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MICROBIAL COMMUNITY PROFILING AND CHARACTERIZATION OF SOME HETEROTROPHIC BACTERIAL ISOLATES FROM RIVER WATERS AND SHALLOW GROUNDWATER WELLS ALONG THE ROUGE RIVER, SOUTHEAST MICHIGAN

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MICROBIAL COMMUNITY PROFILING AND CHARACTERIZATION OF SOME HETEROTROPHIC BACTERIAL ISOLATES FROM RIVER WATERS AND SHALLOW GROUNDWATER WELLS ALONG THE ROUGE RIVER, SOUTHEAST MICHIGAN

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ABSTRACT

This study was conducted to elucidate microbiological characteristics of river water and groundwater communities in order to improve our conceptual and predictive understanding of river and groundwater ecosystem processes, functioning and management. Rouge River bacterial communities from shallow groundwater and river water were screened using Biolog Ecoplates, which test for oxidation of selected carbon sources and by culturing heterotrophic bacteria. The isolates cultured from the samples were also characterized using the 16S rRNA gene-based approach. The patterns of utilization of the groups of carbon substrates by the microbial communities revealed differences between river water and groundwater samples. Carbohydrates, polymers, carboxylic acids and amino acids were highly utilized by the microbial communities in the river samples, while carbohydrates, polymers, amino acids and phenolic compounds were metabolized in the groundwater samples. Sequence comparison results showed that the most prevalent phylum in all sites was the *Firmicutes* (low G+C, mostly gram-positive bacteria). The dominant isolates from this phylum were similar to *Bacillus* spp., (98% nucleotide identity), which represented approximately 62% of the total number of unique isolates. Also prevalent were the γ-*Proteobacteria*, which were dominated by 16S rRNA sequences 98-99% similar to that of *Pseudomonas* spp. The observed profile of carbon sources metabolized reflected the catabolic potential of the river water and groundwater community. Many of the isolates recovered have been known to metabolize several organic substrates, and may have potential use in remediation organic contaminants from the Rouge River. Direct incubation water samples in Biolog Ecoplates produced patterns of metabolic response useful in the classification and characterization of river water and groundwater microbial communities. Heterotrophic bacteria isolated from the sites may play important roles in the fate of many organic and inorganic contaminants from the Rouge River, although future studies are needed to understand their response to these contaminants.

Keywords: Biolog, 16S rRNA, metabolic diversity pattern, carbon source utilization pattern, groundwater bacteria

INTRODUCTION

The Rouge River drainage basin is the oldest and most heavily populated and industrialized area in southeast Michigan [1]. The basin covers 1,210 km^2 of southeastern Michigan, including sections of Wayne, Oakland, and Washtenaw counties. It encompasses 48 municipalities with a population of more than 1.5 million people (Figure 1). The degradation of the Rouge River is representative of that found in many urbanized and industrialized areas within the Great Lakes Basin. Combined sewer overflows (CSOs), urban storm water discharges, non-point source pollution, and municipal and industrial discharges all contribute to the contamination of the Rouge River. A wide variety of pollutants including metals, polychlorinated biphenyls, polyaromatic hydrocarbons, and fecal indicator bacteria were cited as the major sources of contamination [2-5]. Elevated levels of fecal indicator bacteria within the river were also found [5]. It has been suggested that microbes and contaminants can percolate through the soil and contaminate groundwater [6]. Groundwater is an important natural resource worldwide. More than half the water supplied for public use in the United States is groundwater. Previously, scientists believed that groundwater was less vulnerable to pollution compared with surface water. Recent reports, however, indicate that this may not be the case, owing to increasing point and diffuse source of contamination arising from urban, industrial, and agricultural activities [6]. Microorganisms play an important role in the fate of many groundwater contaminants. However, little is known about the bacterial populations that inhabit the Rouge River. Knowledge about microbial community structure and composition is important to improve our conceptual and predictive understanding of river and groundwater ecosystem processes, functioning and management. Hence, this study was conducted to characterize the microbial communities of river water and groundwater communities.

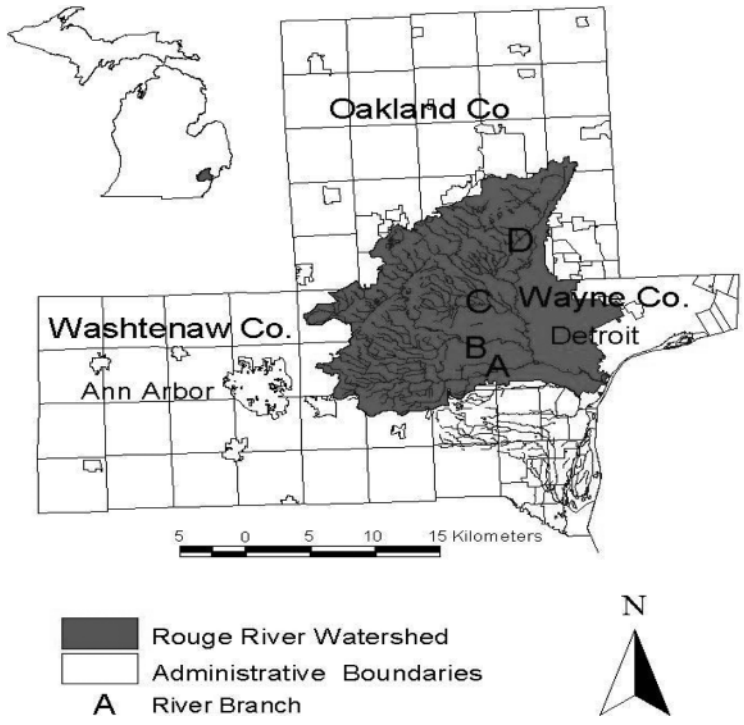

Figure 1. Map showing the study area (Rouge River).

Two complementary microbiological approaches were used in this study: community-level physiological profile (CLLP) analysis, and cultivation and characterization of heterotrophic bacterial strains. CLLP analysis represents functional aspects of microbial communities based on substrate utilization patterns derived from inoculating whole microbial communities into Biolog Eco plates. Using this method, culturable organisms respond to substrates that they can metabolize, providing an overall community fingerprint. CLLP has been used as a rapid method of obtaining a 'fingerprint' for mixed communities of heterotrophic microorganisms [7]. It has also been used as a sensitive and reproducible method for discriminating between microbial communities from a variety of environments [8-12]. To characterize the cultivable heterotrophic bacterial isolates in these ecosystems, the SSU rRNA gene-based approach was used. The latter approach (cultivation), even though it is only capable of detecting a small percentage of microorganisms that are cultivable (between 0.001% and 15%), it is still the reference method for characterization of physiology of bacteria and in understanding the roles of various species and their sensitivity to environmental factors.

