Groundwater Research Report
WR16R001

ANTHROPOGENICALLY DRIVEN CHANGES TO SHALLOW GROUNDWATER IN SOUTHEASTERN WISCONSIN AND ITS EFFECTS ON THE AQUIFER MICROBIAL COMMUNITIES

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Introduction

Placing riverbank inducement (RBI) wells next to riverbanks has been a common practice in Europe since the 1870's and in the United States for the last 60 years. The RBI practice allows for the purification of water entering a pumping well by inducing flow from the riverbed (Ray et al., 2002; Ray, 2008). This method is proposed as a promising and sustainable technology for municipal and public water production across the globe (Ray, 2008; Shamrukh & Abdel-Wahab, 2008). It is often assumed that the RBI practice will remove pollutants before water enters municipal wells, however it has been shown that river filtration often does not produce water that conforms to drinking water standards (Ray et al., 2002; Singh, et al., 2010). In particular, effluent-dominated streams that have strong hydrologic connections between surface water and shallow groundwater can transport contaminants into the shallow groundwater (Bradley et al., 2014; Ray, 2008; Weiss et al., 2005).

Many communities in eastern Wisconsin, including the City of Waukesha, rely on a deep sandstone aquifer for their drinking water. The ever-increasing demand for water has critically depleted this aquifer. The deep aquifer also contains radium concentrations that exceed federal regulations. To counter depletion and excessive radium levels, Waukesha uses three shallow wells that draw water from a shallow unconfined aquifer that contains no radium. The water from these wells is then combined with water from the deep sandstone wells, thereby diluting radium to levels that meet regulation. Three of these shallow wells are located near the Fox River and induce flow from the river and as such are RBI wells. The Fox River receives wastewater treatment plant (WWTP) effluent upstream of the wells; therefore, the RBI wells pump river water containing WWTP effluent. Two RBI wells are located within 200 feet of the river one of which (Well 12) pumps a mixture of groundwater and river water that contains effluent from

three upstream WWTPs. The second RBI well (Well 11) does not pump large amounts of water and will not be discussed further. A third well (Well 13) is located roughly 1500 feet from the river and pumps pristine groundwater. The Wisconsin Department of Natural Resources (WDNR) unique well numbers for the RBI wells are RL255 and RL256, and the unique well number for the pristine well is WK947; the common names for the wells are Well 11, Well 12, and Well 13, respectively.

At the location of Well 12, the Fox River contains large amounts of WWTP effluent under low flow conditions. The purpose of the current project is to investigate if, and to what extent, the microbial community present in the shallow well field is affected by this influx of river water containing treated municipal wastewater effluent. The study merges geochemical and microbial analyses to obtain a more complete picture of the impacted aquifer. Changes in microbial community composition, activity, and genetic functional potential between pristine and impacted groundwater sites were characterized to better understand the impact of anthropogenic activities on native microbial communities. The specific objectives are to:

1. Define differences in the microbial communities and the functional reactivity between pristine and contaminated portions of the shallow sand and gravel aquifer.
    i. Collect and analyze groundwater samples from a shallow sand and gravel aquifer for microbial community composition.
    ii. Calculate free energy yields to determine energetically favorable reactions.
    iii. Assess differences in the microbial communities and geochemical reactivities of the pristine and contaminated locations.

Background

Microorganisms in Groundwater

Subsurface microorganisms utilize, as well as generate, biogeochemical gradients. These microbial metabolism schemes affect changes to important biogeochemical species, directly impacting water quality. Through genome resolution, specific microbial community members have been identified and associated with specific transformations in nutrient and biogeochemical cycles of carbon, nitrogen, and sulfur cycles in the terrestrial subsurface (Brown et al., 2015; Long et al., 2016).

Microbial community composition data indicates which microorganisms are present and in what relative abundances. This data essentially indicates "who" is there, "who" may be contributing to the system, and how environmental conditions impact community structure. Communities become specialized for specific environments under specific environmental conditions, especially in anoxic environments (Vigneron et al., 2018). If nutrient conditions are altered, then the community composition and function of the ecosystem is likely to be altered as well. Community composition can be determined using 16S ribosomal RNA (rRNA) gene sequencing. The16S rRNA gene contains both variable and conserved regions making it a good molecular marker for identifying microorganisms. The hypervariable v4 region was targeted in this study in order to study both *Archaea* and *Bacteria* (Parada, 2016; Walters et al., 2016).

Previously unknown microorganisms discovered from groundwater have recently expanded the tree of life (Hug, et al., 2016). Most of these unknown organisms were discovered from the anoxic subsurface by using rRNA gene sequencing and shotgun metagenomic genome sequencing. These microorganisms include Archaea: *Diapherotrites*, *Parvarchaeota*, *Aenigmarchaeota*, *Nanoarchaeota* and *Nanohaloarchaeota*, known as the DPANN superphylum

and bacteria from a novel group of lineages known as Candidate Phyla Radiation (CPR) (Castelle et al., 2015; Eme & Ford Doolittle, 2015; Hug, et al., 2016; Liu, et al., 2018; Rinke, et al., 2013). Candidate phyla that lack isolated representatives are expected to contribute to, and moderate, nutrient cycling. It has been noted that the rRNA genes of many CPR organisms contain self-splicing introns. This is a rarely recorded bacterial characteristic, and it means these organisms are not detected with standard methods for sequencing 16S rRNA genes (DNA) (Brown et al., 2015). For this reason, we also examined the composition of microbial ribosomal RNA (RNA) in each sample. rRNA, unlike the rRNA gene does not contain introns, and thus it is detectable via common sequence analysis methods. In addition, 16S ribosomal RNA:DNA ratios can also be used to estimate the protein synthesis potential (PSP) of microbes among samples (Denef, et al., 2016).

Many of these novel microbes have ultra-small cell sizes and show limited metabolic capabilities suggesting that many groundwater organisms are likely performing "metabolic handoffs" where single organisms do not contain sufficient cellular machinery to carry out multiple sequential redox transformations (Anantharaman et al., 2016; Castelle et al., 2015; Long et al., 2016). Since resource input is limited and because of the apparent syntrophic nature of these organisms, any resource change could shift the community composition and resultant biogeochemical cycling of chemical resources (Hemme et al., 2015).

Previous Research

Fox River Studies

For over a decade, a monitoring network has been used to investigate the occurrence of Fox River water entering Well 12. This work included geochemical analysis and modeling, as well as trace element and stable isotope analysis, which was used to discriminate among contamination sources in the well (Thorp, 2013; Fields-Sommers 2015). The Fox River water entering Well 12 is enriched in sodium chloride. Differing anthropogenic sources of salt, in particular road salt (derived from seawater) and WWTP effluent can be distinguished through the boron/chloride ratio. WWTP effluent has a higher proportion of boron due to the prevalence of borate-based laundry brighteners in effluent (Vengosh et al., 1991; Thorp, 2013). The red line in Figure 1 shows the mixing line between pristine Well 13 and WWTP effluent. The Fox River lies midway between effluent and pristine groundwater. The yellow square is an average of WWTP samples (n=50), the green circle is an average of Fox River water (n=18), and the orange circle is pristine well water (W13). Brown squares represent RBI Well 12. Well 12 plots along the effluent mixing line, indicating that the sodium and chloride salt in the water is dominated by WWTP effluent. The blue line represents a mixing line between pristine well water and seawater. Thorp (2013) and Field Sommers (2015) both found that sodium and chloride ion concentrations increased over time in Well 12 as Fox River water began entering the well (Figure 2). Feinstein et al. (2010) produced a numerical model that predicted this rise with continued pumpage,

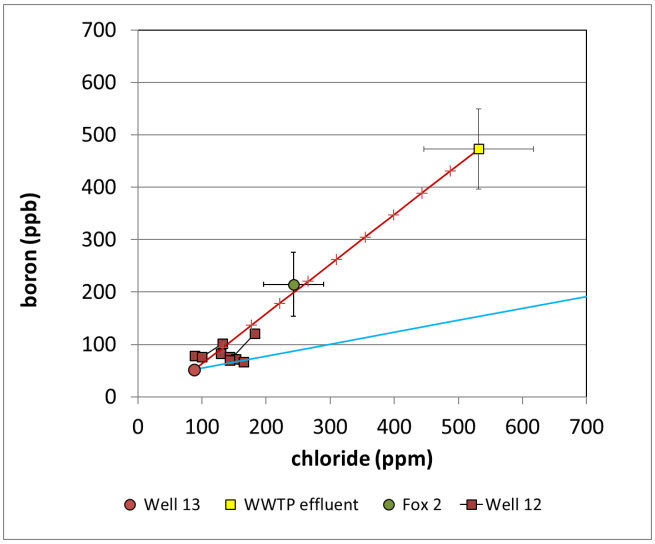


Figure 1. RBI wells pumping a mix of pristine groundwater and WWTP effluent (Thorp, 2013, Fields-Sommers, 2015). Mixing line between seawater and Well 13 is shown in blue. Mixing line between Well 13 and WWTP effluent is shown in red.

because more water is induced to flow toward the RBI well. Unlike the RBI well, the major ion chemistry of the pristine Well 13 remained constant over time regardless of the amount of pumpage (Figure 2). The major ion concentrations in Well 13 corroborate modeling results that indicate it has no hydrologic connection to the Fox River and pumps only pristine groundwater (Feinstein et al., 2010).

