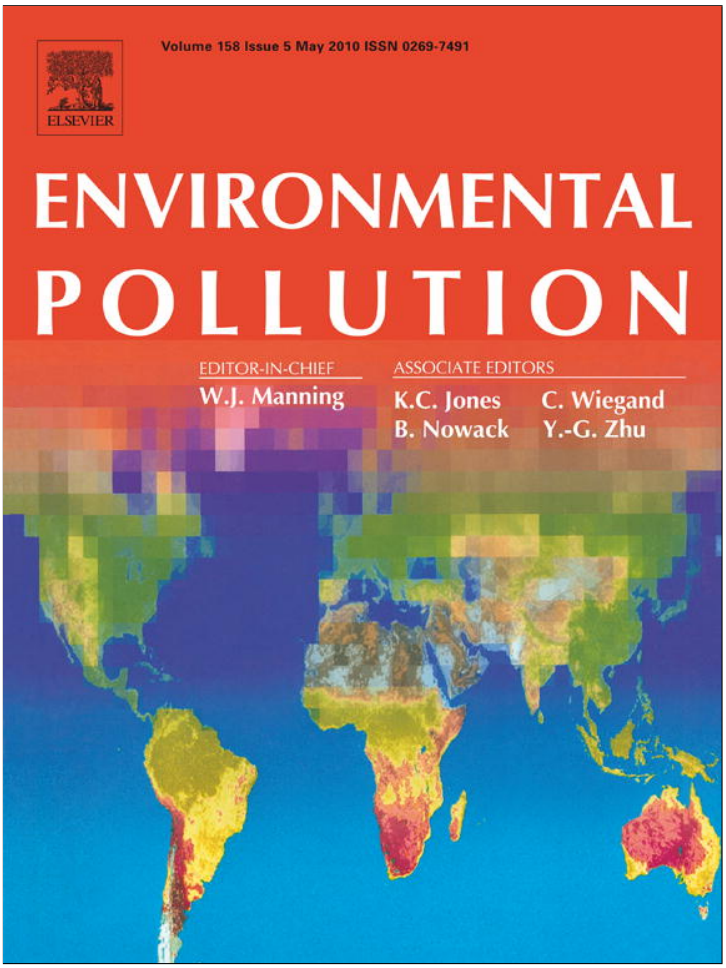

Environmental Pollution 158 (2010) 1435–1443

Contents lists available at ScienceDirect

Environmental Pollution

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

Metabolic diversity of the heterotrophic microorganisms and potential link to pollution of the Rouge River

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BIOLOG differentiated metabolic diversity between microbial communities and can be used as ecological indicator of river function attributable to urbanization and pollution.

ARTICLE INFO

Article history:
Received 19 October 2009
Received in revised form
19 December 2009
Accepted 22 December 2009

Keywords:
Functional diversity
Biolog EcoPlate
Metabolic diversity
Substrate utilization pattern
Community-level physiological profile

ABSTRACT

The heterotrophic microbial communities of the Rouge River were tracked using Biolog Ecoplates to understand the metabolic diversity at different temporal and spatial scales, and potential link to river pollution. Site less impacted by anthrophogenic sources (site 1), showed markedly lower metabolic diversity. The only substrates that were utilized in the water samples were carbohydrates. Sites more impacted by anthrophogenic sources (sites 8 and 9) showed higher metabolic diversity. Higher functional diversity was linked to the physico-chemical and biological properties of the water samples (i.e. higher concentrations of DO, DOC, chlorophyll, and bacterial density). Biolog analysis was found to be useful in differentiating metabolic diversity between microbial communities; in determining factors that most influence the separation of communities; and in identifying which substrates were most utilized by the communities. It can also be used as an effective ecological indicator of changes in river function attributable to urbanization and pollution.