MATERIALS AND METHODS

Site Description and Water Chemistry

The Rouge River is a relatively shallow stream, with an average depth of 1 m during the summer. It has a gentle gradient of about 2 m km^{-1} and an average stream flow of 2 m^3 s^{-1}. Normal spring and summer storms (four to five events per season) will typically increase stream flows to 25 m^3 s^{-1}. On average, the sediment transported and deposited by the Rouge River consists of fine medium sand, although sites in the headwaters region of the watershed are generally characterized by silty to fine sandy substrates.

A network of 54 groundwater monitoring wells (six wells at each of the nine locations) were installed along the lower branch of the Rouge River during the summers of 2000 and 2001. At each of the nine well locations, the wells were installed in clusters of three. Three wells installed on the north side of the river and three wells installed on the south side of the river. The three wells in each cluster were installed in a triangular arrangement to allow a determination of the direction of groundwater flow. The wells, which consist of two-inch diameter PVC well casing, were installed manually using a three-inch diameter hand-auger to depth of 1 m below the water table. The bottom 0.3 m of each well casing was slotted in the laboratory to allow groundwater to flow into the well. Fiberglass screening was wrapped around the slotted part of the casing to filter out fine-grained sediment and the annulus of the well was backfilled with clean sand. Water table depths vary depending upon the proximity of the well to the river, but average depth to the water table was 3 m. Within 24 hours of the installation of each well, the wells were developed by surging the well with a stainless steel bailer and extracting a volume of water equivalent to five well volumes. This procedure was also followed during the sampling of the

wells with the exception that no surging was needed and water extraction was completed using a peristaltic pump instead of a bailer.

Groundwater and river water chemistry have been monitored along the lower branch of the Rouge River on a bimonthly basis since the summers of 1996 and 2000, respectively. River water temperature, dissolved oxygen and electrical conductivity fluctuate on a seasonal basis. For example, winter values of dissolved oxygen were usually in excess of 10 mg l^{-1} with summer values often below 5 mg l^{-1}. Spatial variability of dissolved oxygen was more pronounced during the summer months and appeared to be related to periods of wet weather [13]. Electrical conductivity also displayed seasonal fluctuations, typically higher in the winter and lower in the summer. The high winter values (generally ≈ 10 μS cm^{-1}) were likely related to the runoff of road salt, used as a deicer in the Detroit metropolitan area. The pH of the surface waters averaged about 8 for each of the stations throughout the year, thereby falling into the range for natural waters [14]. Groundwater temperatures averaged between 10 and 13ºC throughout the year, dissolved oxygen as expected, was quite low, but detectable, generally ranging from 0.1 to 0.3 mg l^{-1}.

Sample Collection

In this study, three sites (Lotz, site mw-1; Lilly, site mw-8, and Ford Field, site mw-9) were surveyed along the Rouge River that pass through a heavily commercial and urbanized part of southeast Michigan (Figure 2). At each of these three locations, river water and two groundwater samples were collected. Because the water chemistry of the clustered wells from the sites was very similar, only two groundwater wells at each site were used instead of six: one from the north (gw-1) and one from the south (gw-1). Hence, a total of three river water samples (1-sw, 8-sw, and 9-sw) and six ground water samples (1-gw1, 1-gw2, 8-gw1, 8-gw2, 9-gw1 and 9-gw2) were collected from all three sites.

River water samples were obtained by wading into the river at mid-stream, and manually lowering a sterile glass bottle to a depth approximately equivalent to 0.6 of the depth of the river. At sample sites where the river depth was too great to wade safely (>1.5 m), samples were collected by suspending the sample bottle on a telescoping aluminum pole and collecting water sample from a depth of approximately 1 m. Groundwater samples were collected using a low-flow purging pump. Low-flow purging pumps water from a well at a very low rate, minimizing agitation and mixing with stagnant water contained in solid casing regions of the well. Three samples were collected from each site. These samples were merged together to give one composite sample. All water samples were stored in sterilized media bottles, transported in the laboratory in a cooler, and then stored in a climate-controlled cold room. Samples were analyzed within 8 h of collection to prevent bacterial death.

Community Level Physiological Profile Using Biolog

The Biolog Ecoplate (Biolog, Inc., Hayward, CA) relies on the redox dye tetrazolium violet, to detect respiration activity (NADH formation) on 31 sole carbon sources (Table 1). Thus, any color development presumably indicates utilization of carbon sources inherent in the sample.

Groundwater and river water samples were directly inoculated into Biolog Ecoplates within one day of sample collection. Each well of Ecoplates plates was inoculated with 125 μl of this suspension incubated at 22°C. Color development in the microplate wells were measured every 2 h at 590 nm using an automated Sunrise microplate reader (Tecan, Research Triangle Park, NC). Absorbance readings were continuously recorded between one and seven days following inoculation. To avoid possible

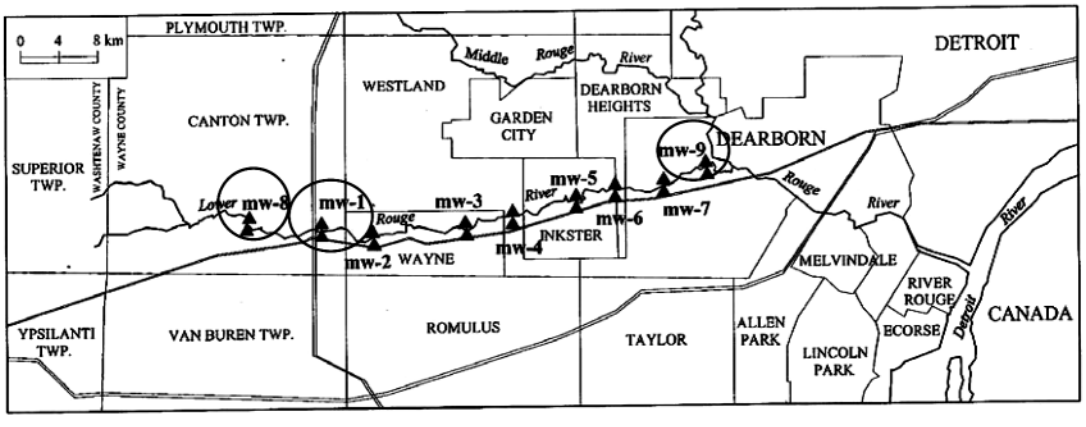

Figure 2. Location of sampling sites along the lower branch of the Rouge River. The ▲ symbol indicates the locations of the groundwater wells. Three wells are installed in each of these wells. The encircled sites (site mw-1, Lotz; site mw-8, Lilly and site mw-9, Ford Field) are associated to the sites selected for this study.