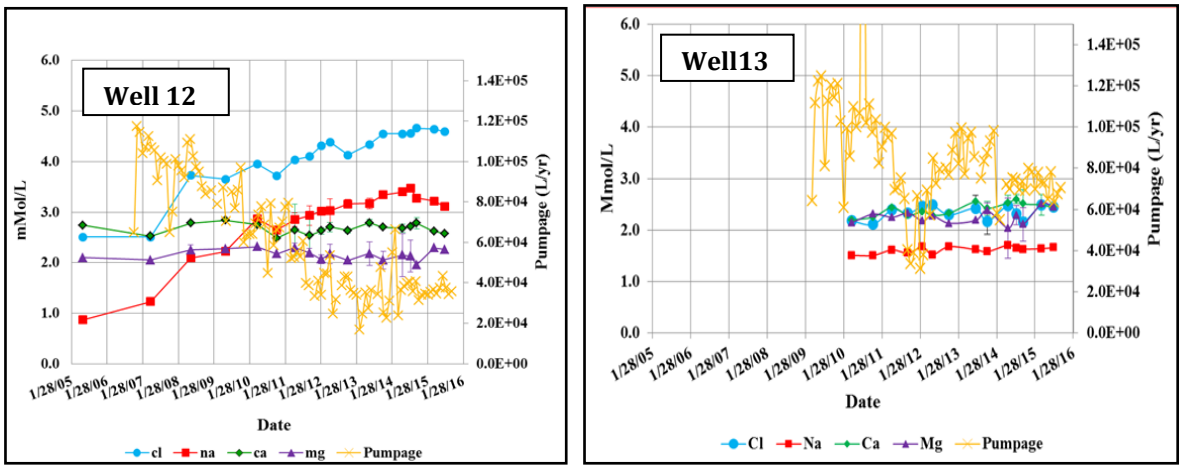


Figure 2. Major ion chemistry and pumpage trends in the Waukesha Wells 12 and 13 from 2005 through 2015 (Fields-Sommers, 2015).

Microbial Studies

As sequencing technologies have improved, the number of extensive microbial community and metagenomic datasets has grown rapidly, including several representing subsurface environments. For example, Smith et al. (2012) examined the extent of variation between the composition and function of microbial communities in two aquifer systems. Lin et al. (2012) and Luef et al. (2015) used metagenomics to estimate the functional potential of microbes in groundwater. Amend and Shock (2012) formulated 370 possible reactions that are related to microbial metabolism in subsurface environments. Lisle (2014) examined the 370 possible reactions laid out by Amend and Shock (2012) and identified five energetically favorable reactions in the Floridian aquifer system. Similarly, Davidson, et al. (2011) calculated energetically favorable reaction in a South African aquifer system. In these studies the researchers identified the biogeochemical reactions most likely to proceed by calculating Gibbs free energy for each well examined. Then the free energy yields of redox reactions driven by microbial activity were applied to constrain the list of the possible biogeochemical reactions to those that were relevant to the study environment. A similar approach is used in this study, but here we attempt to link microbial community composition to the reactions and identify differences in the communities and reactants in a pristine versus well impacted by river water infiltration.

Methods

Monitoring Network

The City of Waukesha, WI operates a wellfield in the shallow sand and gravel aquifer in the southern portion of the City. An RBI well, (Well 12) is located 83 feet from the riverbank

(Fox River). A pristine well (Well 13) is located 1,500 feet from the riverbank. Well 12 pumps as much as 50% river water, which contains treated wastewater effluent from the three upstream WWTPs, whereas Well 13 pumps pristine groundwater. Both wells are screened in a shallow gravel layer at depths ranging from 60 to 150 feet. See Figure 3 for detailed locations. Water from both wells was analyzed for geochemical components (major ions, nutrients, dissolved gases and DOC), and for microbial community composition (16S rRNA gene) and community activity (16S rRNA).

Field Methods and Equipment

Nitrile gloves were used in sample preparation and during sampling. All sample bottles, syringes, and filter holders were washed in a hydrochloric acid bath for a minimum of 12 hours. Sampling equipment was single use and disposed of after use to prevent cross contamination. Tubing used at each well was not replaced, but was thoroughly cleaned while the wells were purged. All water samples were filtered through 0.2 μm regenerated cellulose filters in the field. Samples for cation analysis were preserved with trace metal grade nitric acid. Anion, nutrient (nitrate, nitrite, ammonium, and phosphate) and DOC samples were not acidified. Major ion samples were refrigerated until analysis, while nutrient and DOC samples were frozen.

Hydrogen gas was sampled following gas equilibration. Groundwater was pumped through a cell with headspace for 10 minutes after which a sample of headspace gas was withdrawn and sent to a commercial lab (Pace Analytical) for analysis. Dissolved oxygen, specific electrical conductivity, pH and temperature were measured using field probes. Chemetrics colorimetric kits were employed to verify oxygen readings. Parameters that are

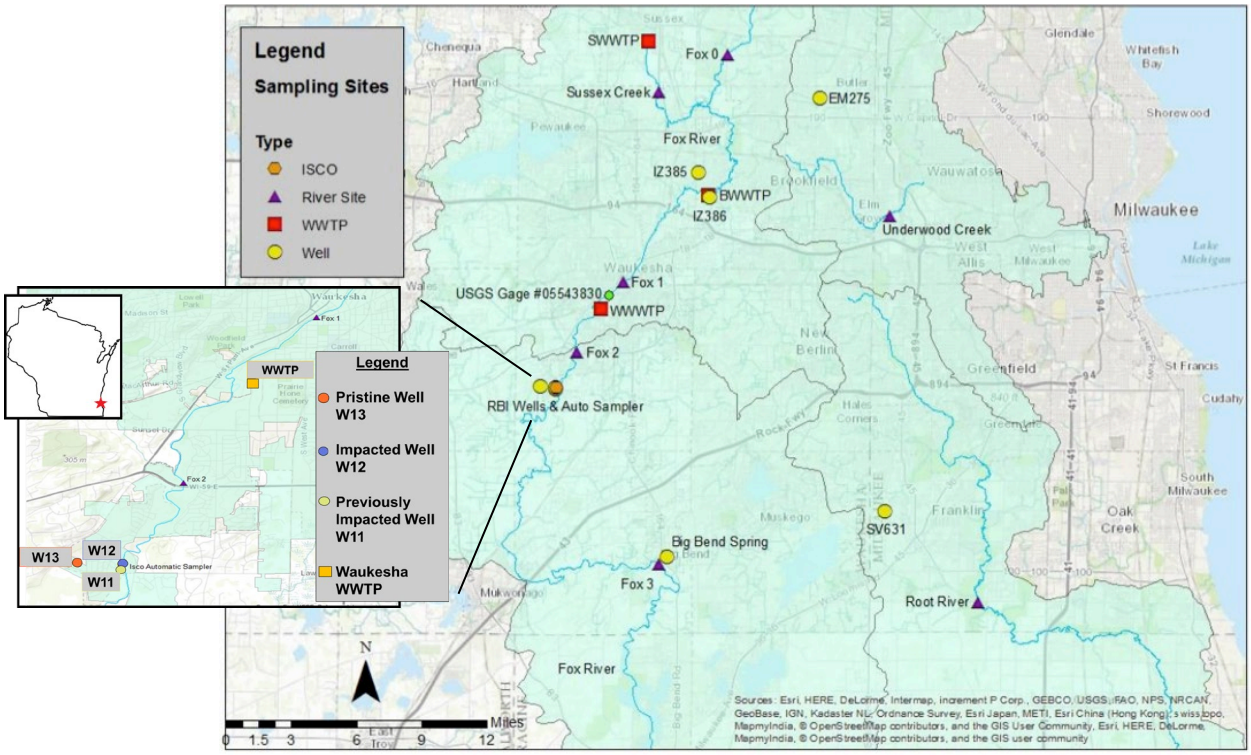


Figure 3. Map of the overall monitoring network, with light green indicating the watersheds of the sampling sites (Fields-Sommers, 2015). Inset map shows the locations of the three wells in the Waukesha wellfield.

subject to rapid change (alkalinity, ferrous iron) were measured in the field by titration, or CHEMetrics colorimetric kit respectively.

