks. The FC2 site is surrounded by livestock, which could explain the origin of these coliforms.

Many different *Bacillus* sp. from the *Firmicutes* phylum were enriched from groundwater. Under stressful environmental conditions, these bacteria can produce endospores and remain in a dormant state for long periods. Fermentative *Firmicutes* may be important transitional community members producing labile substrates from organic compounds or formation constituents that support energy. Another interesting fact is that *Bacillus* sp. were detected in sample FC8 where high levels of strontium were detected. Previously it was shown that these microorganisms are capable of removing strontium from water (Chaalal et al., 2015). The presence of this microorganism in groundwater with elevated strontium (5.0 mg/L) may be a reflection of its tolerance for this heavy metal. Unfortunately, historical groundwater quality measurements were not available to compare strontium levels over time; however, subsequent measurement will elucidate the degree, if any, of bacterial strontium metabolism.

Bacteroidetes is a Phylum composed of three large classes of Gram-negative, non-spore forming, anaerobic or aerobic, and rod-shaped

Table 1
Summary of the 19 groundwater constituents in relation to detected bacterial communities. All groundwater constituents are summarized within the contexts of the federal Safe Drinking Water Act (SDWA) guidelines.

Sample #	Summary of basic water quality[a]	Summary of anionic abnormalities	Summary of metal ion abnormalities	VOCs/SVOCs detected	Unretained natural gas area (C1–C3) arb. units	TOC (mg/L)	TN (mg/L)	Bacterial communities detected by MALDI-TOF MS with degree of confidence (%)
FC1	Elevated TDS (747.5 mg/L)	Elevated chloride (1090.0 mg/L), elevated nitrate (148.0 mg/L), elevated sulfate (847.0 mg/L)	No abnormalities detected	No abnormalities detected	2662	6.0	11.6	*Pseudomonas spp.* (99.9%), *Pseudomonas aeruginosa (99.9%)*, *Aeromonas sp. (99.9%)*, *Escherichia coli* (99.9%), *Pseudomonas putida* (92.8%), *Alcaligenes faecalis* (96.4%), *Acinetobacter sp.* (84.0%), *Citrobacter sp.* (92.9%), *Citrobacter amalonaticus* (91.2%), *Klebsiella oxytoca* (99.9%), *Sphingobacterium multivorum* (76.5%)
FC2	Elevated TDS (780.0 mg/L)	Elevated chloride (447.0 mg/L), elevated sulfate (278.0 mg/L)	No abnormalities detected	No abnormalities detected	2937	3.3	0.68	*Sphingobacterium multivorum* (84.1%), *Brevundimonas vesicularis* (75.9%), *Escherichia coli* (95.4%), *Klebsiella oxytoca* (99.9%), *Citrobacter sp.* (92.8%)
FC3	Elevated TDS (838.5 mg/L)	No abnormalities detected	No abnormalities detected	No abnormalities detected	3018	6.0	0.47	*Pseudomonas aeruginosa* (99.9%)
FC4	Elevated TDS (747.0 mg/L)	No abnormalities detected	No abnormalities detected	No abnormalities detected	7482	4.5	0.9	*Alcaligenes faecalis* (78.0%), *Stentrophomonas maltophilia* (99.9%), *Rhizobium* sp. (84.0%)
FC5	Elevated TDS (715.0 mg/L)	No abnormalities detected	No abnormalities detected	No abnormalities detected	3732	5.0	0.43	*Alcaligenes faecalis* (87.5%)/*Stentrophomonas maltophilia* (84.0%)