Table 1. Substrates in Biolog® Ecoplate.

Amines	**Carbohydrates**	**Carboxylic acids**	**Polymers**
Phenyl ethylamine	α-D-lactose	α-Ketobutyric acid	α-Cyclodextrin
Putrecine	β-Methyl D-glucoside	D-Galacturonic acid	Glycogen
	D-Cellobiose	D-Glucosaminic acid	Tween 40
Amino acids	D-Mannitol	D-Malic acid	Tween 80
L-Arginine	I-Erythritol	Itaconic acid	
L-Asparagine	Glucose-1-phosphate	γ-Hydroxybutyric acid	**Phenolic compounds**
L-Phenylalanine	D-Xylose	Pyruvic acid methyl ester	2-Hydroxy benzoic acid
L-Serine	D-Galactonic acid γ-lactone		4-Hydroxy benzoic acid
L-Threonine	N-Acetyl-D-glucosamine		
Glycyl-L-glutamic acid	D,L-α-Glycerol phosphate		

confounding effects of inoculum density, a standardization was made to select an appropriate time of reading for each plate. Such point of time was selected when the average of continuous absorbance data is 0.6 [15]. Average well color data (AWCD) were calculated according to Garland and Mills [7], i.e. AWCD = Σ $(C - R)/n$, where C is color production within each well (optical density measurement), R is the absorbance value of the plates' control well, and n is the number of substrates (n = 31). The relative abundance of the substrate was determined based on the amount (absorbance value) of each substrate that was utilized relative to the total amount of substrates (sum of absorbance values of each plate) used by the microbial community, tested based on the measured densities of reduced tetrazolium dye in wells.

The absorbance readings obtained from the Biolog Ecoplates with AWCD equal to 0.6 were further analyzed and compared using principal components analysis (PCA). PCA is the multivariate ordination technique that was used on the correlation matrix of the carbon source utilization patterns. PCA is a statistical method used to reduce the number of variables to a smaller number of new variables called principal components (PCs). Differences in CLLP patterns in groundwater and river water samples were tested statistically by comparing principal component scored among different samples using analysis of variance. The plots of the PC scores for each sample were used to display differences in metabolic diversity pattern. Correlation analysis was used to relate the original variables (substrates) to the principal components. Interpretation of the principal components was based on significant factor loading of the individual substrates on each of the principal components. Principal components 1 and 2 were analyzed using a two-way analysis of variance (ANOVA). Tukey's post hoc test was used to determine significant differences ($p < 0.05$) between individual pairs of means. Data were log-transformed as necessary to conform to the ANOVA assumption of homogeneity of variance. Statistical analyses were calculated using SYSTAT statistical computing package (SYSTAT Version 9.0, SPSS Inc., Chicago, IL, USA).

Pure Culture Isolation and Characterization

Serial dilutions of groundwater and river water samples were plated on several media: 0.1X trypticase soy agar (TSA), nutrient agar (NA), Luria-Bertani (LB) and R2A medium. Plates were incubated aerobically at 10°C. Morphologically distinct isolates were picked, re-streaked to purify, and stored on 50% glycerol at −80°C. Gram staining characteristics and cell morphologies were determined by standard methods [16]. Motility was observed by wet mounts. Physiological characterization of strains was based on API 20E testing (BioMereux, Vitek, Inc.). Test results were obtained as specified by the manufacturer.

Representative isolates of distinct morphologies were identified by SSR rRNA sequence analysis. Isolates were subcultured on 0.1 X TSA. Genomic DNA from these isolates were extracted using DNEasy kit (Qiagen, Inc., Valencia, CA). The SSU rRNA genes were amplified with FD1 and 1506 primers [17]. PCR was performed in an iCycler Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA) as 50-µl reactions containing 1x PCR buffer (50 mM KCl, 10 mM Tris, 0.1% Triton X-100, pH 9.0), 1mM dNTPs, 1.5 mM $MgCl_2$, 1 µM of each primer, 4 µg bovine serum albumin (BSA), and 2.5U *Taq*. The thermal cycling protocol used included initial denaturation at 94°C for 2 min, followed by 25 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 1 min. A final extension step at 72°C for 7 min was also used. The PCR amplicons were checked by electrophoresis on 1% agarose gels. Three amplicons were pooled for each set of 16S rRNA sequencing reactions in order to maximize sequencing template concentration and to allow for the detection of possible microheterogeneity in any rRNA gene copies. Amplicons were purified with Montage PCR 96 filter plates (Millipore Corporation, Bedford, MA). Flanking and internal primers 350r, 519f, 788f, 925r and 1099f [17] were used for direct sequencing of the amplicons.

DNA sequencing was performed with ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster, CA), and the ABI PRISM 3700 DNA Analyzer (PE Applied Biosystems, Foster, CA). All sequence fragments generated from a given template were

Table 2. Accession numbers of 16S rRNA sequences of isolates deposited in Genbank.

Isolates	Accession numbers	Isolates	Accession numbers
1-gw1-9	DQ990019	8-gw2-9	DQ990045
1-gw2-2	DQ990020	8-gw2-10	DQ990046
1-gw2-5	DQ990021	8-gw3-4	DQ990047
1-gw2-8	DQ990022	8-gw3-5	DQ990048
1-gw-10	DQ990023	8-gw3-7	DQ990049
1-gw3-2	DQ990024	8-gw3-8	DQ990050
1-gw3-3	DQ990025	9-gw1-3	DQ990051
1-gw3-4	DQ990026	9-gw1-4	DQ990052
1-gw3-7	DQ990027	9-gw1-5	DQ990053
1-gw3-8	DQ990028	9-gw1-6	DQ990054
1-gw3-10	DQ990029	9-gw1-8	DQ990055
1-gw1-3	DQ990030	9-gw1-9	DQ990056
8-gw1-1	DQ990031	9-gw1-10	DQ990057
8-gw1-2	DQ990032	9-gw2-3	DQ990058
8-gw1-3	DQ990033	9-gw2-4	DQ990059
8-gw1-5	DQ990034	9-gw2-6	DQ990060
8-gw1-6	DQ990035	9-gw2-7	DQ990061
8-gw1-8	DQ990036	9-gw2-8	DQ990062
8-gw1-9	DQ990037	9-gw2-9	DQ990063
8-gw1-10	DQ990038	9-gw2-10	DQ990064
8-gw2-1	DQ990039	9-gw3-6	DQ990065
8-gw2-2	DQ990040	9-gw3-7	DQ990066
8-gw2-4	DQ990041	9-gw3-8	DQ990067
8-gw2-5	DQ990042	9-gw3-9	DQ990068
8-gw2-6	DQ990043	9-gw3-10	DQ990069
8-gw2-7	DQ990044		