For each sampling event, the well line was flushed for 15 minutes, and then two to three liters of water (filtering stopped at 30 minutes) was filtered to collect cells for microbial molecular analyses. Filters were then flash frozen in liquid nitrogen to prevent degradation/alteration of the nucleic acids. All samples were filtered sequentially through 3 µm, 0.2 µm, and 0.1 µm polyether sulfone (PES) filters. The 3 µm filter was used as a pre-filter to remove larger particles.

Laboratory Methods

All geochemical analyses were conducted at the University of Wisconsin - Milwaukee School of Freshwater Sciences. Complete analytical results can be found in Salo (2019). Major anions, were analyzed using ion chromatography. Major cations, were analyzed using atomic absorption spectroscopy. Nitrite (NO_2^-) and ammonium (NH_4^+) were measured using the molybdenum blue method on an AutoAnalyzer. Total dissolved phosphorus was measured on a UV-VIS spectrophotometer using the molybdate method after photo-oxidative conversion of dissolved organic phosphorus compounds into orthophosphate. DOC was analyzed via high temperature combustion after removal of inorganic carbon by conversion to dissolved carbon dioxide and purging.

Simultaneous DNA and RNA extraction was performed with Qiagen's AllPrep Powerviral DNA/RNA kit. Promega's RQ1 RNase-Free DNase (Cat #M6101) and GoScript™ Reverse Transcription System were used to treat RNA samples and reverse transcribe RNA to complementary DNA (cDNA). The reverse primer 806Rb for the v4 16S rRNA gene region was used in the cDNA synthesis (Apprill et al., 2015). Polymerase Chain Reaction (PCR) was used to target and amplify the V4 16S rRNA gene region in the DNA and cDNA samples using 515Fb (Parada et al., 2016) and 806Rb primers with Invitrogen's™ Platinum™ Taq DNA Polymerase. Samples were run in triplicate PCR reactions and the products were pooled before further DNA purification with the Agencourt AMPure XP magnetic bead kit. After purification samples were sent to the Great Lakes Genomics Center (GLGC) for library preparation and sequencing on the illumina MiSeq using 2 x 250 bp chemistry

Sequence Data Processing

16S rRNA gene sequence data was processed using Mothur (Schloss et al., 2009), and DADA2 (Callahan et al., 2016). Low quality sequences, according to illumina standards, were filtered out. illumina primers were removed utilizing cutadapt (Martin, 2011). DADA2 was used to merge reads, denoise sequence reads, and remove chimeras to create an amplicon sequence variant (ASV) table. Mothur was used to remove primers from merged reads that were binned incorrectly as Forward and Reverse, and these were added to the existing ASV count table. Mothur was also used to remove sequences that were 5% shorter or longer than the median length of all sequences, which was 253 bp. All ASVs were taxonomically classified using SILVA v132. ASVs were removed from the final dataset if they: 1) were a member of the Mock community, 2) had a lower mean count in samples than in the negative control, or 3) were classified as mitochondria, chloroplast, or Eukaryote.

We analyzed and visualized the data in R (R Development Core Team, 2016). After performing sequence data processing and rigorous quality control, the sequence dataset (RNA and DNA, 0.2 μm and 0.1 μm fractions, from W12, W13, and the Fox River sites) included 46,887 unique amplicon sequence variants (ASVs). We then removed very low abundance ASVs, those occurring at an abundance of <0.01% summed across all samples. The threshold of 0.01% was chosen to be stringent enough to remove cross contamination sequences (i.e. between samples during PCR and sequence library; as observed from Mock community sequence cross-contamination), but to also allow for rare community members to be included. This dataset was used in all sample and statistical comparisons. It consisted of 23,100 ASVs.

Thermodynamic Calculations

Seven groundwater samples were collected from both groundwater wells over a 14-month period spanning from November 2016 through January 2018 and analyzed for common groundwater constituents for biogeochemical analyses. The constituents were averaged between the seven samples to create a composite sample representative of the groundwater in each well (Table 1). Sulfide and ferrous iron were below detection limits in both wells. For thermodynamic calculations, both ferrous iron and sulfide concentrations were set to the limit of detection. In order to assess the potential metabolic pathways being carried out by the microbial consortia, free energy calculations were performed with 23 representative biogeochemical reactions (Table 2). The reactions are all driven by microbial metabolisms in groundwater systems (Davidson et al., 2011; Lisle, 2014).

The equilibrium constants (K_{eq}) for all 23 reactions were calculated using Amend and Shock (2011) values of standard free energies of formation ($\Delta G°_f$). The activity of each constituent was calculated using PHREEQC version 3.1.7.9213 (Parkhurst and Appelo, 2005) using the Wateqf.dat database (Ball and Nordstrom, 1991). Free energy values under in-situ conditions (ΔG_r) were calculated using the $\Delta G°_r$ values for each reaction, groundwater temperature, and activities of the reactants and products. Free energy flux (FEF, kJ $cell^{-1}$ $second^{-1}$) is the amount of energy a microbial cell can potentially generate from performing each reaction assuming that the reaction proceeds until one reactant (the limiting reactant) is fully consumed. This represents the total FEF available for microbial use and is defined by:

$$FEF = 4\pi * r * D_c * C * \Delta G_r \quad (1)$$

Table 1. Composite water quality data for two shallow groundwater wells from November 2016 through January 2018 with the respective standard deviations. *Ferrous iron and sulfide values are set to the LOD.

Parameter	Units	RBI	Pristine
		Well 12	Well 13
Temperature	°C	10.6 ± 0.2	10.5 ± 0.1
pH		7.0 ± 0.2	7.1 ± 0.6
Calcium	mg/L	90.5 ± 20	83.7 ± 10
Chloride	mg/L	201.3 ± 60	97.2 ± 30
Magnesium	mg/L	53.3 ± 2	56.7 ± 4
Potassium	mg/L	3.2 ± 0.5	2.6 ± 0.5
Sodium	mg/L	81.1 ± 3	39.8 ± 2
Dissolved oxygen	mg/L	0.15 ± 0.2	0.14 ± 0.2
Ferrous Iron*	mg/L	0.1	0.1
Ammonium	mg/L	0.07±0.01	0.03±0.01
Nitrate	mg/L	0.3 ± 0.7	1.7 ± 1.1
Nitrite	mg/L	0.003	0.04
Sulfate	mg/L	68.2 ± 10	96.9 ± 10
Sulfide*	mg/L	0.1	0.1
Total dissolved phosphorus	mg/L	nd	nd
Dissolved organic carbon	mg/L	0.93 ± 0.3	0.65 ± 0.4
Bicarbonate	mg/L	462.3 ± 100	411.3 ± 100
Hydrogen	µmol/L	0.005	0.004
Methane	µmol/L	0.45±0.14	0.04±.02

where r (meters cell^{-1}) is the radius of the microbial cell, D_c (m^2 sec^{-1}) is the diffusion coefficient of the limiting reactant, C (moles m^{-3}) is the concentration of the limiting reactant, and ΔG_r (kJ $mole^{-1}$) is the free energy of reaction under in-situ conditions for each reaction.

Free energy calculations were related to the 23 reactions based on the relationship of free energy yields for the production of adenosine triphosphate (ATP) (Schink, 1997; Thauer et al., 1977). The following assumptions were made when relating free energy to ATP production or microbial activity;

1) conservation of energy occurs during the electron transport process for all reactions

2) the conversion of energy to ATP proceeds with maximum efficiency, creating a minimum free energy yield needed for ATP production, which was set at –20 kJ mol^{-1} of limiting reactant for ΔG_r. Only reactions whose ΔG_r were less than –20 kJ mol^{-1} were considered to be energetically favorable.

3) the maximum rate that energy could be gained is dependent on diffusion rates (Onstott, 2005), and subsurface microorganisms are immobile.