1. Introduction

The Rouge River watershed located in southeastern Michigan consists of over 200 km of streams and is Detroit's primary drainage system, with over 70% of the watershed being developed into residential, commercial and industrial sites (Murray et al., 1997). The area it encompasses is highly urbanized with populations of over 2 million people. Increased pollution and physical alteration of the river, bank erosion, abandoned dumpsites, combined sewer overflows (CSO), and contaminated ground and storm water runoff, are all consequences of the urbanization of Metro Detroit (Murray et al., 1997). Previous investigations of the Rouge River have shown that the industrial activities in Metro Detroit have lead to an increase in harmful environmental pollutants specifically heavy metals, organic pollutants and salts (Kannan et al., 2001; Murray et al., 1997). These pollutants can percolate through the soil and contaminate groundwater. Measuring river health has become a popular way to monitor and direct restoration. Water-quality regulatory guidelines stipulate that a water system must meet criteria to be considered healthy. These criteria include physico-chemical characteristics (i.e. suspended particulate matter, dissolved oxygen, pH, nutrients and salinity) (USEPA Water Quality Criteria, section 304 (a)(1) of the Clean Water Act). More recently, assessment has considered microorganisms as biological indicators of river health (Payment et al., 2000; Murray et al., 2001).

Microbial communities respond rapidly to environmental changes, and are responsible for the regeneration of nutrients through mineralization of organic matter (Allan, 1995). To date, microbiological studies of river waters and shallow groundwater wells along the Rouge River have focused on the enumeration on indicator bacteria that impact surface water quality (Murray et al., 2001) as well as isolation and characterization of bacterial isolates (Tiquia et al., 2007, 2008). The extent of spatial variation of microbial communities, and their possible link to the pollution of the Rouge River and the adjacent groundwater system have not yet been studied. Groundwater and rivers have traditionally been treated as distinct entities in most ecological research, mainly due to differences in accessibility and nature. Hydrologically, surface waters and groundwaters are very closely connected (Castro and Hornberger, 1991). Groundwater ecosystems depend on energy from the surface in the form of dissolved and particulate organic matter (Ghiorse and Wilson, 1988; Madsen and Ghiorse, 1993) and conversely, many streams receive considerable inputs of nutrients from groundwater (Ford and Naiman, 1989; Fiebig and Lock, 1991). While many studies have been conducted on river and microbial communities using molecular tools (Crump et al., 1999; Zwart et al.,

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doi:10.1016/j.envpol.2009.12.035

2002; Araya et al., 2006; Böckelmann et al., 2006; Maksimenko et al., 2008), there were very few reports on the metabolic diversity and their link to pollution in rivers (Harbott and Grace, 2005).

Organic carbon is a key driver of river and groundwater food webs, therefore characterization of the biologically important classes of organic carbon is needed to better understand how the ecosystem processes are affected by urbanization. Heterotrophic bacteria possess enzymes that breakdown a variety of organic compounds into smaller ones so that previously unavailable carbon can be assimilated (Findlay et al., 1997). As the composition of dissolved organic carbon (DOC) changes in aquatic environments associated with perturbation, the production of enzymes by heterotrophic bacteria should change in response to changes in DOC compounds (Moorehead and Sinsabaugh, 2000). Here, we investigated the carbon substrate utilization patterns of microbial communities as a whole, at three different, spatially close sites along the Rouge River using Biolog EcoPlate. Using this method, mixed communities of heterotrophic microorganisms respond to different carbon substrates that they can metabolize, thus providing information about the metabolic diversity of the communities. The ability of a microorganism to utilize different carbon sources is dependent on enzymes that they produce. Some microorganisms use many carbon sources, while others may only use a few. Accordingly, microorganisms may be dependent on just a few key carbon sources, while others are able to use a broader range of them. The main goal of this study was to determine the potential of CLPP (Community-Level Physiological Profile) methodology as an assay for resolving the metabolic diversity of river water and groundwater samples on temporal and spatial scales, and to link the metabolic diversity patterns to river pollution. Three sampling sites (Lotz, Lilley, and Ford Field) were chosen in order to reflect spatial differences. These three sites were sampled during the spring and summer of 2008. Metabolic fingerprints obtained from selected samples were used to understand functional diversity implied by carbon substrate shifts. The DOC and chlorophyll contents of the river water and groundwater samples were also measured to assess the potential of the Biolog Ecoplate technique to discriminate C bioavailability and productivity among sites of contrasting urbanization.