edited against electropherograms and then assembled into contigs using SeqMan (Lasergene DNASTAR, Inc., Madison, WI). For most sequences, two to four overlapping fragments (from both coding and non-coding strand) were used to assemble the contigs. Constextual 16S rRNA sequences for all unknown sequences were identified from published databases using BLAST [18], and by comparison with sequences deposited in the Ribosomal Database Project [19]. All sequences were manually aligned using MegAlign (Lasergene DNASTAR, Inc., Madison, WI). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.0 [20]. Phylogenetic trees were constructed with distance matrices and minimum evolution method using the Jukes-Cantor distance model. The 16S rRNA full sequences reported in this study have been deposited in GenBank. The accession numbers for each sequence are reported (Table 2).

RESULTS

Metabolic Diversity Pattern

Inoculum density was a major issue on interpreting the Biolog Ecoplate data. It was noted the color development was correlated with the cell density [15]. The microbial numbers were low across all samples and the variations between samples were relatively small (≤ one order of magnitude) (Figure 3). To reduce the effects of differential rates of color development owing to inoculum density on the classification of samples, two methods were used. Firstly, the data were transformed by dividing the raw difference (substrate well-control well value) of each well by the AWCD of the plate, giving greater indication of difference in sole-carbon-source utilization patterns between samples [7]. Secondly, similar AWCD was used to determine the reading time for plate comparisons [15]. This was achieved by taking a series of readings across a prolonged period, then selecting each plate reading closest to the reference AWCD. Therefore, the results of the Biolog test in this study reflect the metabolic potential of river and groundwater microbial communities.

Results of the Biolog Ecoplate test indicated that the carbon sources utilized by the microbial communities were similar between sites but the relative abundance of the metabolized substrates are different (Figure 3). The data also showed that the culturable members of the microbial communities in river and groundwater samples used carbohydrates, amino acids, amines, carboxylic acids, polymers and phenolic compounds (Table 3). Correlation analyses showed that the microbial communities of the river waters used β-methyl-D-glucoside, D-mannitol, glycogen, D-glucosaminic acid, tween 80, glucose-1-phosphate, L-serine, N-acetyl glucosamine and itaconic acid to a greater degree than the communities of groundwater. Microbial communities of the groundwater used tween 40, D-galactonic acid γ-lactone, 4-hydroxy benzoic acid, D-cellobiose, L-serine and L-arginine to a greater degree than the river water communities. The number of carbon substrates utilized was least diverse in groundwater samples from site 1 (1-gw1) compared with all samples surveyed (Figure 3). It appears that only six substrates were used by the microbial communities and about 21% of the total density measured in the wells was from D-galacturonic acid.

The ordinate plot of PCA for river and groundwater samples is shown (Figure 4). PCA of different samples reduced the data into two principal components (PC1 and PC2) that explained a large amount (80%) of the total variation in the substrates. A comparison of the metabolic diversity patterns between river and groundwater microbial communities showed a significant separation along PC1 (p = 0.0062) and PC2 (p = 0.05). Carbon sources with high correlation coefficients for PC1 and PC2 are listed in Table 3. Separation of the river and groundwater samples from the three sites along PC1 was affected by amines, amino acids, carbohydrates, carboxylic acids, polymers and phenolic compounds. Substrates affecting PC2 were carbohydrates, carboxylic acids and phenolic compounds (Table 3). Comparison of the metabolic diversity patterns of microbial communities between sites provided information concerning the effect of spatial variation on microbial communities. The PCA of site 9 had a higher degree of variability (scattered data points) in their metabolic diversity patterns compared with sites 1 and 8