4) Acetate was used as a surrogate for DOC on a carbon-for-carbon basis. This allowed for the use of a single $\Delta G°_f$ for the electron donor term in heterotrophic reactions.

Results & Discussion

Geochemical Analyses

Chloride concentrations are much higher in Well 12 (RBI) compared to Well 13 (pristine). Both wells are near calcite saturation (SI = -0.10 ± 0.02). The geochemical data in Table 1 were used to determine which of the biogeochemical reactions were applicable to the groundwater in this study. Reactions that are not energetically favorable are shown in italics. Reactions with N_e gas was a product (N_e gas was not measured) are also shown in italics. Sulfide and ferrous iron were not detected in either well; therefore, the detection limits for the Chemet kits were used in thermodynamic analyses. The resultant ΔG_r and FEF values are maximum estimates for reactions involving sulfide and ferrous iron (Table 2). The ΔG_r of all reactions are normalized to 8 moles of electrons transferred per reaction. Of the 23 biogeochemical reactions listed, 17 reactions were determined to be thermodynamically feasible in the shallow groundwater wells using the minimum free energy yield of –20 kJ mol^{-1}. FEF values for the energetically favorable reactions range from -6.2×10^{-18} to -1.0×10^{-14} kJ $cell^{-1}$ s^{-1}.

Groundwater Well Differentiation

The 17 favorable biogeochemical reactions were compared between the two shallow groundwater wells to see if the influx of WWTP effluent altered the geochemical processing being performed by the microbial community in the well field. Figure 4 depicts the relative FEF

Table 2: The free energy of reaction and free energy flux for a set of biogeochemical reactions.

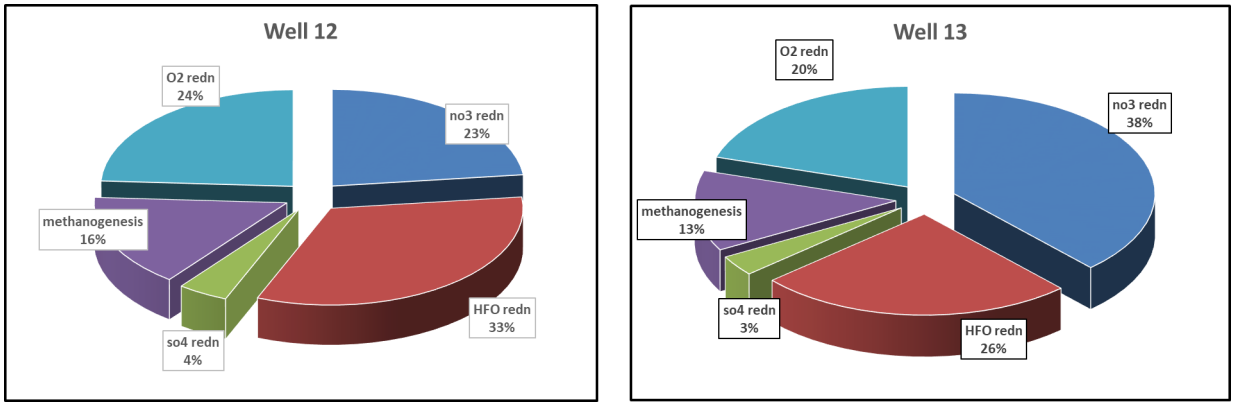
Redox Reaction	RBI Well 12		Pristine Well 13	
	ΔG_r[1]	FEF[2]	ΔG_r	FEF
$CH_4 + SO_4^{2-} \rightarrow H_2O + HCO_3^- + HS^-$	*-5*	*n.a.*[3]	*-1*	*n.a.*
2. Acetate + NO_3^- + $H_2O \rightarrow 2HCO_3^- + NH_3$	-485	-1.3×10^{-14}	-492	-3.9×10^{-15}
3. *$4H_2 + 1.6NO_3^- + 1.6H^+ \rightarrow 0.8N_2 + 4.8H_2O$*	-	-	-	-
4. *Acetate + $1.6NO_3^- + 0.6H^+ \rightarrow 2HCO_3^- + 0.8H_2O + 0.8N_2$*	-	-	-	-
5. $4H_2 + NO_3^- + H^+ \rightarrow NH_3 + 3H_2O$	-396	-6.23×10^{-18}	-400	-5.1×10^{-18}
6. Acetate + $SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$	-58	-1.1×10^{-15}	-59	-1.1×10^{-15}
7. $4H_2 + H^+ + SO_4^{2-} \rightarrow HS^- + 4H_2O$	*21*	*n.a.*	*23*	*n.a.*
8. 4Acetate + $4H_2O \rightarrow 4CH_4 + 4HCO_3^-$	-212	-5.6×10^{-16}	-231	-6.3×10^{-17}
9. *$4H_2 + H^+ + HCO_3^- \rightarrow CH_4 + 3H_2O$*	*26*	*n.a.*	*24*	*n.a.*
10. *$4H_2 + H^+ + 2HCO_3^- \rightarrow$ Acetate + $4H_20$*	*79*	*n.a.*	*81*	*n.a.*
11. Acetate + $8Fe(OH)_3 + 15H^+ \rightarrow 8Fe_2^+ + 20H_2O + 2HCO_3^-$	-421	-1.8×10^{-15}	-414	-1.8×10^{-15}
12. $HS^- + 8Fe(OH)_3 + 15H^+ \rightarrow SO_4^{2-} + 8Fe^{2+} + 20H_2O$	-363	-1.5×10^{-15}	-356	-1.6×10^{-15}
13. $4H_2 + 2O_2 \rightarrow 4H_2O$	-737	-1.2×10^{-17}	-735	-9.3×10^{-18}
14. Acetate + $2O_2 \rightarrow 2HCO_3^- + H^+$	-816	-2.4×10^{-14}	-816	-2.3×10^{-14}
15. $CH_4 + 2O_2 \rightarrow HCO_3^- + H^+ + H_2O$	-763	-2.0×10^{-15}	-758	-2.1×10^{-16}
16. $HS^- + 2O_2 \rightarrow SO_4^{2-} + H^+$	-758	-2.2×10^{-14}	-758	-2.1×10^{-14}

17. $(^4/_3)NH_3 + 2O_2 \rightarrow (^4/_3)NO_2^- + (^4/_3)H^+ + (^4/_3)H_2O$	-373	-4.4 x 10^{-16}	-361	-2.9 x 10^{-15}
18. $H_2S + 4NO_3^- \rightarrow SO_4^{2-} + 4NO_2^- + 2H^+$	-510	-6.0 x 10^{-16}	-502	-8.0 x 10^{-15}
19. $3H_2S + 4NO_2^- + 2H^+ + 4H_2O \rightarrow 3SO_4^{2-} + 4NH_4^+$	-1251	-1.5 x 10^{-15}	-1283	-1.0 x 10^{-14}
20. $(^4/_3)NH_4^+ + 2O_2 \rightarrow (^4/_3)NO_2^- + (^8/_3)H^+ + (^4/_3)H_2O$	-340	-9.9 x 10^{-15}	-328	-2.6 x 10^{-15}
21. $4NO_2^- + 2O_2 \rightarrow 4NO_3^-$	-247	-2.9 x 10^{-16}	-254	-4.6 x 10^{-15}
22. $8Fe^{2+} + 2O_2 + 20H_2O \rightarrow 8Fe(OH_3) + 16H^+$	-395	-1.7 x 10^{-15}	-402	-1.8 x 10^{-15}
23. $4NO_3^- + 4H_2 \rightarrow 4NO_2^- + 4H_2O$	-561	-8.9 x 10^{-18}	-552	-7.0 x 10^{-18}

1. Free energy of the reaction (ΔG_r), kilojoules per mole (kJ mol^{-1})
2. Free energy flux (FEF), kilojoules per cell per second (kJ $cell^{-1}$ s^{-1}).
3. Italicized reactions are not pertinent to this study either because ΔG_r>-20 kJ mol^{-1} or because N_2 gas is a reactant (N_2 was not measured).