2. Materials and methods

2.1. Site description

The Rouge River is a 203-km watershed that runs through Washtenaw, Oakland and Wayne counties in Southeastern Michigan. Collectively, these three counties occupy approximately 3058 km^2. The Rouge River is primarily the drainage for the City of Detroit and over 66% of the watershed has been developed, ranging from residential and commercial to heavy industrial. The site locations that were surveyed run along the lower branch of the river where the most land use and salt runoff are likely to occur. Throughout much of the watershed, the Rouge River is a relatively shallow stream (average depth in summer is 1 m) with 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 (4–5 events per season) typically increase stream flows to 25 m^3 S^{-1}. On average, the sediment transported and deposited by the Rouge River consists of fine to medium sand, although sample sites in the headwaters of the watershed are generally characterized by silty to fine sandy substrates. The average air temperature in the spring (May–June 2008) was 16 °C (8°C_{min} to 27°C_{max}) whereas it was 20 °C (14 °C_{min} to 26 °C_{max}) in the summer (July–August 2008). Average precipitation in spring was twice higher (0.4 cm) than in summer (0.2 cm). Wind speed (10 km h^{-1}) and barometric pressure (1013 kPa) averages were similar in both seasons.

Three sites surveyed along the Rouge River pass through a heavily commercial and urbanized part of southeast Michigan that parallels a major highway (Michigan Avenue). Sites 1 (Lotz) and 8 (Lilley) represent the locations of major intersections along Michigan Avenue. These two sites are characterized by woods and farmland. Site 9 (Ford Filed) is further east of Michigan Avenue, it is located close to a large recreational site used by the City of Dearborn for sporting and other events. Sites 8 and 9 are located immediately down-gradient of one or more combined sewer overflow (CSO) sites.

Each site contains six groundwater wells, three replicate wells were located on the north side and the other three replicate wells were located on the south side. Water samples were taken from the groundwater wells and river. Nine water samples were collected from each location: three water samples were from groundwater wells located at the north side of the river, and three samples from the south side, and three composite samples from surface (river) water. Collectively, the river water samples are referred to as sw (1-sw, 8-sw, and 9-sw). The groundwater samples are referred to as gw1 and gw2 (1-gw1, 1-gw2, 8-gw1, 8-gw2, 9-gw1, and 9-gw2). The gw1 samples were collected at the north side whereas gw2 samples were taken from the south side of the river. Water samples were taken 4 times during periods of high (spring; May–June) and 4 times during low-flows (summer; July–August) of 2008.

To collect the groundwater samples, a low-Flow purging pump (Geo Scientific, Ltd., Vancouver, British Columbia) was used. This pump drives out water from a well at a very low rate, minimizing agitation and mixing with stagnant water contained in solid casing regions of the well. Groundwater samples were collected at a depth of 1.9–2.1 m (Table 1). On the other hand, surface water samples were collected manually by lowering a sterile glass bottle at a depth of 1 m. At each sampling location, one-hour composite samples were collected. The hourly composite samples were collected by combining 4 individual 400-ml grab samples collected every 15 min into a single container. Following collection, all water samples were placed in coolers and maintained on ice during transport to the laboratory, and stored in a climate-controlled cold room. Since the population of the bacteria in the water samples are likely to change during transport and storage, the samples were, all the samples were analyzed upon arrival to the laboratory (within 8 h of sampling).

2.2. Field data analysis

Dissolved oxygen (DO) was measured in the field at each location using a YSI meter (Model 50B, YSI Inc., Yellow Springs, OH). Water temperature, pH, and electrical conductivity (EC) was recorded using a portable Horiba multimeter (Horiba Model D-24, Equipco, Concord, CA). Ancillary data were also collected at each sampling period, including air temperature, rainfall, wind speed and direction, and presence of animals and debris in the water.

2.3. Dissolved organic carbon (DOC), chlorophyll content and prokaryotic cell density

Water samples for DOC were filtered through 47-mm diameter Whatman GF/C filters and acidified to pH3. Samples were kept in the dark at 4 °C until analyzed on a Shimadzu TOC5000 with platinum-catalyzed high-temperature combustion to CO_2 and infrared detection. Chlorophyll concentration was determined by filtering the water samples through 47-mm diameter Whatman GF/C filters. The filters were extracted in 10 ml of buffered 90% acetone (1 mg°$MgCO_3$°l^{-1}) in the dark at room temperature for 24 h. Chlorophyll was measured by a Turner Designs 10-Au-005CE fluorometer configured with a chlorophyll optical kit. Prokaryotic cell concentration was counted directly with 4′,6′-diamidino-2-phenylindole (DAPI) (Hobbie et al., 1977; Porter and Feig, 1980).