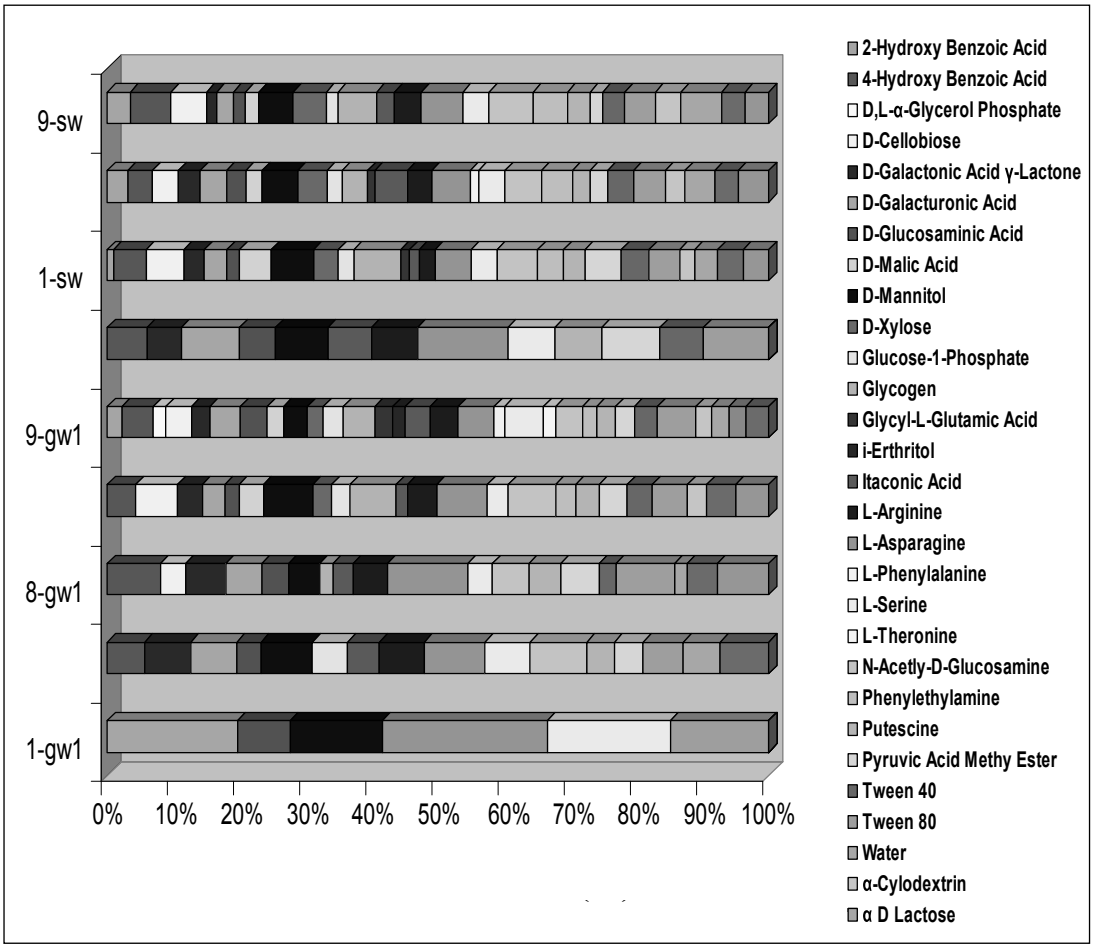

Figure 3. Relative abundances of metabolized substrates in Biolog Ecoplate and microbial counts of groundwater (1-gw1, 1g-w1, 8-gw1, 8-gw2, 9-gw1, and 9-gw2) and river water (1-sw, 8-sw, and 9-sw) samples from three different sites (site 1, Lotz; site 8, Lilly and site 9, Ford Field) of the Rouge river. Relative abundance refers to the amount of each substrate that was utilized relative to the total amount of substrates used by the microbial community tested based on the measured densities of reduced tetrazolium dye in wells.

(Figure 4). This indicated that the metabolic diversity patterns of the microbial community at this site were more variable compared with sites 1 and 8. However, ANOVAs of PC1 scores obtained from sites 1, 8 and 9 showed that PC1 scores were not significantly different (p = 0.150). ANOVAs of the PC2 scores also indicated no significant difference (p = 0.253) existed among the three sites.

Characterization of Viable Heterotrophic Bacteria

Approximately 63 colonies having visibly different colony morphologies were selected from TSA, R2A, nutrient broth and Luria-Bertani plates, and were purified. These isolates were further characterized by cell morphology, gram staining and API 20E assay. Thirty-four isolates were gram negative and 29 were gram positive. The majority of the isolates were motile rods (54 isolates) and the rest were motile spirilli (two isolates) and non-motile cocci (five isolates). The results of the API tests are summarized in Table 4. The majority of the isolates were able to metabolize carbohydrates, proteins and amino acids. Overall, the test result profiles show very subtle differences (p = 0.98) between isolates recovered from river water and groundwater samples that utilized various substrates. Most of the isolates from the river water showed positive results in the TDA, GEL, VP, ONPG, ADH and CIT API 20E tests. Isolates from the groundwater were typically positive for TDA, GEL, VP, ONPG, ARA, MAN, GLU, SAC, AMY, RHA, MEL, INO API 20E tests (Table 4).

Of the 63 isolates, 52 unique isolates were subjected to molecular analyses. Thirty-seven of these isolates were obtained from groundwater samples and 15 isolates from

Table 3. Correlation between principal components (PC1 and PC2) and single variables (Biolog Ecoplate substrates) for the PCAs described in the text.

Carbon source	PC 1	PC 2
Amines		
Putrecine	0.954	
Amino acids		
L-Arginine	0.880	
L-Asparagine	0.940	
L-Serine	0.960	
Glycyl-L-glutamic acid	0.876	
Carbohydrates		
α-D-lactose		
β-Methyl D-glucoside	0.954	
D-Cellobiose	0.903	
D-Mannitol	0.938	
I-Erythritol	0.821	
Glucose-1-phosphate	0.980	
D-galactonic acid γ-lactone		0.971
N-acetyl-D-glucosamine	0.865	
D,L-α-glycerol phosphate	0.841	
Carboxylic acids		
α-Ketobutyric acid		0.971
D-Glucosaminic acid	0.914	
D-Malic acid		–0.835
γ-Hydroxybutyric acid		0.921
Pyruvic acid methyl ester	0.879	
Polymers		
α-Cyclodextrin	0.930	
Tween 40	0.976	
Tween 80	0.966	
Phenolic compounds		
2-Hydroxy benzoic acid		0.932
4-Hydroxy benzoic acid		–0.835

Compounds that did not fall into PC1 and PC2: **Amine** Phenyl ethylamine; **Amino acids** L-Phenylalanine and L-Threonine; **Carbohydrates** D-Xylose; **Carboxylic acids** D-Galacturonic acid and Itaconic acid.

river water samples. The phylogenetic tree (Figure 5) shows the relationship of these 52 isolates based on their 16S rRNA gene sequences. These sequences clustered to two lineages of bacteria: α and γ-*Proteobacteria* and *Firmicutes* (low G+C, mostly gram-positive bacteria). BLAST searches revealed that the 16S rRNA gene sequences of the isolates that fell in *Proteobacteria* were closely related (98-99% related) to a number of *Brevundimonas* (α), *Yersinia* (γ), *Serratia* (γ), *Buttiauxella* (γ), *Escherichia* (γ), *Haemophilus*, *Aeromonas* (γ) and *Pseudomonas* (γ) spp. (Figure 5, Table 5). Most of these sequences were recovered from groundwater samples. The 16S rRNA gene sequences of the isolates that fell into the phylum *Firmicutes* demonstrated a very close relationship (98-99%) with 16S rRNA gene sequences of several *Bacillus*, *Exiguobacterium* and *Staphylococcus* spp., which represented approximately 64% of the total number of unique isolates. Many of the sequences recovered from river water samples clustered into this group, together with 21 16S rRNA gene sequences derived from groundwater samples.