FC6	Elevated TDS (741.0 mg/L), low pH (6.02)	No abnormalities detected	No abnormalities detected	No abnormalities detected	3785	3.0	0.19	–
FC7	Elevated TDS (780.0 mg/L)	Elevated chloride (404.0 mg/L), elevated sulfate (549.0 mg/L)	No abnormalities detected	No abnormalities detected	4620	2.0	0.54	*Aeromonas sp.* (99.9%), *Serratia marcescens* (87.6%), *Rhizobium* sp. (89.1%), *Pseudomonas aeruginosa* (99.9%)
FC8	Elevated TDS (988.0 mg/L)	Elevated chloride (581.0 mg/L), elevated sulfate (251.0 mg/L)	Elevated strontium (5.0 mg/L)	No abnormalities detected	2949	2.6	1.4	*Bacillus cereus* group (90.0%), *Aeromonas sp.* (90.0%), *Aeromonas hydrophila* (95.0%)
FC9	Elevated TDS (773.5 mg/L)	Elevated chloride (431.0 mg/L)	No abnormalities detected	No abnormalities detected	4401	3.3	1.7	*Pseudomonas aeruginosa* (99.9%), *Pseudomonas stutzeri* (92.5%), *Alcaligenes faecalis* (78.0%)
FC10	Elevated TDS (760.5 mg/L)	No abnormalities detected	No abnormalities detected	No abnormalities detected	5171	8.0	1.3	*Pseudomonas sp.* (78.5%), *Pseudomonas stutzeri* (78.7%)
FC11	Elevated TDS (591.5 mg/L)	No abnormalities detected	No abnormalities detected	150 μg/L methanol, 20 μg/L ethanol, and 90 μg/L isopropyl alcohol detected, 2 unknown peaks detected	4195	3.4	1.0	*Aeromonas sobria* (76.0%), *Aeromonas sp.* (93.1%)
FC12	Elevated TDS (676.0 mg/L), low pH (6.12)	No abnormalities detected	No abnormalities detected	No abnormalities detected	2916	1.8	3.0	*Cupriavidus pauculus* (90.5%), *Bacillus cereus* (99.9%), *Pseudomonas* sp. (84.1%)
FC13	Elevated TDS (604.5 mg/L)	No abnormalities detected	No abnormalities detected	No abnormalities detected	5355	3.6	1.0	*Pseudomonas aeruginosa* (96.0%), *Bacillus cereus* (99.9%), *Citrobacter amalonaticus* (91.7%), *Rhizobium* sp. (83.5%)
FC14	Elevated TDS (637.0 mg/L)	No abnormalities detected	No abnormalities detected	No abnormalities detected	4462	3.3	0.8	*Pseudomonas aeruginosa* (93.4%), *Achromobacter xylosoxidans* (86.3%), *Bacillus cereus* (99.9%)
FC15	Elevated TDS (554.0 mg/L)	No abnormalities detected	No abnormalities detected	No abnormalities detected	3207	2.5	0.45	*Bacillus cereus* (83.5%)
FC16	Elevated TDS (565.5 mg/L)	Elevated chloride (475.0 mg/L), elevated sulfate (506.0 mg/L)	No abnormalities detected	No abnormalities detected	2529	4.2	0.6	*Bacillus cereus* (87.3%)
FC17	No abnormalities detected	No abnormalities detected	No abnormalities detected	No abnormalities detected	52,438	4.1	0.29	*Bacillus coagulans/megaterium* (82.6%), *Rhizobium* sp. (83.9%)
FC18	Elevated TDS (500.5 mg/L)	Elevated chloride (392.0 mg/L), elevated sulfate (335.0 mg/L)	No abnormalities detected	No abnormalities detected	3879	9.0	1.8	*Stenotrophomonas maltophilia* (76.0%), *Pseudomonas aeruginosa* (99.9%), *Pseudomonas stutzeri* (99.9%)
FC19	Elevated TDS (533.0 mg/L)	No abnormalities detected	No abnormalities detected	No abnormalities detected	2818	4.1	0.29	*Bacillus anthracis* (96.9%), *Bacillus subtilis* (99.9%), *Bacillus megaterium* (99.9%), *Brevundimonas vesicularis* (82.5%)