for all 17 favorable reactions classified by electron acceptor. The potential FEF that exists in the system (panel A) assumes all reactions proceed to completion, i.e. limiting species are always reactants. The actual FEF used in the system (panel B) takes into account reactions that have not run to completion, i.e. limiting species can be either reactants or products. As such, this represents the FEF actually used by the microbial consortium and allows comparison of the free energy available to the microbes versus the free energy actually used. Reactions in which solid hydrous ferric oxides (HFO) serve as the electron acceptor (reactions 11 and 12), assume HFO concentration is large and the limiting reactant is set to the electron donor. Although there is significant free energy flux available from all the common electron acceptors, (oxygen, nitrate, HFO, sulfate, methane), the actual free energy flux used is concentrated in the upper redox couples (oxygen, nitrate and to a lesser extent HFO) where the energy gain per mole is the greatest. Little processing of the lower redox couples occurs. Well 12 contains 38% more organic carbon than Well 13 as a driving force for heterotrophic redox reactions and acetate

Panel A



Panel B

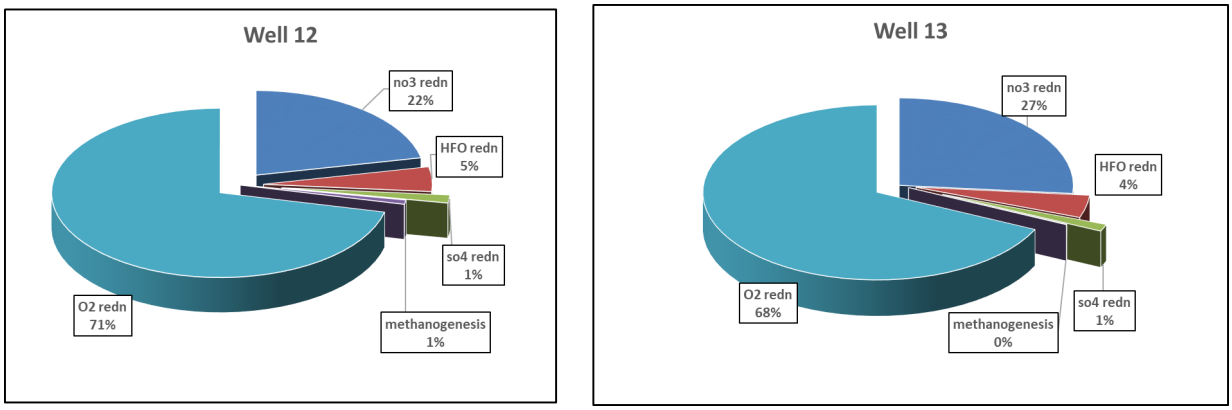


Figure 4: Percentage of FEF classified by electron acceptor. Panel A) Potential FEF available for microbial consortia. Panel B) FEF actually used by the microbial consortia. Reactions in each category correspond to those listed in Table 2. NO_3 reduction = reactions 2, 5, 18, 19, 23. HFO reduction = reactions 11, 12. SO_4 reduction = reaction 6. Methanogenesis = reaction 8. O_2 reduction = reactions 13-17, 20-22.

fermentation. The resulting difference in FEF can be seen in Figure 5. The percentage of FEF that is due to heterotrophic/fermentation reactions is significantly greater in Well 12 than in Well 13 (57% versus 36%) and is largely driven by a 3.4-fold increase in FEF due to nitrate reduction (-3.9×10^{-15} versus -1.3×10^{-14} kJ cell^{-1} s^{-1} respectively). This observation is supported by the low

oxidized nitrate and nitrite concentrations in well 12 with respect to well 13. The total heterotrophic/fermentative FEF in Well 12 is -4.1×10^{-14} kJ cell^{-1} s^{-1} and is -3.0×10^{-14} kJ cell^{-1} s^{-1} in Well 13.

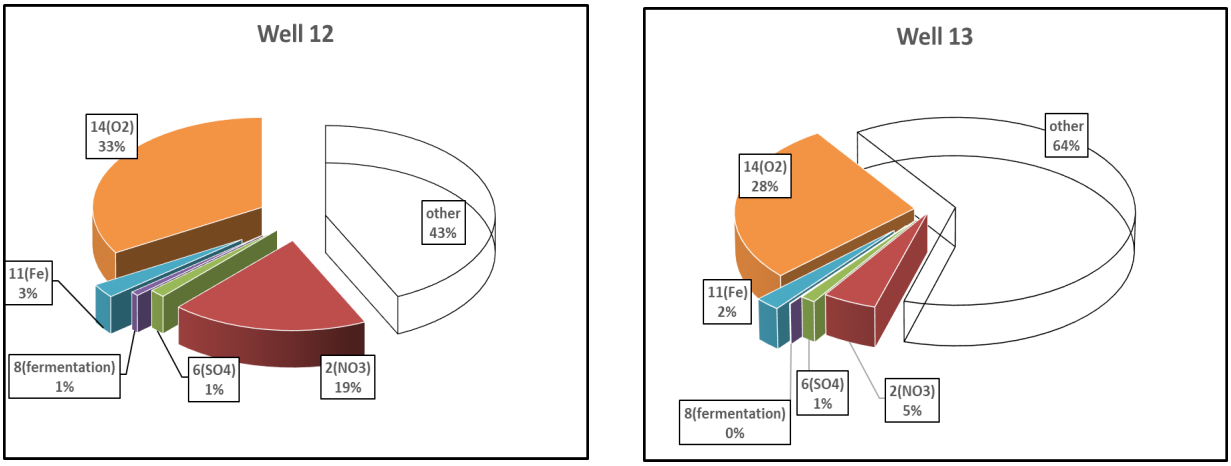


Figure 5: Percentage of FEF due to heterotrophic and fermentation reactions. Specific reaction numbers are indicated for each category.

It should be noted that this FEF analysis is limited to the high energy heterotrophic and fermentation reactions and, because N_2 gas was not measured, excludes denitrification reactions. Many autotrophic reactions are likely occurring, especially in the impacted RBI well (Well 12). The genomic data indicate the presence of several taxa capable of autotrophic nitrogen cycle reactions. Well 12 also exhibits a high FEF for ammonium oxidation (reaction 20 in Table 2). Taxa capable of performing these reactions are found in the genomic data.

Microbial Community Composition

The Fox River and groundwater samples harbored completely distinct microbial communities (Figure 6), an indication that overall there is little overlap in organisms between the two environments. The groundwater samples also had greater community variability than the

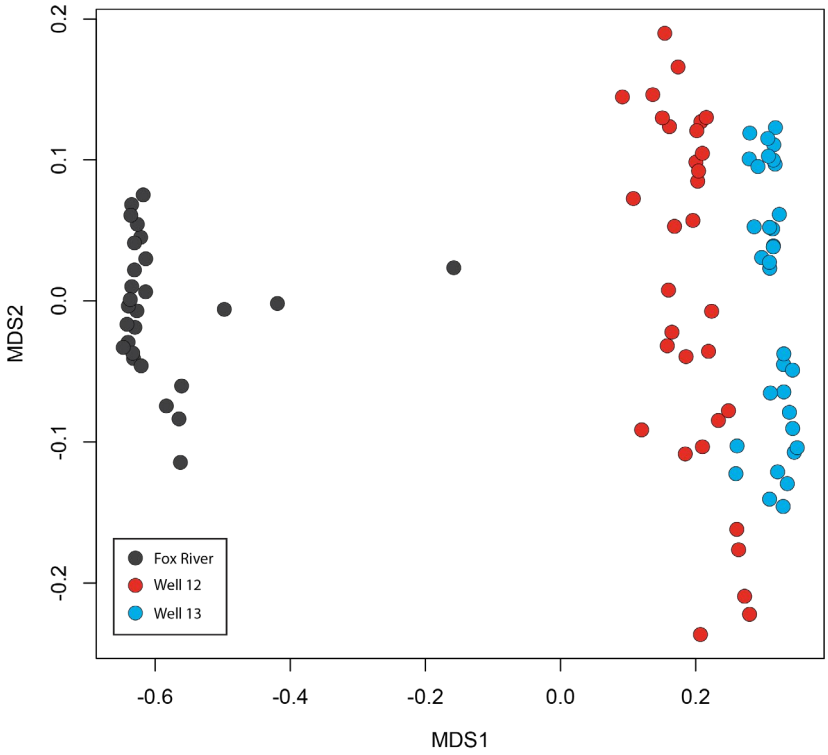


Figure 6: Non-metric multidimensional scaling (nMDS) ordination of Fox River and Groundwater Microbial Community Samples. Each dot depicts the microbial community in a sample. Colors indicate sample group. All filter size (0.1 µm and 0.2 µm) and nucleic acid based communities are represented

river samples (Figure 6). This result may be related to the presumed greater niche heterogeneity in below ground versus surface water environments.