2.4. Assessment of substrate utilization potential

In this study, Biolog EcoPlate™ (Biolog, Inc., Hayward, CA) was used to determine the "metabolic fingerprint" of heterotrophic microorganisms present in the water samples based on the carbon source they utilize. Biolog EcoPlates™ composed of 31 different carbon compounds and a control. It contains three replicates of the carbon source and control wells. A redox dye (tetrazolium violet) is added in each well, which turns purple when the carbon source is used by the microbial communities present in the sample.

Groundwater and river water samples were inoculated into Biolog EcoPlates within 1 day of sample collection. Each well of EcoPlates plates was inoculated with 125 μl of this suspension and incubated at 15 °C. Color development in the microplate wells were measured at 590 nm using an automated Sunrise microplate reader (Tecan, Research Triangle Park, NC). Biolog plates from the spring sampling were read at frequent intervals in order to generate relationship between average well color development and time (data not shown). On the basis of this relationship, 48 h was chosen as an optimum time to compare different water samples, because the average well color development was increasing rapidly (linear phase). Analysis of substrate utilization at different time point readings produced the same results with respect to significance of between samples (groundwater and river samples), sites, (Lotz, Lilley and Ford Field) and seasons (spring and summer) throughout a 24–74 h incubation period.

2.5. Constructing the CLPP

Raw absorbance data were corrected by blanking each response well against its own first reading (immediately after inoculation). This blanking not only avoids the intrinsic absorbance of the carbon sources but also the negative values when compared to subtracting the control well from the response well (Insam and

Table 1
Physico-chemical data, chlorophyll content, and bacterial density of the water samples collected at three different sites during spring and summer.

Sites	Samples	Water depth[a] (m)	Dissolved oxygen (mg l^{-1})	pH	Water temperature (°C)	Electrical conductivity (μS cm^{-1})	Dissolved organic carbon (mg l^{-1})	Chlorophyll content (μg l^{-1})	Bacterial density × 10^5 ml^{-1}
Spring (May–June 2008)									
Site 1	1-gw1	2.05 ± 0.0	3.33 ± 3.06	6.34 ± 0.00	13.8 ± 0.1	3999 ± 101.21	4.97 ± 1.36	0.02 ± 0.01	0.53 ± 0.0028
	1-gw2	1.75 ± 0.0	4.33 ± 0.58	6.47 ± 0.09	13.5 ± 0.1	2216 ± 351.18	3.17 ± 0.35	0.03 ± 0.01	0.27 ± 0.0064
	1-sw	1.00 ± 0.0	12.67 ± 3.06	6.80 ± 0.08	15.8 ± 0.0	1535 ± 24.66	8.66 ± 1.75	2.50 ± 0.50	0.95 ± 0.0071
Site 8	8-gw1	2.07 ± 0.0	4.67 ± 1.15	7.21 ± 0.02	15.6 ± 0.0	1488 ± 11.01	3.73 ± 1.10	0.02 ± 0.01	0.14 ± 0.0053
	8-gw2	2.03 ± 0.0	1.00 ± 1.00	6.55 ± 0.00	12.0 ± 0.0	2155 ± 173.22	3.83 ± 1.76	0.04 ± 0.02	0.13 ± 0.0064
	8-sw	1.00 ± 0.0	18.67 ± 2.31	6.88 ± 0.01	16.0 ± 0.1	1402 ± 28.62	7.60 ± 0.89	3.77 ± 0.23	3.50 ± 0.0212
Site 9	9-gw1	1.91 ± 0.0	4.33 ± 2.08	7.12 ± 0.00	11.4 ± 0.1	3447 ± 226.14	3.77 ± 1.88	0.01 ± 0.01	0.47 ± 0.0099
	9-gw2	1.97 ± 0.0	2.67 ± 1.15	6.78 ± 0.06	11.8 ± 0.0	1357 ± 45.08	3.83 ± 0.64	0.02 ± 0.01	0.25 ± 0.0070
	9-sw	1.00 ± 0.0	4.33 ± 3.51	6.66 ± 0.00	15.9 ± 0.0	1455 ± 41.29	9.67 ± 2.05	3.80 ± 0.72	2.00 ± 0.0414
Summer (July–August 2008)									
Site 1	1-gw1	1.90 ± 0.0	3.20 ± 0.26	6.67 ± 0.00	15.1 ± 0.0	2695 ± 101.28	2.97 ± 0.31	0.02 ± 0.01	1.75 ± 0.0354
	1-gw2	1.80 ± 0.0	3.67 ± 0.35	6.08 ± 0.00	15.8 ± 0.0	2115 ± 192.54	3.00 ± 0.26	0.02 ± 0.00	1.90 ± 0.0141
	1-sw	1.00 ± 0.0	7.67 ± 2.52	7.08 ± 0.01	17.6 ± 0.1	1738 ± 7.93	7.90 ± 0.40	2.17 ± 0.29	33.00 ± 0.0414
Site 8	8-gw1	1.75 ± 0.0	2.07 ± 0.21	7.00 ± 0.01	15.3 ± 0.0	1585 ± 37.52	3.13 ± 0.06	0.03 ± 0.01	1.55 ± 0.0071
	8-gw2	1.90 ± 0.0	2.83 ± 0.76	6.38 ± 0.03	14.1 ± 0.0	2052 ± 126.57	4.07 ± 1.01	0.03 ± 0.01	1.80 ± 0.0283
	8-sw	1.00 ± 0.0	4.00 ± 1.73	6.73 ± 0.01	16.6 ± 0.0	1801 ± 25.89	7.13 ± 0.21	5.01 ± 0.01	2.75 ± 0.0354
Site 9	9-gw1	1.95 ± 0.0	2.80 ± 0.20	7.11 ± 0.00	13.4 ± 0.1	2947 ± 13.07	2.53 ± 0.15	0.03 ± 0.01	2.10 ± 0.0269
	9-gw2	1.98 ± 0.0	3.03 ± 0.95	6.35 ± 0.00	13.9 ± 0.1	1357 ± 16.80	2.17 ± 0.12	0.03 ± 0.01	21.00 ± 0.0919
	9-sw	1.00 ± 0.0	3.33 ± 0.58	6.58 ± 0.00	16.1 ± 0.0	1455 ± 27.83	9.73 ± 0.68	6.87 ± 0.25	78.00 ± 0.0325
T-tests									
Spring vs. summer		0.28^{ns}	0.14^{ns}	0.31^{ns}	0.003^{**}	0.41^{ns}	0.020^{*}	0.17^{ns}	0.019^{**ns}
sw vs. gw		0.0001^{***}	0.004^{**}	0.39^{ns}	0.0001^{***}	0.009^{**}	0.0001^{***}	0.0001^{***}	0.044^{**}