[a] Basic water quality measurements include temperature, dissolved oxygen, conductance, total dissolved solids, salinity, pH, and oxidation-reduction potential. Anions measured include bromide, chloride, fluoride, nitrate, sulfate, sulfide, bicarbonate, and carbonate. Metal ions analyzed include arsenic, barium, beryllium, cadmium, chromium, copper, iron, lead, manganese, mercury, nickel, selenium, silver, sodium, strontium, and zinc. VOCs/SVOCs selected for analysis are from pertinent list of compounds commonly used in hydraulic fracturing (Waxman et al., 2011). VOCs, volatile organic compounds; SVOCs, semi volatile organic compounds; TOC, total organic carbon; TN, total nitrogen.

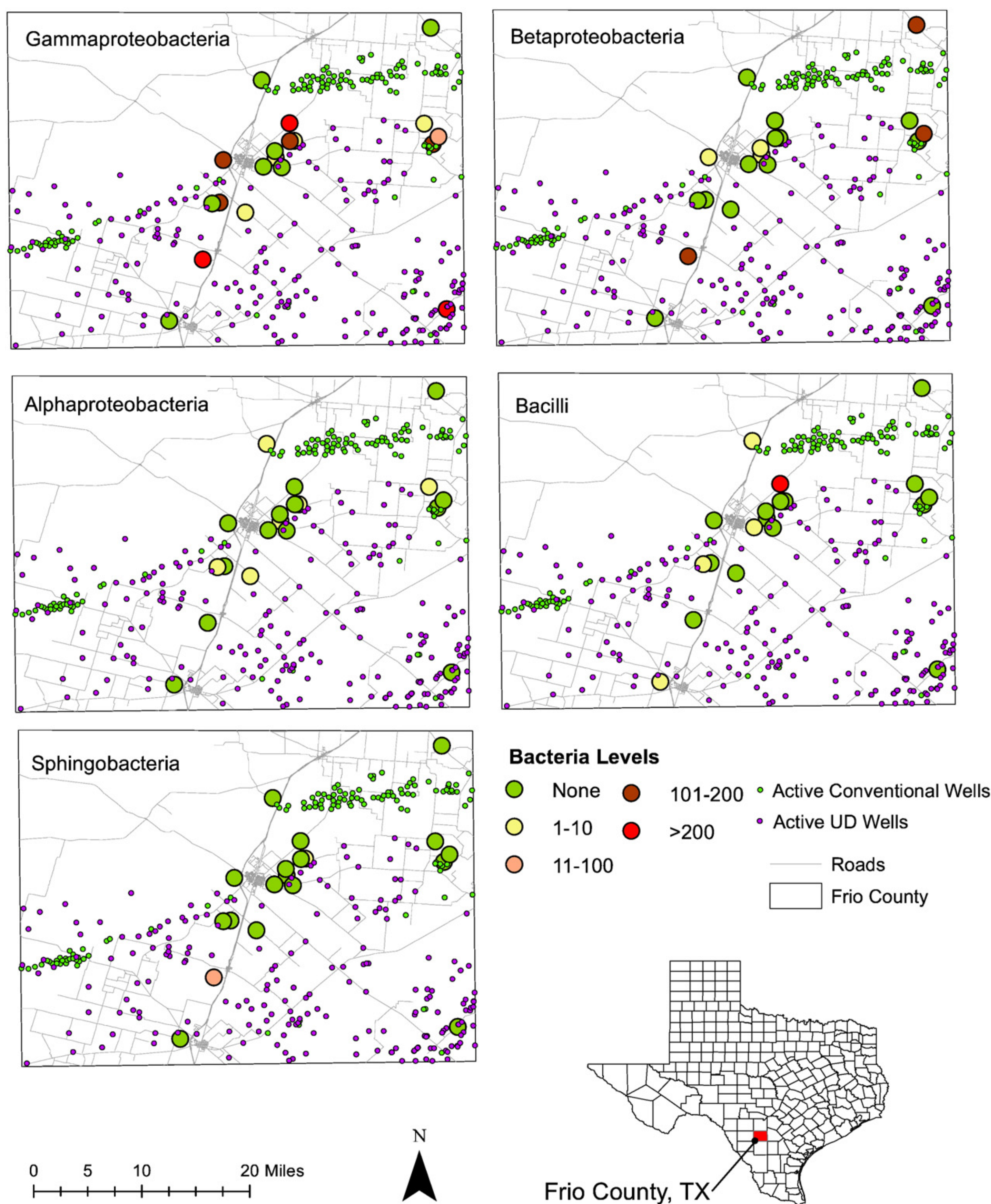
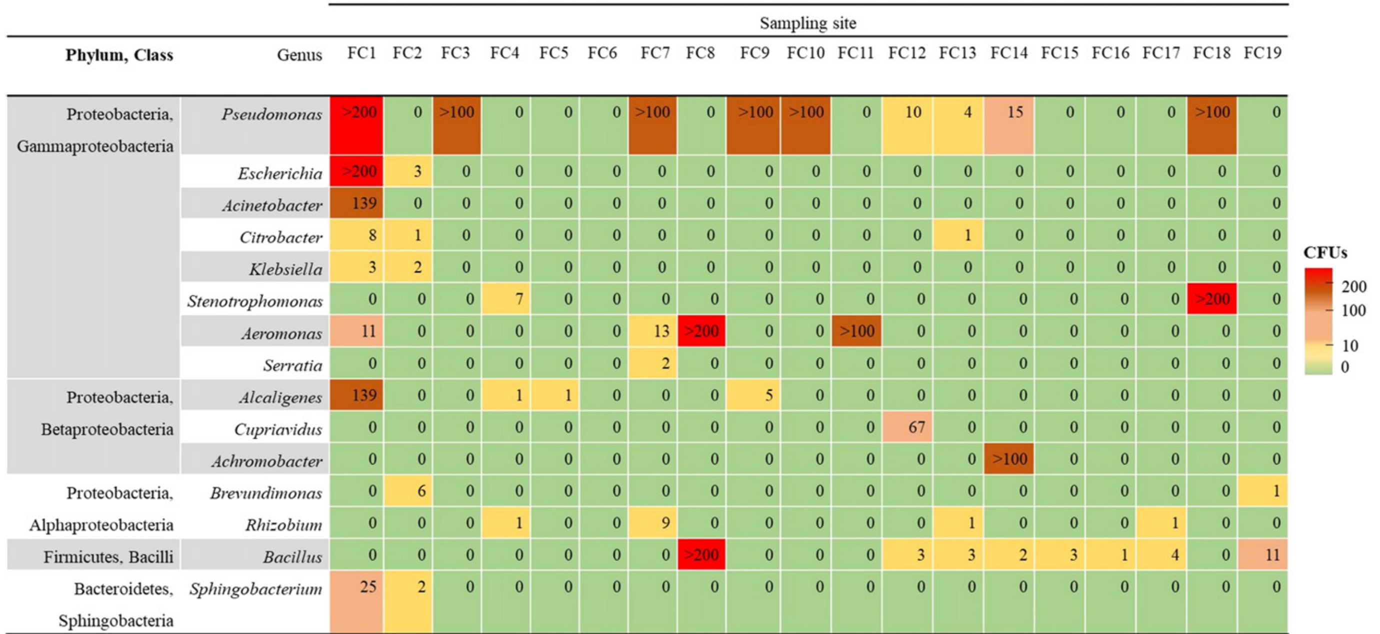