The microbial communities of the groundwater wells also were distinct from each other, PERMANOVA p = 0.001) (Figure 7). Within the wells, the microbial community composition exhibited several distinct patterns related to our sampling scheme. There was a large difference in the communities captured on filters representing two size fractions: 1) 3 µm to 0.2 µm and 2) 0.2 µm to 0.1 µm; PERMANOVA P = 0.001). Similarly, there was a clear distinction between communities generated from 16S rRNA genes (DNA) versus those generated from the 16S rRNA (RNA; Figure 7). Although significant, assessing the community via organism presence (DNA) versus organism presence and activity (RNA) had less of an influence on community

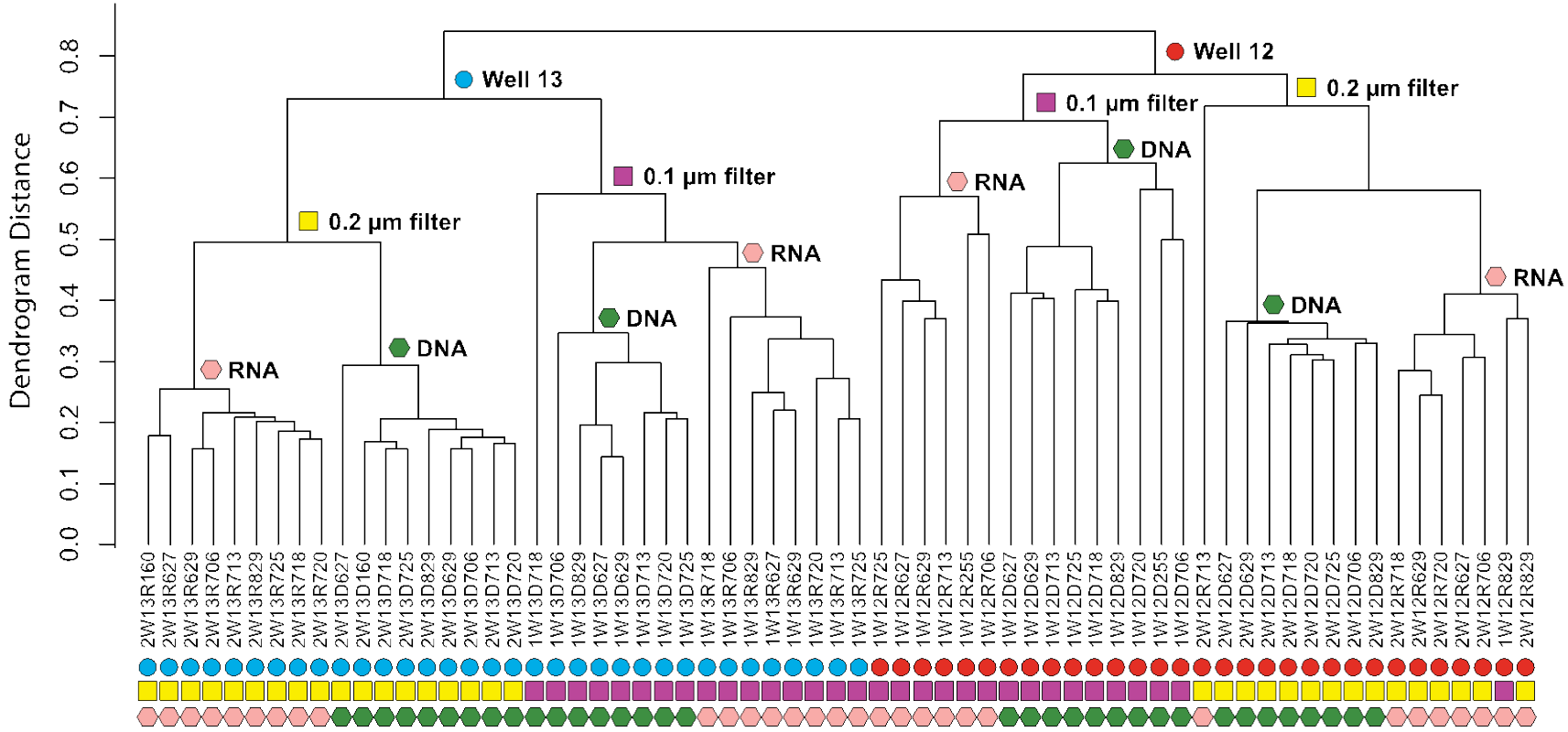


Figure 7: Groundwater Microbial Community Composition Comparison. A dendrogram constructed from community Bray-Curtis dissimilarity with average group linkage is depicted. Each sample labeled with the well, filter size fraction, and nucleic acid type that was used to generate the community composition. Major breakpoints in the dendrogram are labeled with sample groups.

composition than either the sample well or the filter size. Our sample groups (well, filter size, and nucleic acid type) each had a clear influence on the community composition observed. It is clear that there are a significant number of small cells in the groundwater well field, the taxonomic composition of the small cells is distinct from that of larger cells, and that measuring organism activity versus presence will provide similar reproducible but distinct organism abundance relationships.

Taxonomic Affiliations of Groundwater Microorganisms

A high proportion of ASVs identified in the well samples were classified taxonomically as "Unclassified" Figure 8). Further, in all defined sample groups (i.e. filter size and nucleic acid) the groundwater had a much larger proportion of sequences as taxonomically unclassified

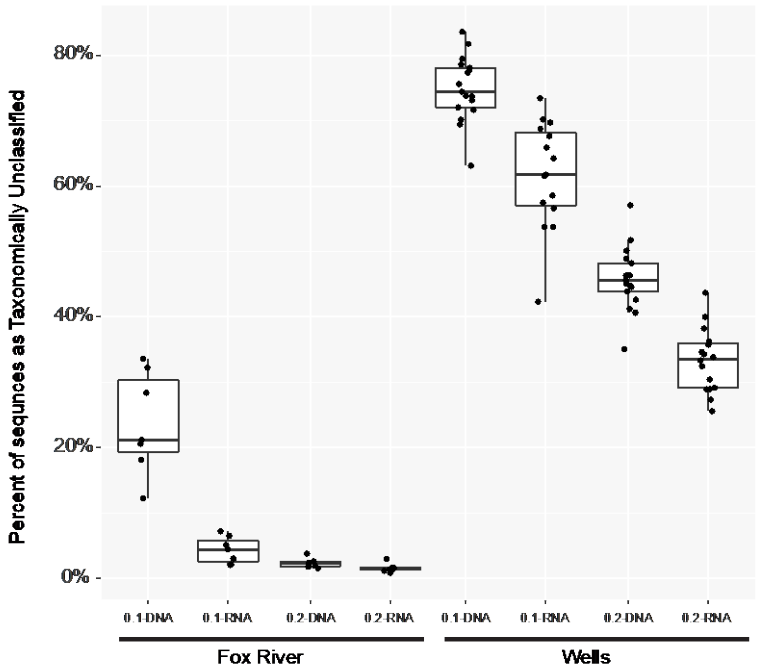


Figure 8: Proportion of Unclassified ASVs in Groundwater and River Water Samples. Sample groups for filter size (0.1 µm and 0.2 µm) and nucleic acid (DNA and RNA) are depicted within each sample type (Fox River or Well). Boxes indicate 1st and 3rd quartiles and the median and lines indicate ranges up to 1.5 standard deviations. All sample values are depicted as points.

as compared to the river samples. A high proportion of Unclassified sequences is unusual, as numerous studies over the past few decades have created extensive 16S rRNA gene databases (Quast et al., 2012). For instance, on average <5% of the total sequences were Unclassified in the Fox River samples, and this is typical in most modern microbial community analyses. The percentage of Unclassified increased significantly in the 0.1 μm filtered Fox River samples, as up to 30% of the sequences were Unclassified in the DNA-based communities. An Unclassified designation indicates the sequences cannot be distinguished to even the Domain level, i.e. Bacteria, Archaea, or Eukarya. This can be the result of methodological error, such as non-targeted PCR amplification or the creation of new artificial DNA sequences during nucleic acid processing and sequencing. However our data supports that many of these Unclassified sequences represent real 16S rRNA gene sequences; 1) Our methodology produced an error rate of 0% in a mock community of 20 different organisms (i.e. all final sequences were a perfect match to the expected sequences), 2) Several Unclassified ASVs were highly abundant (top 25 most abundant ASVs in the dataset) and were prevalent (present in all or nearly all well samples); this consistency is unlikely to be a product of errors in the process, which tend to accumulate more stochastically across samples, and 3) a blastn comparison of several Unclassified ASV sequences against the NCBI nr database (Johnson et al., 2008) identified other studies had obtained similar sequences (>94% identity), and these were typically from groundwater samples (data not shown). Together our validation results suggest many of the Unclassified ASVs are true *Bacteria* or *Archaea* 16S rRNA genes, but currently they are too divergent from known sequences to be given any taxonomic designation. These data also revealed several interesting patterns for future work. The 0.1 μm filter consistently recovered a higher percentage of Unclassified sequences in samples, including in the Fox River samples,

which indicates many very small microorganisms are underrepresented in traditional water sampling procedures. Second, DNA-based communities as compared to RNA-based communities consistently produced a higher percentage of Unclassified ASVs. It is unclear why this would be the case, but could indicate increased chimeric or mutated sequences in the DNA fraction, which is likely given the comparable stability of DNA in the environment. The result could also indicate some of the Unclassified microorganisms produce a comparably low number of rRNAs, thus reducing their representation in the overall pool of rRNA molecules produced by the community. More work is needed here to further understand this extremely large pool of organisms in groundwater that have no comparable information in the now decades old databases.