gw = groundwater; sw = river water; vs. = versus *t*-test with $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; ns = not significant.
[a] The water depth (m) corresponds to the depth at which the water samples were collected.

Goberna, 2004). Average well color data (AWCD) were calculated according to Garland and Mills (1991). The relative abundance of the different substrates was determined based on the amount (AWCD value) of substrate that was utilized relative to the total amount of substrates (sum of AWCD values of each plate) used by the microbial community, tested based on the measured densities of reduced tetrazolium dye in wells.

2.6. Statistical analyses

Analysis of variance (ANOVA) was calculated to determine differences between three sites based on the AWCD data from each carbon source. When ANOVA showed significant difference between sites ($P = \leq 0.05$), means were separated using Bonferroni's *t*-test ($P = \leq 0.05$). Paired *t*-test was also performed to compare CLPP patterns between two seasons (spring and summer) and two water samples (river water and groundwater).

Principal components analysis (PCA) was used to determine differences between patterns of the substrate utilization profiles. PCA is a statistical method used to reduce the number of variables to a smaller number of new variables called principal components (PCs). To compare the utilization profiles, the samples were compared with the 31 variables (C substrates). This was accomplished by projecting the original data onto new axes, or PCs (Lebart et al., 1984). These PCs were ranked according to the amount of variance of the original sample that is accounted for by each PC. PC1 accounts for most of the variance, PC2 accounts for the next greatest amount of variance. Basically, the farther apart two samples were in the original (31-dimensional) space, the farther apart they were in 2-dimensional space. Differences in CLPP patterns in groundwater and river water samples were tested statistically by comparing principal component scores 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.