DISCUSSION

The results of this research demonstrated that direct incubation of river and groundwater samples in Biolog Ecoplates produces patterns of metabolic response useful in the classification and characterization of microbial communities. The ability to rapidly visualize community structure as a combination of metabolic potentials enables us to compare microbial communities across a range of spatial scales and identify ecologically relevant functional differences among communities. The microbial community structure of river water was different from the groundwater. This result is not

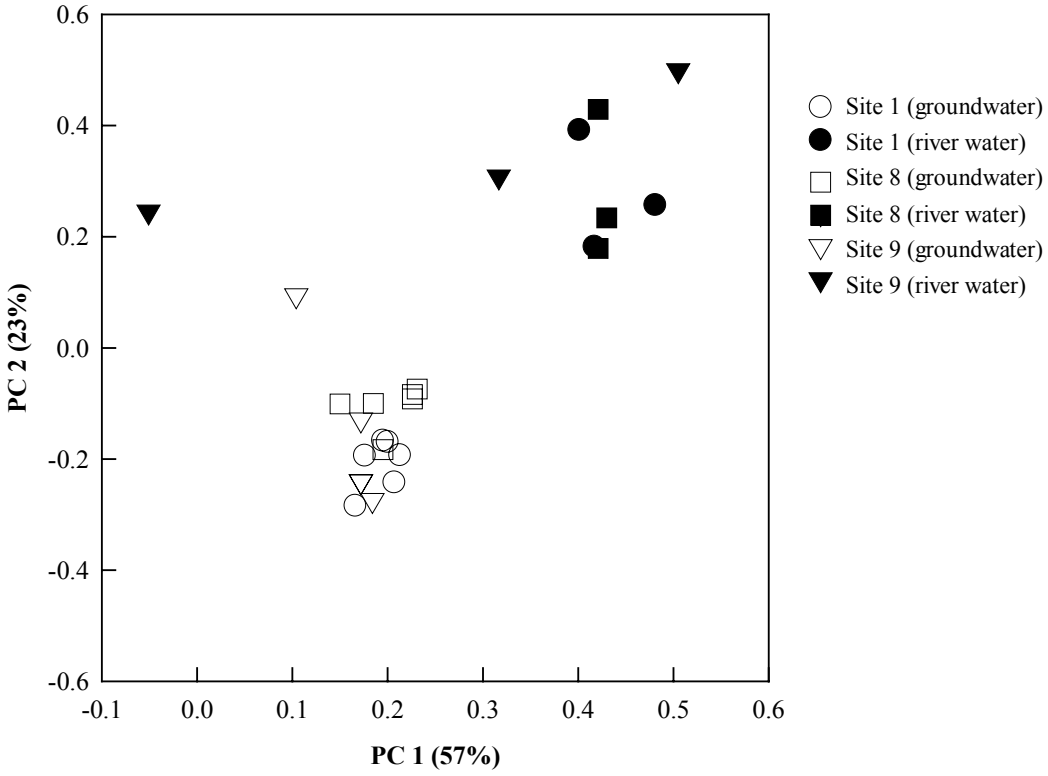

Figure 4. Ordination produced from principal components analysis (PCA) of groundwater and river water samples from the Rouge River. Scores for each sample for the first (PC 1) and second (PC 2) principal components are plotted. Symbol type distinguishes the sample sites associated with groundwater and surface water samples.

surprising since river water often contains a wider range of organic substances than the groundwater, and thus it exhibits higher functional diversity. The grossly differing redox states of the groundwater and the river water also contribute to the differences in microbial communities. Since inoculum density was consistent among all samples with relatively small variations (Figure 3), it is likely that the differences observed are due to changes in microbial community composition. The existence of organisms unable to metabolize substrates on Biolog Ecoplates, many of which are likely to be present in river and groundwater samples, may complicate any attempt to extrapolate from community Biolog profiles to *in situ* community metabolic capacity [8]. Nonetheless, the present study and others [7-12] found Biolog patterns for specific communities to be highly reproducible.

The observed profile of carbon sources metabolized reflected the catabolic potential of the river water and groundwater community. Carbohydrates, carboxylic acids and amino acids are of particular interest since they form the most readily available carbon sources in the environment and represent majority of Biolog Ecoplates. These carbon sources were utilized by the microbial populations from all sites to a greater degree than other carbon sources. Whatever the sampling site studied, carbohydrates tended to be the most utilized substrates by bacteria from the river and groundwater samples. The patterns of utilization of the groups of carbon substrates by the microbial communities revealed differences between river water and groundwater samples. Carbohydrates, polymers, carboxylic acids and amino acids were highly utilized by the microbial communities in the river samples, while carbohydrates, polymers, amino acids and phenolic compounds were profusely metabolized in the groundwater samples. The stimulation of organisms capable of utilizing phenolic compounds in groundwater samples suggests that the plant species on the grasslands along the groundwater wells may release more of these compounds in their rhizosphere. Several *Bacillus pumilus, Brevundomonas diminuta* and *Pseudomonas* spp. isolates (Figure 5, Table 5) known to utilize phenolic compounds were isolated from groundwater samples.

Heterotrophic microbial communities inhabiting the river and groundwater mediate key processes that control ecosystem carbon and nutrient cycling. Although heterotrophic microbes have been resuscitated from river and groundwater systems in many places, the description of these communities from the Rouge River has not yet been documented. The data presented here supplement a few reports on microbes inhabiting river waters [21-23] and groundwater systems [24-27]. The majority of the bacterial isolates in these studies were gram-negative. For example, in

Table 4. Number of heterotrophic isolates showing positive results on 20 different enzymatic assays on API 20E strip.

	Numbers of isolates that tested positive											
	Site mw-1			Site mw-8			Site mw-9					
Enzymatic activity/substrate	1-gw1	1-gw2	1-sw	8-gw1	8-gw2	8-sw	9-gw1	9-gw2	9-sw	gw[a]	sw[b]	Total
Tryptophane deaminase (TDA)	3/3	6/6	7/7	10/10	8/9	7/7	7/8	8/8	4/5	95%	95%	60/63
Gelatinase (GEL)	1/3	4/6	5/7	5/10	6/9	6/7	7/8	4/8	4/5	61%	79%	42/63
Acetoin production (VP)	1/3	3/6	6/7	6/10	7/9	4/7	3/8	0/8	3/5	46%	68%	33/63
β-galactosidase (ONPG)	1/3	5/6	6/7	6/10	5/9	3/7	4/8	0/8	3/5	48%	63%	33/63
Arginine dihydrolase (ADH)	1/3	0/6	2/7	3/10	3/9	1/7	7/8	2/8	2/5	36%	26%	21/63
Citrate utilization (CIT)	0/3	2/6	0/7	0/10	1/9	0/7	4/8	4/8	1/5	25%	5%	12/63
Indole production (IND)	0/3	0/6	0/7	2/10	1/9	0/7	0/8	3/8	2/5	14%	11%	8/63
Fermentation/ Oxidation (arabinose; ARA)	0/3	1/6	2/7	3/10	1/9	1/7	6/8	3/8	2/5	32%	26%	19/63
Fermentation/ Oxidation (mannitol; MAN)	0/3	1/6	1/7	4/10	3/9	0/7	5/8	0/8	2/5	30%	16%	16/63
Fermentation/ Oxidation (glucose; GLU)	0/3	1/6	1/7	3/10	3/9	0/7	3/8	0/8	2/5	23%	16%	13/63
Fermentation/ Oxidation (sucrose; SAC)	0/3	0/6	2/7	1/10	0/9	0/7	6/8	0/8	3/5	16%	26%	12/63
Fermentation/ Oxidation (amygdalin; AMY)	0/3	1/6	1/7	2/10	1/9	0/7	4/8	0/8	2/5	18%	16%	11/63
Fermentation/ Oxidation (rhamnose; RHA)	0/3	2/6	1/7	3/10	2/9	0/7	0/8	0/8	0/5	16%	5%	8/63
Fermentation/ Oxidation (sorbitol; SOR)	0/3	1/6	1/7	1/10	0/9	0/7	2/8	0/8	2/5	9%	16%	7/63
Fermentation/ Oxidation (melibiose; MEL)	0/3	1/6	0/7	1/10	0/9	0/7	0/8	1/8	0/5	7%	0%	3/63
Fermentation/ Oxidation (inositol; INO)	0/3	1/6	0/7	1/10	0/9	0/7	0/8	0/8	0/5	4%	0%	2/63
Ornithine decarboxylase (ODC)	0/3	2/6	0/7	2/10	0/9	1/7	1/8	0/8	1/5	11%	11%	7/63
Lysine decarboxylase (LDC)	0/3	1/6	0/7	1/10	0/9	0/7	0/8	0/8	3/5	5%	16%	5/63
Urease (URE)	0/3	1/6	0/7	0/10	0/9	0/7	1/8	0/8	1/5	5%	5%	3/63
Sodium thiosulfate production (H_2S)	0/3	0/6	0/7	0/10	0/9	0/7	0/8	0/8	0/5	0%	0%	0/63