Fig. 1. Total colony forming units (CFU's) of the bacterial classes found in water samples taken from the Eagle Ford Shale region in Frio County, Texas. The locations of nearby active conventional production wells (green dots) and unconventional production wells (purple dots) are also shown.

bacteria that are widely distributed in the environment (Thomas et al., 2011). *Sphingobacterium multivorum* was the only microorganism isolated from this Phylum. It has an aerobic metabolism, obtains hydrogen or electrons from organic substrates (organotroph), and has the ability to reduce nitrogen to nitrogen gas.

No microorganisms were recovered from sample FC6. This does not mean that no water microbiome exists in this sample. Most likely, the appropriate media needs to be used to isolate the types of bacteria present in the water. Additionally, a range of environmental conditions such as nutrient starvation or toxic chemical concentrations, can cause growing bacteria to produce spores or reduce their metabolism to a dormant state. Furthermore, the exposure of microorganisms to environmental stresses may result in a decline in their cultivability making them viable but non-culturable (VBNC) microorganisms. In this case, though they are metabolically active, most cells are either injured or genuinely unculturable (Dworkin and Shah, 2010). Therefore, the



Phylum, Class	Genus	FC1	FC2	FC3	FC4	FC5	FC6	FC7	FC8	FC9	FC10	FC11	FC12	FC13	FC14	FC15	FC16	FC17	FC18	FC19
Proteobacteria, Gammaproteobacteria	*Pseudomonas*	>200	0	>100	0	0	0	>100	0	>100	>100	0	10	4	15	0	0	0	>100	0
	Escherichia	>200	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Acinetobacter	139	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Citrobacter	8	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	Klebsiella	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Stenotrophomonas	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	>200	0
	Aeromonas	11	0	0	0	0	0	13	>200	0	0	>100	0	0	0	0	0	0	0	0
	Serratia	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Proteobacteria, Betaproteobacteria	*Alcaligenes*	139	0	0	1	1	0	0	0	5	0	0	0	0	0	0	0	0	0	0
	Cupriavidus	0	0	0	0	0	0	0	0	0	0	0	67	0	0	0	0	0	0	0
	Achromobacter	0	0	0	0	0	0	0	0	0	0	0	0	0	>100	0	0	0	0	0
Proteobacteria, Alphaproteobacteria	*Brevundimonas*	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	Rhizobium	0	0	0	1	0	0	9	0	0	0	0	0	1	0	0	0	1	0	0
Firmicutes, Bacilli	*Bacillus*	0	0	0	0	0	0	0	>200	0	0	0	3	3	2	3	1	4	0	11
Bacteroidetes, Sphingobacteria	*Sphingobacterium*	25	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Fig. 2. Heat map illustrating the different bacterial classes in 19 groundwater samples from Frio County, TX, 2016. Numbers on heat map indicate CFUs.

recovery of VBNC microorganisms is harder as they do not grow under many laboratory conditions, leading to the results observed.