Hierarchical cluster analysis (HCA) was used to detect differences among the patterns of substrate utilization at the 3 sites sampled. The data were assembled into a large matrix. Each sample was placed into one of the *n* rows with *p* columns of variables, in this case the AWCD data. The samples were treated as *n* points in a *p*-dimensional space. HCA groups data into clusters with similar attributed by determining the relative distance between pairs of samples in a space (Lebart et al., 1984). Euclidean distance and centroidal clustering were used in this analysis. Data from HCA were used to construct dendrogram. The shorter the branch lengths, the greater the similarity and therefore, the smaller the distance are between samples. Distance was measured from 0 to 1.5, with 0 corresponding to an exact duplicate and 1.5 indicating maximum relative dissimilarity within the data set. All Statistical analyses were calculated using SYSTAT statistical computing package (SYSTAT Version 9.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Field data and physico-chemical properties of the water samples

Water depth between seasons remained constant. Surface water temperature showed slight seasonal variation with average values ranging from 11.4 °C to 16.0 °C in the spring, and 13.4–17.6 °C in the summer (Table 1). DO and DOC concentrations were higher in the spring (DO = 6.22 ± 5.68 mg l^{-1}; DOC = 5.47 ± 2.48 mg l^{-1}) than in summer (DO = 3.62 ± 1.61 mg l^{-1}; DOC = 4.74 ± 2.77 mg l^{-1}) with pH values (sprig = 6.76 ± 0.29; summer = 6.66 ± 0.36), EC (summer = 2117 ± 974 μS cm^{-1}; spring = 1972 ± 545 μS cm^{-1}), and chlorophyll contents (spring = 1.13 ± 1.71 μg l^{-1}; summer = 1.58 ± 2.61 μg l^{-1}) WERE relatively close between seasons. Bacterial densities were significantly higher ($P = 0.019$) in the summer (0.92 ± 1.73 × 10^5 m^{-1}) than in spring (15.98 ± 25.83 × 10^5 m^{-1}).

The river water samples generally had higher DO (sw = 7.69 ± 5.81 mg^{-1}; gw 3.01 ± 1.03 mg^{-1}), water temperature (sw = 16.3 ± 0.03 °C; gw = 13.6 ± 1.49 °C), and DOC (sw = 8.38 ± 1.04 mg l^{-1}; gw = 3.26 ± 0.75 mg l^{-1}) chlorophyll content (sw = 4.24 ± 1.65 μg l^{-1}; gw = 0.03 ± 0.01 μg l^{-1}) and bacterial density (sw = 25.99 ± 29.51 × 10^5 m^{-1}; gw = 3.44 ± 5.83 × 10^5 m^{-1}) than the groundwater samples. Groundwater samples, on the other hand, had higher EC than the river

water samples (gw = 2190 ± 844.60 μS cm^{-1}; sw = 1598 ± 157.99 μS cm^{-1}), but their pH values were similar (gw = 6.68 ± 0.37; sw = 6.79 ± 0.17).

The physico-chemical properties of the river water samples were significantly different from the groundwater samples (Table 1). Seasonal variation (spring and summer) did not significantly change the properties of the water samples with the exception of water temperature and bacterial populations, which showed statistically higher values in the summer than in spring (Table 1).

3.2. Community-level physiological profiles

Previous studies using Biolog assays suggested aggregating responses to substrates within functional classes such as amines, amino acids, carbohydrates, carboxylic acids, polymers, and phenolic compounds, to highlight general patterns of substrate utilization (Lehman et al., 1995; Zak et al., 1994). In this study, the 31 carbon sources were grouped to sum utilization responses to all substrates within these six functional classes. A complete list of the substrate with class is noted in Table 2. The CLPP from different water samples showed that the measured utilization typically differed among substrates, and revealed differences and similarities among type of water sample, seasons, and sites. Although all 31 substrates were used by the microbial communities at each site (Fig. 1), the relative abundance based on AWCD for each substrate was different. Substrates that produced consistently low relative responses were probably poorly degraded by the microbial communities studied here. However, most substrates apparently supported at least some bacterial activity. In this study, we considered 4% absorbance of the total absorbance per plate as the threshold for substrate utilization (Fig. 1).

Table 2
Results of *t*-test showing the effect of season (spring vs. summer) and type of water sample (river water vs. groundwater) on the 31 carbon sources.