Note: Total number of isolates 63 tested. gw=groundwater; sw=river water.

[a]Percentage of groundwater isolates from all sites that utilized the various carbon substrates.

[b]Percentage of river water isolates from all sites that utilized the various carbon substrates.

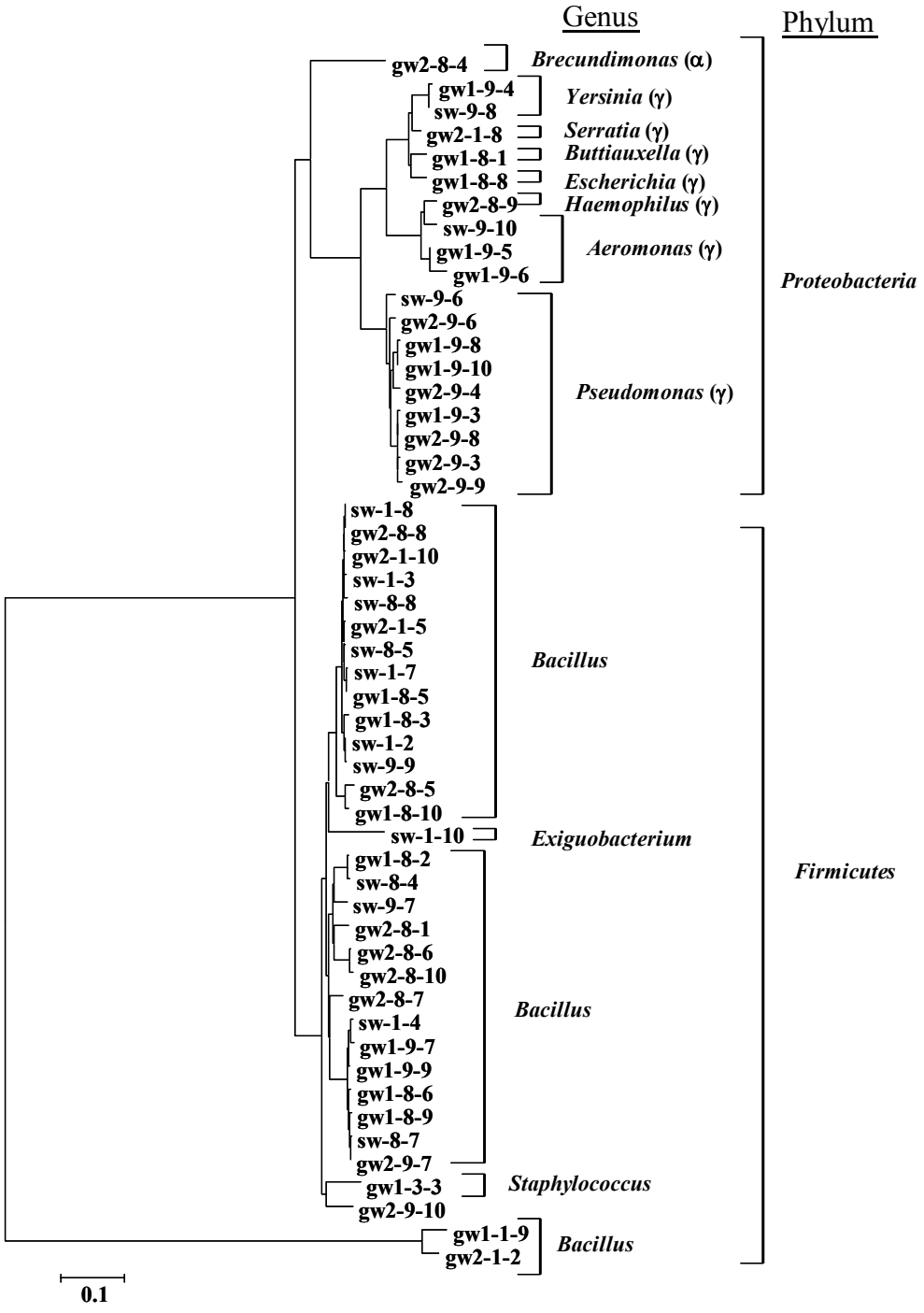

Figure 5. Phylogenetic relationships of bacterial isolates from groundwater and river water samples and reference sequence from Genbank. Phylogenetic trees were constructed in MEGA with Jukes-Cantor distance model. The scale bar indicates the expected number of changes per sequence position. The nearest relatives of each isolate in Genbank are listed (Table 4).

Table 5. Sequence comparisons between 16S rRNA genes sequences of bacterial isolates obtained from groundwater and subsurface water samples of the Rouge River and closest 16S rRNA genes sequences from Genbank.