The presence of methanol in sample FC11 is interesting given that some microorganisms can perform aerobic methane-assimilation. In fact, *Aeromonas* sp., isolated from this water sample, is a methanotroph and therefore is able to oxidize methane to form methanol (Liao et al., 2016). However, the additional detections of ethanol and isopropyl alcohol in samples FC11 suggest that the methanol may also be attributed to an anthropogenic source, as these alcohols have been detected in contaminated groundwaters overlying the Barnett and Cline shale formations (Hildenbrand et al., 2017, 2015b) and are additives used extensively in UD (Waxman et al., 2011). This is further substantiated by the fact that the FC11 samples exhibited a cumulative natural gas signal (4195 arbitrary units) that was only slightly elevated above the sample set median (3785 arbitrary units), which is ultimately representative of the water with low levels of C1–C3 gases (<1.0 mg/L). If *Aeromonas* sp.

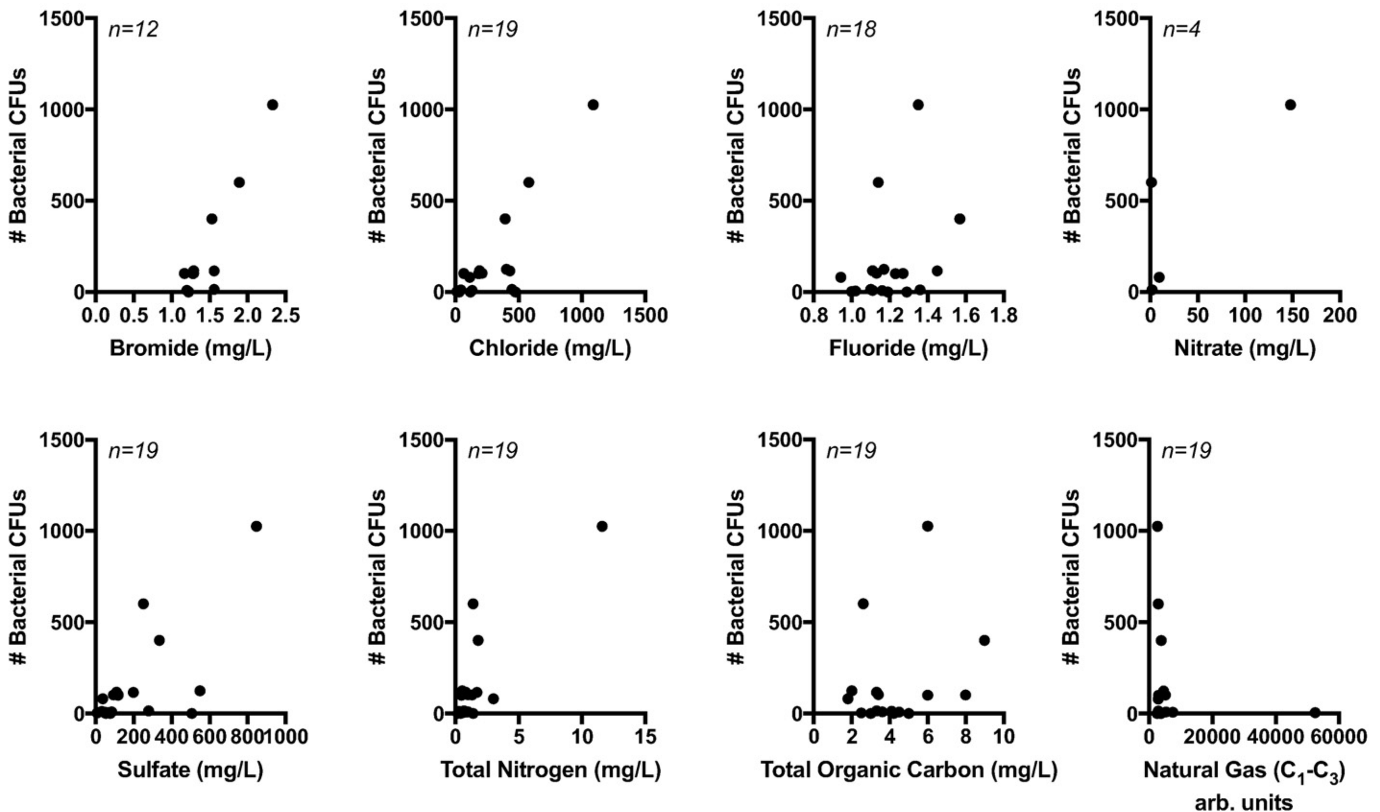


Fig. 3. Number of CFUs relative to concentrations of different chemical compounds for samples with detectable chemical values.

were to be the source of the methanol detected in the FC11 sample, one would likely detect a more substantial feed source of methane and a medium of significant complexity to facilitate this enzymatic conversion (Poole, 1998).