In addition to the Unclassified ASVs, many known taxa were common to the well samples. These taxa include 16S rRNA gene sequences classified to both *Bacteria* and *Archaea* and include many recently discovered CPR and DPANN organisms such as *Woesarchaeota, Candidatus* Parcubacteria and *Candidatus* Nomurabacteria (Table 3). Many CPR and DPANN organisms are known only by their genomic signatures as they have not been brought into culture (Castelle et al., 2018). To date, they are found primarily in oxygen-limited or anoxic environments like that found in wells 12 and 13. Although many of the most abundant well microbes had limited taxonomic information, some common genera included: *Sulfurifustis*, *Sulfuricurvum*, *Thauera*, *Ferritrophicum*, and *Brocadia* (Table 3). The majority of abundant taxa are classified to taxonomic lineages that generate energy from low oxygen or chemolithotrophic processes, such as nitrification, sulfur oxidation, fermentation, iron oxidation, anaerobic ammonium oxidation, and sulfate reduction. All of these processes were identified as energetically favorable in our free energy flux calculations (Figure 4). This shallow groundwater

system is clearly dominated by a diverse array of microorganisms capable of using non-oxygen electron acceptors to generate energy. It is likely many unusual and perhaps not yet discovered energy-generating mechanisms are employed in this environment. Similarly, deep aquifers, which have only recently been studied extensively for their microbial inhabitants, have revealed an abundance of novel microorganisms and microbial energy generating and metabolic processes such as fermentation using iron or methane, use of iron hydrogenases to produce molecular hydrogen, and nitrogen fixation in anoxic environments (Castelle et al., 2018; Di Rienzi et al., 2013; Jaffe et al., 2019).

Previous studies also documented that deep groundwater systems contain a large proportion of very small cells that pass through the typical 0.2 µm filters used to assess microbial communities (Brown, et al., 2015; Long et al., 2016). In future studies, we recommend that 0.1-µm filters be used to assess the entire microbial community present in both shallow and deep

Table 3: Relative abundance of the top 20 most abundant (on average) taxa in the well samples.

	Taxonomy[1]	**% Abundance 0.2 µm filter [2]**	**% Abundance 0.1 µm filter**
1	Unclassified	39.7%	68.8%
2	Bacteria; Nitrospirae; 4-29-1	9.1%	2.7%
3	Bacteria; Proteobacteria; Gammaproteobacteria; Acidiferrobacterales; Acidiferrobacteraceae; Sulfurifustis	5.9%	7.5%
4	Bacteria; Proteobacteria; Deltaproteobacteria; MBNT15	3.6%	0.5%
5	Bacteria; Proteobacteria; Deltaproteobacteria; DTB120	3.4%	0.5%
6	Archaea; Nanoarchaeaeota; Woesearchaeia	3.0%	6.3%
7	Bacteria; Omnitrophicaeota	2.5%	0.4%
8	Bacteria; Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Rhodocyclaceae	1.5%	0.5%
9	Bacteria; Gemmatimonadetes; Gemmatimonadetes; Gemmatimonadales; Gemmatimonadaceae	1.5%	0.06%
10	Bacteria; Nitrospirae; HDB-SIOI1093	1.4%	0.1%

11	Bacteria; Epsilonbacteraeota; Campylobacteria; Campylobacterales; Thiovulaceae; Sulfuricurvum	1.3%	0.2%
12	Bacteria; Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Gallionellaceae	1.2%	0.6%
13	Bacteria; Proteobacteria; Gammaproteobacteria; Betaproteobacteriales	1.1%	0.1%
14	Bacteria; Planctomycetes; Brocadiae; Brocadiales; Brocadiaceae	0.8%	0.1%
15	Bacteria; Nitrospirae; Thermodesulfovibrionia	0.7%	0.1%
16	Bacteria; Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Nitrosomonadaceae; GOUTA6	0.6%	0.07%
17	Bacteria; Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Rhodocyclaceae; Thauera	0.6%	0.2%
18	Bacteria; Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Sulfuricellaceae; Ferritrophicum	0.6%	0.05%
19	Bacteria; Proteobacteria; Gammaproteobacteria; Legionellales; Legionellaceae; Legionella	0.6%	0.1%
20	Bacteria; Patescibacteria; Parcubacteria; Candidatus Nomurabacteria	0.2%	0.9%

1. Silva (Quast et al., 2012) database taxonomy assignments (to genus) are listed
2. Relative abundances are listed separately for samples collected with the 0.2 um and 0.1 um filters. All taxa with average relative abundances of ≥0.5% in either filter group are listed.

aquifer samples. Additionally, we found the RNA and DNA fractions in each well-filter grouping were distinct (PERMANOVA $p = 0.001$). The RNA fraction represents those microorganisms that are active, while the DNA fraction represents all microorganisms present. The clear composition difference between these groups indicates there are active microbes in this ecosystem and some have disproportionate production of ribosomes compared to their abundance. We also found numerous examples of ASVs that were abundant and prevalent in the RNA samples (n=137 ASVs with ≥0.1% community max abundance), but were not present in any DNA sample. Some common aquifer microbes are known to have spliced ribosomal RNA genes (Castelle et al., 2018). This is an unusual feature and one that prevents the detection of these organisms using standard DNA methods. It is clear that access to the full diversity of microbes in these systems is only available via non-targeted methods like metagenomics and/or combinations of methods targeting both RNA and DNA. Much is left to be discovered about the

interactions of microbes and their environment in below ground fluid systems and how these interactions impact water quality.

Microorganisms & Energetics Differentiating the Pristine & RBI Wells

Although the groundwater and Fox River microbial communities were significantly different, there were a few community members present in both sets of samples. This is not surprising as the two aquatic systems are connected via hydrologic flow, where the groundwater typically flows to the Fox River. In our previous preliminary work, we found no traces of fecal bacteria in the RBI Well 12, which suggested that microorganisms were not entering the well from the river (Fields-Sommers, 2015). However, fecal organisms make up a very small percentage of the microbial community in the Fox River, and typically have large cell sizes, so they are not sensitive tracers of transport of microbes into the wells. To identify whether any river microbes where infiltrating the wells, we classified all sequences as to whether they belong to a group of typical surface freshwater microorganisms (TaxAss using the FreshTrain database; Rohwer et al., 2018). All sequences in each of our four partitioned community types (by filter size and nucleic acid type) were classified for the river and well samples. We found the common surface freshwater microorganisms made up 50-75% of typical river sample communities (Figure 9). In contrast the wells had a very low percentage of these microorganisms, usually <0.5%, but all samples did contain some common surface freshwater microbes. There was a significant difference in the concentration of surface organisms present in the RBI (Well 12) versus the pristine well (Well 13), where the RBI well had, on average, 10-times more surface microbes per sample (Figure 9). Also these river indicator organisms were found in 29 out of 31 Well 12 samples versus only 14 out of 34 Well 13 samples. According to these results, it is likely

both wells have some hydrologic connection to surface water, but the connection is much greater for the RBI well. We suggest continued work is needed to understand how these surface water microorganisms are making it into the well field and whether this represents any risk to water quality.