Sample ID	Site		Closest match	Accession number	Percent nucleotide identity (%)
1-gw1-9	1	groundwater	*Bacillus cereus*	AY224388	99%
1-gw2-5	1	groundwater	*Bacillus pumilus*	AM062682	99%
1-gw2-10	1	groundwater	*Bacillus pumilus*	AB098578	99%
1-gw2-2	1	groundwater	*Bacillus subtilis*	AJ276351	99%
1-gw2-8	1	groundwater	*Serratia fonticola*	AJ233429	98%
1-gw1-3	1	groundwater	*Staphylococcus epidermidis*	AJ717377	99%
8-gw2-9	8	groundwater	*Aeromonas salmonicida*	X74681	98%
8-gw1-6	8	groundwater	*Bacillus cereus*	AM062683	99%
8-gw1-9	8	groundwater	*Bacillus cereus*	AM062683	99%
8-gw2-5	8	groundwater	*Bacillus licheniformis*	AB039328	99%
8-gw2-6	8	groundwater	*Bacillus megaterium*	AJ717381	99%
8-gw2-10	8	groundwater	*Bacillus megaterium*	AJ717381	99%
8-gw2-1	8	groundwater	*Bacillus niacini*	AB021194	98%
8-gw1-5	8	groundwater	*Bacillus pumilus*	AM062682	99%
8-gw2-2	8	groundwater	*Bacillus pumilus*	AM062682	99%
8-gw1-2	8	groundwater	*Bacillus sp.*	AJ315057	99%
8-gw2-7	8	groundwater	*Bacillus sp.*	AJ315068	99%
8-gw1-3	8	groundwater	*Bacillus sp.*	AJ315067	99%
8-gw1-10	8	groundwater	*Bacillus subtilis*	AJ276351	99%
8-gw1-1	8	groundwater	*Buttiauxella agrestis*	AJ233400	99%
8-gw2-4	8	groundwater	*Brevundimonas diminuta*	X87274	97%
8-gw1-8	8	groundwater	*Escherichia coli*	Z83205	99%
9-gw1-5	9	groundwater	*Aeromonas media*	X60410 S42863	99%
9-gw1-6	9	groundwater	*Aeromonas media*	X74679	98%
9-gw1-7	9	groundwater	*Bacillus cereus*	AJ310098	99%
9-gw1-9	9	groundwater	*Bacillus cereus*	AM062683	98%
9-gw2-10	9	groundwater	*Bacillus sphaericus*	AB116123	99%
9-gw2-8	9	groundwater	*Pseudomonas fluorescens*	AY538263	99%
9-gw2-3	9	groundwater	*Pseudomonas frederiksbergensis*	AJ249382	99%
9-gw2-9	9	groundwater	*Pseudomonas frederiksbergensis*	AJ249382	99%
9-gw2-6	9	groundwater	*Pseudomonas libaniensis*	AF057645	99%
9-gw1-8	9	groundwater	*Pseudomonas marginalis*	AB021401	98%
9-gw1-10	9	groundwater	*Pseudomonas marginalis*	AB021401	99%
9-gw2-4	9	groundwater	*Pseudomonas sp.*	AJ002801	98%
9-gw1-3	9	groundwater	*Pseudomonas syringae*	AB001448	98%
9-gw1-4	9	groundwater	*Yersinia intermedia*	X75279	98%
1-sw-2	1	river water	*Bacillus pumilus*	AM062682	99%
1-sw-3	1	river water	*Bacillus pumilus*	AM062682	99%
1-sw-4	1	river water	*Bacillus cereus*	AJ310100	99%
1-sw-7	1	river water	*Bacillus pumilus*	AM062682	98%
1-sw-8	1	river water	*Bacillus pumilus*	AM062682	99%
1-sw-10	1	river water	*Exiguobacterium sp.*	AB219055	99%
8-sw-7	8	river water	*Bacillus cereus*	AM062683	99%
8-sw-5	8	river water	*Bacillus pumilus*	AM062682	99%
8-sw-8	8	river water	*Bacillus pumilus*	AM062682	98%
8-sw-4	8	river water	*Bacillus sp.*	AJ315057	99%
9-sw-10	9	river water	*Aeromonas sp.*	AJ223179	97%
9-sw-9	9	river water	*Bacillus pumilus*	AM062687	99%
9-gw2-7	9	river water	*Bacillus cereus*	AJ310098	99%
9-sw-7	9	river water	*Bacillus sp.*	AB043854	98%
9-sw-6	9	river water	*Pseudomonas sp.*	AJ002813	99%
9-sw-8	9	river water	*Yersinia intermedia*	AF366380	99%

a shallow water table aquifer in Oklahoma [25], about two-thirds of the bacteria isolated were gram-negative. In a carbonate aquifer in the Atlantic Coastal Plain [26], about 70% of the isolates recovered were gram negative. Finally in aquifers of the Atlantic Coastal Plain [24], anywhere from 60% to 90% of the isolates recovered were gram-negative. Among the dominant species in these studies are the *Pseudomonas* spp. In the present study 17% of the isolates were 99% similar to the 16S rRNA sequence of *Pseudomonas* spp. and found both in river water and groundwater samples (Table 5). Pseudomonads are very common in river water and shallow water table aquifers in that they are extraordinarily versatile in the kinds of organic substrates on which they can grow [28, 29]. In one study, a species of *Pseudomonas* was shown to grow on 127 different organic compounds [30]. In addition, some strains of Pseudomonads are capable of dissimilatory Fe (III) reduction.

The gram-positive bacteria isolated from river and groundwater samples were dominated by *Bacillus* spp. Out of 52 strains, 32 were identified as *Bacillus* spp. based on 16S rRNA sequences (Table 5). Members of the genus *Bacillus* have been isolated previously from groundwater systems by Chapelle *et al.* [26]. These spore forming bacteria are widely distributed in soils and are capable of both fermentative and respirative metabolism. Several species of *Bacillus* spp. are important degraders of organic pollutants. For example, *Bacillus cereus* has been found to degrade 1,3-dichlorobenzene derived from town-gas industrial influent [31]; *Bacillus subtilis* has been used to degrade p-aminobenze from textile industry wastewater [32]; and *Bacillus licheniformis* has been shown to degrade organic hydrocarbons in soil [33]. These organisms may play important roles in the fate of many contaminants (both organic and inorganic); although future studies are needed to understand the response of these bacteria to different contaminants from the Rouge River.

The metabolic analysis of microbial communities using Biolog plates has several caveats. First, it is strongly biased towards the culturable, fast-growing species such as Pseudomonads. Second, the plates are biased by including more carbon sources favoring gram-negative over gram positive bacteria. Third, the plate chemistry only detects aerobic respiration. Even with these limitations, Biolog can still be useful in making generalized comparisons of the metabolic potential of the culturable fraction of the community.

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