Figs. 3 and 4 show the relationship between bacterial abundance and bacterial diversity with respect to pertinent groundwater constituents. Higher bacterial concentrations and increased diversity were both observed for the water samples with higher values of total nitrogen, chloride, bromide, and sulfate. On the contrary, fluoride, natural gas constituents (as represented by the measurement of unretained natural gas, C1–C3) and total organic carbon (TOC) values had no apparent impact on bacterial abundance and diversity. This higher abundance may be explained by the presence of different elements used in different metabolic pathways such as nitrate, sulfate, and organic carbon compounds (Brochier-Armanet and Moreira, 2011). Previous studies have shown that the presence of high levels of nutrients is related with a higher bacterial abundance (Pinto et al., 2012). Furthermore, nitrate levels appeared to influence diversity within the microbiomes (Fig. 4), while having no relationship with total bacterial concentrations (Fig.Many different3). However, total nitrogen levels showed strong relationships in terms of both diversity and abundance, suggesting that nitrogen-bearing species other than nitrate (i.e., nitrite and/or ammonium) are the primary drivers of bacterial abundance within the groundwater microbiome. Though statistically significant correlations were found with several constituents for both bacterial concentrations and diversity, these trends also appear to be driven by a single data point. However, this data point cannot be excluded from our analyses as it provides valuable insight into the relationships between prominent groundwater constituents and bacterial abundance and diversity.

Overall, most of the bacteria isolated from the collected groundwater samples were heterotrophic meaning they are able to degrade organic compounds such as hydrocarbons as a source of carbon. Furthermore, some isolates also show the ability to tolerate high levels of metals. These are important characteristics since only these types of adaptable bacteria are able to survive and proliferate.

It is also important to note that a majority of the detected bacteria are considered opportunistic pathogens (Rusin et al., 1997), meaning that they can cause severe infections in people with a compromised immune system or on antibiotic treatment. *P. aeruginosa* and *S. maltophilia* are of particular concern as they are considered environmental global multiple-drug-resistant organisms (MDRO) (Brooke, 2012). Some authors suggest that *P. aeruginosa* should be absent in 100 mL of finished drinking water (Mena and Gerba, 2009); however, according to Bartram et al. (Exner et al., 2003), *P. aeruginosa* as an infectious dose of $10^{8}-10^{9}$ cells and *S. maltophilia* of 10^{6}–10^{9} cells. Nevertheless, this also means that the infectious dose is even lower for immunocompromised people.

3.2. 16S rRNA gene sequence analysis

In a previous work, the bacteria isolated from groundwater and identified by MALDI-TOF MS were sequenced using 16S rRNA gene sequence to confirm the identification (Martin et al., 2017). Nevertheless, in the present work, different microorganisms were isolated and their MALDI-TOF MS identification was confirmed by performing 16S rRNA gene sequence analysis. The comparison is shown in Table 2.

All the identifications made by MALDI-TOF MS were confirmed by the 16S rRNA gene sequence analysis, which shows the accuracy of the results. However, it is possible to see that the 16S rRNA gene