We initially established that Well 12 harbored a fundamentally different microbial community than Well 13 (Figures 5 & 6) and that the groundwater samples harbored diverse microbial communities with many small cells and a composition quite unique from that in surface water. Following these analyses, we examined further what taxa were differentiating the RBI and pristine wells and how these organisms relate to the measured nutrient concentrations and estimated free energy available from microbial chemical transformations. Since the Well was the largest differentiator of the microbial communities, any environmental parameter that

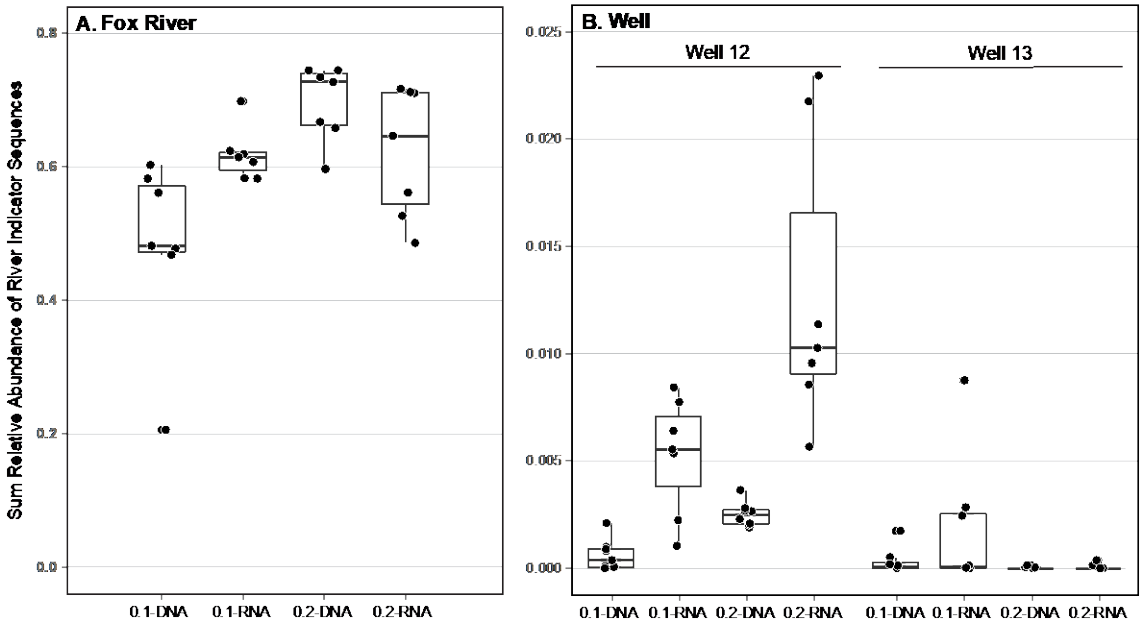


Figure 9: Box plots of the surface freshwater indicator microorganism sequences in each river and well community group. The data is divided by the A) Fox River and B) well - Well 12 (RBI) and Well 13 (pristine). Within each sample group, the communities are broken down further by filter size 0.1 or 0.2 (μm) and nucleic acid type (DNA or RNA). The y-axis is scaled uniquely to each plot so the data may be visualized. The boxes in the plots represent the 25% to 75% percentiles (the inter-quartile range or IQR). The horizontal line through the IQR box indicates the median. Whiskers show the lowest and highest values no further than 1.5x IQR away from the IQR, and all other points above/below the IQR are indicated by points.

was also distinct between the wells was correlated to the microbial community composition. This included significantly higher concentrations in Well 12 of chloride, sodium, and ammonium and significantly lower concentrations in Well 12 of nitrate, nitrite, and sulfate (Table 4). There was no significant relationship between the groundwater microbial community composition and sample date (Table 4).

On an individual taxa level, Well 12 is expected to have less nitrifying microorganisms or an increase in nitrate reducing microorganism (higher ammonia and lower nitrate) than Well 13. The lower sulfate would also predict a lesser role for sulfur oxidizers or increased sulfate reducers. Although nitrifying and sulfur oxidizing taxa are present in all well samples

Table 4: Environmental Parameter Correlation to Groundwater Microbial Community Composition.

Parameter	Correlation envfit (r^2)[1]
Sample Date	0.039
Nitrate	0.896 ***
Nitrite	0.844 ***
Ammonium	0.822 ***
Total Dissolved P	0.274 **
Sulfate	0.805 ***
Calcium	0.064[2]
Magnesium	0.142
Sodium	0.980 ***
Chloride	0.922 ***
Potassium	0.832 ***

1. Correlation between microbial community ordination (nMDS) and environmental data is reported from the envfit function in the vegan package in R
2. Only two dates were used in the correlation of ions (sulfate, calcium, magnesium, sodium, chloride, and potassium) to the microbial community composition.

samples, these taxa were differentiators in the communities between the wells. We performed a Multinomial Species Classification Method (CLAM; (Chazdon et al., 2011)) from the vegan package in R to identify ASVs that differentiated the Well 12 and Well 13 communities.

Stringent conditions were used to discriminate specialists to each well. The conditions included a specialization threshold of 5/6, which indicates a specialist organism/sequence has to occur at least 5x more in one well versus the other. For example, we found: 1) Sulfate was at higher concentrations in Well 13. *Desulfocapsa* was more abundant in W12; this group is composed of sulfate reducers. There was also a difference in sulfur oxidizers between the wells *Sulfurifustis* was more abundant in Well 13, and *Sulfuricurvum* was more abundant in Well 12. 2) Several abundant ASVs (top 100 most abundant organisms) classified as *Nitrospirae* were more abundant in Well 13 than in Well 12. These microbes are known nitrifiers, and there was significantly less ammonium and more nitrate in Well 13; 3) *Thauera*, a known nitrate reducer was found only in Well 12, which was predicted to have more than 3 times the potential for nitrate reduction, 4) *Ferritrophicum* and *Gallionellaceae*-affiliated microbes were more abundant and potentially more active in W13 than W12. Both are known iron oxidizers. 5) Many more abundant microbes in Well 12 were from taxa with fermentative or unknown metabolic functions (*Amesbacteria*, *Amnitrophicaeota*, and *Woesarchaeia*); and 6) Legionella were 2X more abundant in Well 12. The ASVs and associated taxonomic affiliations for the specialists of both wells A can be found in Gayner (2018).

Conclusions

Thermodynamic analysis of the groundwater wells indicates that there is potential FEF available from a wide variety of electron acceptors, but microbes largely use the most energetically favorable acceptors; oxygen, nitrate and to a lesser extent ferric oxides. Diverted river water that is entering the RBI well (Well 12) contains more dissolved organic carbon and as a result, Well 12 has a higher total free energy flux available for microbes to access than the

pristine well (Well 13). The additional flux results largely in an increase in nitrate reduction in the RBI well. The FEF analysis generally agrees with differences in the taxonomic composition between the two wells, where the RBI Well 12 has increased abundance of nitrate and sulfate reducers and more organisms predicted to have fermentative capabilities. In contrast, Well 13 has more microorganisms likely oxidizing ammonia and sulfur. Well 12 also harbored significantly more common surface water taxa than Well 13, which suggests the RBI is creating unrecognized connections between the surface and well water. We did not find that either well had significant wastewater microorganism contamination, but given microbes may be entering from the river, this should be monitored going forward. It is clear that river bank inducement is altering the typical microbial community in this shallow aquifer.

Although our FEF and microbial analysis revealed several compelling differences between the RBI and pristine wells, the understanding of these systems is largely incomplete. More than half of the identified microbes are either Unclassified or have unknown metabolisms, which limits our ability to understand energetic differences occurring in the RBI. In addition to the taxonomic differences, our data indicates these shallow groundwater systems require additional atypical sampling in order to assess microbial diversity. We found extensive microbial diversity and unique taxa in 1) the RNA fraction, which was not contained in the traditional sampling of DNA, presumably due to spliced ribosomal operons, and 2) very small cells that pass through commonly used 0.2 μm filters. Future sampling of groundwater must consider this extensive and atypical diversity to develop a more complete understanding of theses systems. Overall, we found shallow groundwater to be a complex microbial ecosystem, containing numerous unknown or only recently discovered microorganisms that harbor a myriad of metabolic capabilities and unusual genetic structures. RBI creation appears to alter this microbial

ecosystem by introducing organic carbon, which shifts some energy generation toward fermentative and reductive capabilities rather than oxidation of ammonia/nitrite and sulfur. More work is needed to understand how anthropogenic alterations to hydrologic flow impacts aquifer microbial communities and in turn how these changes impact water quality.

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