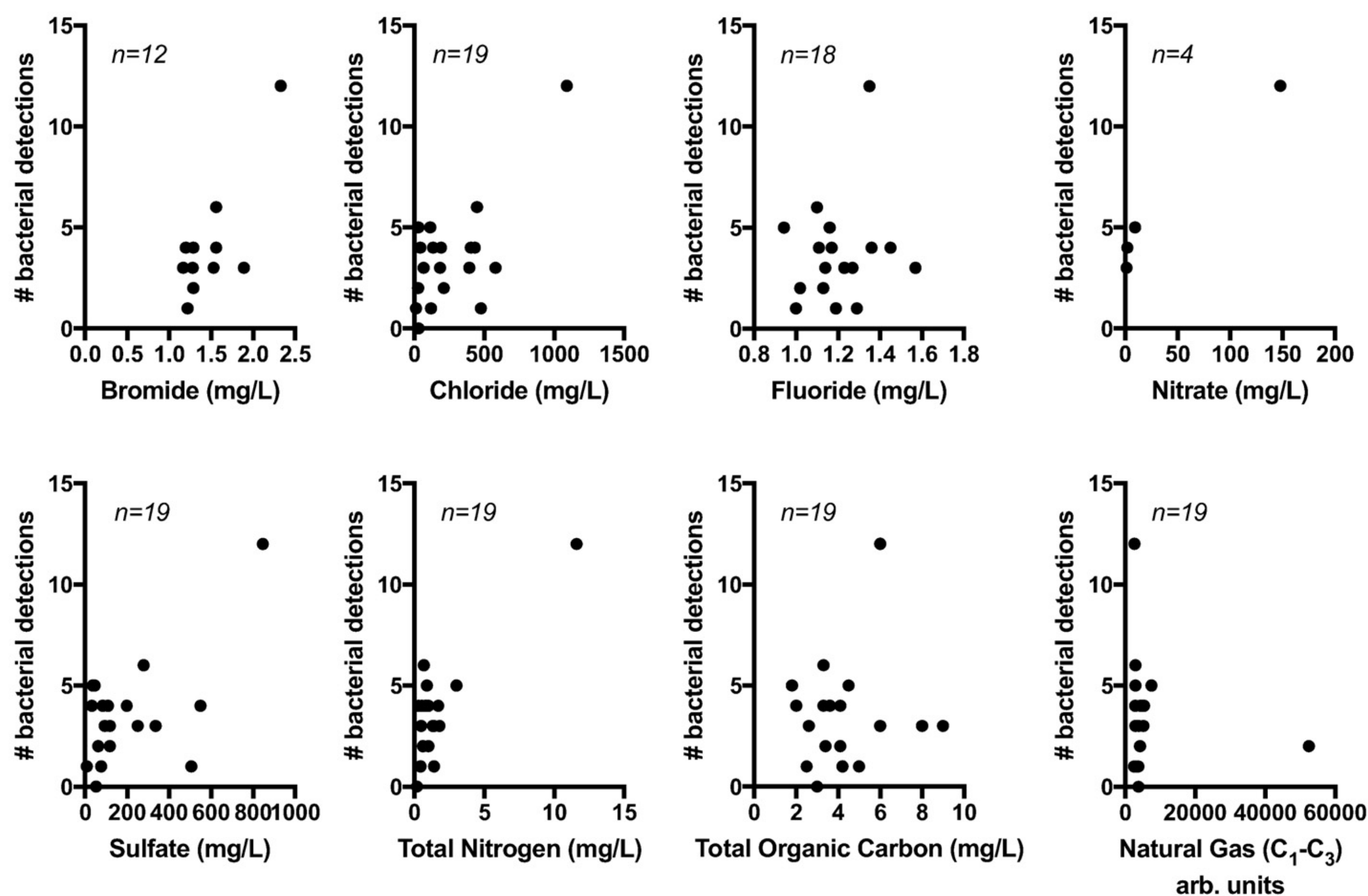


Fig. 4. Bacterial diversity relative to concentrations of different chemical compounds in water samples with detectable chemical values. The number of bacterial detection includes the microorganisms that were detected by MALDI-TOF MS (as unidentified) then characterized by 16S rRNA sequencing.

Table 2
Comparison of the MALDI-TOF MS identifications with the 16S rRNA gene sequence identifications.

MALDI-TOF MS identifications	Match score	16S rRNA gene sequence identifications
Klebsiella oxytoca	99.90%	*Citrobacter sp./Klebsiella oxytoca/Enterobacter sp.*
Sphingobacterium multivorum	84.10%	*Sphingobacterium sp.*
Serratia marcescens	87.60%	*Serratia marcescens*
Citrobacter amalonaticus	91.50%	*Citrobacter sp./Klebsiella oxytoca/Enterobacter sp.*
Brevundimonas vesicularis	82.50%	*Brevundimonas vesicularis*
Bacillus anthracis	96.90%	*Bacillus cereus group*
Cupriavidus pauculus	90.50%	*Cupriavidus sp./Ralstonia sp.*
Alcaligenes faecalis	99.90%	*Alcaligenes faecalis*

sequence results for *Klebsiella* sp. and *Citrobacter* sp. are not conclusive since different identifications with a 99% match were obtained for the same microorganism. The genus *Klebsiella*, *Citrobacte*r, and *Enterobacter* are all members of the same Family and therefore genetically very similar (Sanderson, 1971).

B. anthracis was identified as *B. cereus* group. As shown in a previous work (Martin et al., 2017), this microorganism genotypically and phenotypically resembles both *B. cereus and B. thuringiensis*, and therefore, their differentiation is very difficult. However, in the future we expect to develop a method to differentiate closely-related microorganisms and overcome the uncertainty in the identification of these microorganisms. Some of the isolated bacteria provided no identification using the MALDI-TOF MS – SARAMIS library and therefore were sequenced to obtain an identification (Table 3). It is worth nothing that the SARAMIS library is currently tailored toward microorganisms commonly found in a clinical setting. As such, the protein spectra of these previously unidentified organisms will be added to expand the functionality of the SARAMIS library for subsequent environmental investigations.

Some of the bacteria identified with the RNA sequencing, *Aeromonas* sp. and *Bacillus* sp., had been previously identified using MALDI-TOF MS, which shows that these bacteria should have been identified with the latter technique. The unknown results may be explained by the sample preparation, which can have an impact on the identification of bacteria. The other unknowns identified are commonly found in the environment. For example, *Arthrobacter* sp. has been found to survive in stressful environments such as chemically contaminated sites and can also handle starvation (Mongodin et al., 2006). In the work by Spear et al. (Spear et al., 1988), *Achromobacter xylosoxidans* was found to cause community-acquired infection due to its presence in a untreated drinking water well source. *Brevundimonas* sp. are typical aquatic organisms and have been previously isolated from mineral water (Jayasekara et al., 1999) as *Microbacterium* sp. that has also been previously isolated from bottled mineral water (Falcone-Dias et al., 2015).

Table 3
Microorganisms detected by MALDI-TOF MS but characterized by 16S rRNA sequencing.

Sample #	RNA identification
FC2	*Brevundimonas spp.*
FC3	*Bacillus spp.*
FC4	*Arthrobacter spp.* *Microbacterium spp.*
FC9	*Aeromonas spp.*
FC10	*Bacillus sp.*
FC12	*Bacillus sp.* *Microbacterium spp.*
FC14	*Achromobacter spp.*

4. Conclusions

Each of the sampled water wells reported in this study revealed a unique microbiome. The bacterial dynamics are complex and not fully understood; however, coupling measurements of the groundwater microbiome with the analysis of organic and inorganic groundwater constituents enables us to make some powerful inferences. For example, *Pseudomonas* was the predominant and robust bacteria as it is adaptable to different environmental conditions. The presence of various forms of nitrogen promoted the presence of denitrifying bacteria. Certainly, the bacteria adapt to their environment and changes are observed when changes in the chemical composition occur. Very little evidence of groundwater contamination was found that could be attributed to conventional and/or unconventional oil and gas development, with the exception of FC11 and the three alcohols that were detected. While the sampling sites were all surrounded by UD activities, UD production wells do not contribute to groundwater contamination in a systematic fashion (Hildenbrand et al., 2017, 2015). In fact instances of well casing failure and/or out-of-zone stimulation (via hydraulic fracturing or 'fracking') have been found to be isolated (Darrah et al., 2014; Digiulio and Jackson, 2016; Ingraffea et al., 2014; Sherwood et al., 2016). As such, these data do not provide a definitive link between UD or agricultural activities and the groundwater microbiome; however, they do provide a baseline measurement of bacterial diversity and quantity in groundwater located near these anthropogenic activities. In the future, studies will be performed to systematically examine how the frequency, intensity, or the size of chemical disturbances affect bacterial diversity, to better understand the relationship between anthropogenic activities and bacterial communities. These proposed time-series analyses would also account for seasonal variations as these can also have an impact on the structure of the groundwater microbiome.

The majority of the bacteria isolated from water wells in this study are heterotrophic bacteria meaning they are able to degrade organic compounds such as hydrocarbons. The presence of natural gas constituents (C1–C3) suggests that the detected heterotrophic bacteria are amenable to organic contaminants and may be suitable for use in bioremediation. While the data presented here provide a snap shot of the interplay between organic and inorganic groundwater constituents, and the resulting microbiome, additional time series measurements would further elucidate the extent to which the concentrations of the target analytes have been affected by the detected bacterial species. As such, a study of the degradation or accumulation of metals and organic compounds should be performed to fully understand how these compounds are assimilated and into what products they are converted. In this line of thinking, more work needs to be performed to better understand the impacts of different stress conditions (e.g. high-level of metals and hydrocarbons) in the survival and adaptation of different bacteria. Furthermore, as these stressful conditions can damage microorganisms (VBNC) or cause them transition into a dormant state, improvements should be made to recover these microorganisms from water. Approaches such as using the sample as culture media, and testing different culture media and conditions should be explored to further understand the ecology within the groundwater microbiome.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2017.10.264.

Acknowledgements

This research was partially supported by donations from concerned mineral rights owners to the Collaborative Laboratories for Environmental Analysis and Remediation at the University of Texas at Arlington. These efforts were supported by charitable donations by the Earth Day Texas organization. The project described was also supported by the National Center for Advancing Translational Sciences, National Institutes of Health, through the Institute for Integration of Medicine & Science/Clinical and Translational Science Award (IIMS/CTSA) UL1 TR001120. The

content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Conflicts of interest

The authors declare no conflict of interest.

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