Carbon source[a]	Spring vs. summer		River water vs. groundwater	
	P-values	Significance	*P*-values	Significance
Amines				
Putrecine	0.595	ns	0.011	*
Phenylethylamine	0.631	ns	0.032	*
Amino acids				
L-Arginine	0.461	ns	0.019	*
L-Asparagine	0.222	ns	0.018	*
L-Phenylalanine	0.043	*	0.013	*
L-Serine	0.754	ns	0.004	**
Glycyl-L-glutamic acid	0.533	ns	0.018	*
L-Threonine	0.733	ns	0.010	*
Carbohydrates				
α-D-lactose	0.685	ns	0.001	***
β-Methyl D-glucoside	0.631	ns	0.241	ns
D-Cellobiose	0.433	ns	0.008	**
D-Mannitol	0.657	ns	0.017	*
I-Erythritol	0.733	ns	0.006	**
Glucose-1-phosphate	0.503	ns	0.004	**
D-galactonic acid γ-lactone	0.519	ns	0.005	**
N-acetyl-D-glucosamine	0.048	*	0.020	*
D,L-α-glycerol phosphate	0.604	ns	0.010	*
D-Xylose	0.550	ns	0.004	**
Carboxylic acids				
α-Ketobutyric acid	0.309	ns	0.010	*
D-Glucosaminic acid	0.290	ns	0.009	**
D-Malic acid	0.026	*	0.310	*
γ-Hydroxybutyric acid	0.722	ns	0.004	**
Pyruvic acid methyl ester	0.414	ns	0.040	*
D-Galacturonic acid	0.414	ns	0.008	**
Itaconic acid	0.636	ns	0.004	**
Polymers				
α-Cyclodextrin	0.698	ns	0.018	*
Tween 40	0.901	ns	0.011	*
Tween 80	0.028	*	0.049	*
Glycogen	0.697	ns	0.061	ns
Phenolic compounds				
2-Hydroxy benzoic acid	0.021	*	0.005	**
4-Hydroxy benzoic acid	0.971	ns	0.002	**

T-test with $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; ns = not significant.
[a] Substrates present in the Biolog ECO microplates are divided into categories according to the nutrients they provide.

3.3. Analysis of substrate utilization data from groundwater and river water

Inspection of the CLPPs suggests that some differences between the groundwater samples and the river water samples (Fig. 1). In general, fewer numbers of substrates were used in the groundwater samples (9–13) than the river water samples (1–28). The river water samples varied considerably with regard to the number of utilized substrates, with higher number in the spring (15–28) compared to summer (1–11). The groundwater samples showed lower number of substrate utilization, with only the spring samples showing signals higher than the threshold for substrate utilization (4%). *T*-test results showed significant difference between two water samples. The river water samples were characterized by significantly higher (*t*-test, $P \leq 0.001$) utilization of 13 substrates than the groundwater samples: 1 amino acid (L-serine); 6 carbohydrates (α-D-lactose, D-cellobiose I-erythritol, Glucose-1-phosphate, D-galactonic acid γ-lactone and D-xylose); 4 carboxylic acids (D-glucosaminic acid, γ-hydroxybutyric acid, D-galacturonic acid, and Itaconic acid); and 2 phenolic compounds (2-hydroxy benzoic acid and 4-hydroxy benzoic acid) (Fig. 1).

3.4. Seasonal patterns of substrate utilization

The breakdown of the percent utilization of the carbon sources into six substrate guilds showed very similar use between two seasons (Fig. 2). *T*-test results indicated no significant seasonal trends for all 31 carbon sources with the exception of 5 carbon sources: L-phenylalanine, N-acetyl-D-glucosamine, D-malic acid, Tween 80 and 2-hydroxy benzoic acid (Table 2). Although the differences between seasons were not significant for most carbon sources, more substrates were utilized in summer (13–28) than in spring (9–11) (Fig. 1). Another key difference included the higher utilization of carbohydrates, polymers, and phenolic compounds in the summer, and carboxylic acids and amino acids in the summer (Fig. 2). Interestingly, neither D-glucosamic acid nor D-malic acid were utilized in both seasons.

3.5. Comparison of substrate utilization data at different sites

Frequency distribution data based on substrate categories of the river water and groundwater samples showed some differences among the three sites (Fig. 3). River water samples from sites 1 and 9 were very similar and showed significantly higher amino acid ($P = 0.043$) and carbohydrate ($P = 0.031$) utilization than site 8 (Fig. 3A). At these two sites, the river water samples most often used amino acids were L-arginine, L-asparagine, and L-serine, whereas the most common used carbohydrates were N-acetly-D-glucosamine, D-galactonic acid γ-lactone, D-mannitol, β-methyl-D-glucoside, and α-D-lactose. The number of amine, carboxylic acid, polymer and phenolic acid utilization of the river water samples was similar among all three sites (Fig. 3A). Significant differences between the three sites were observed from the groundwater

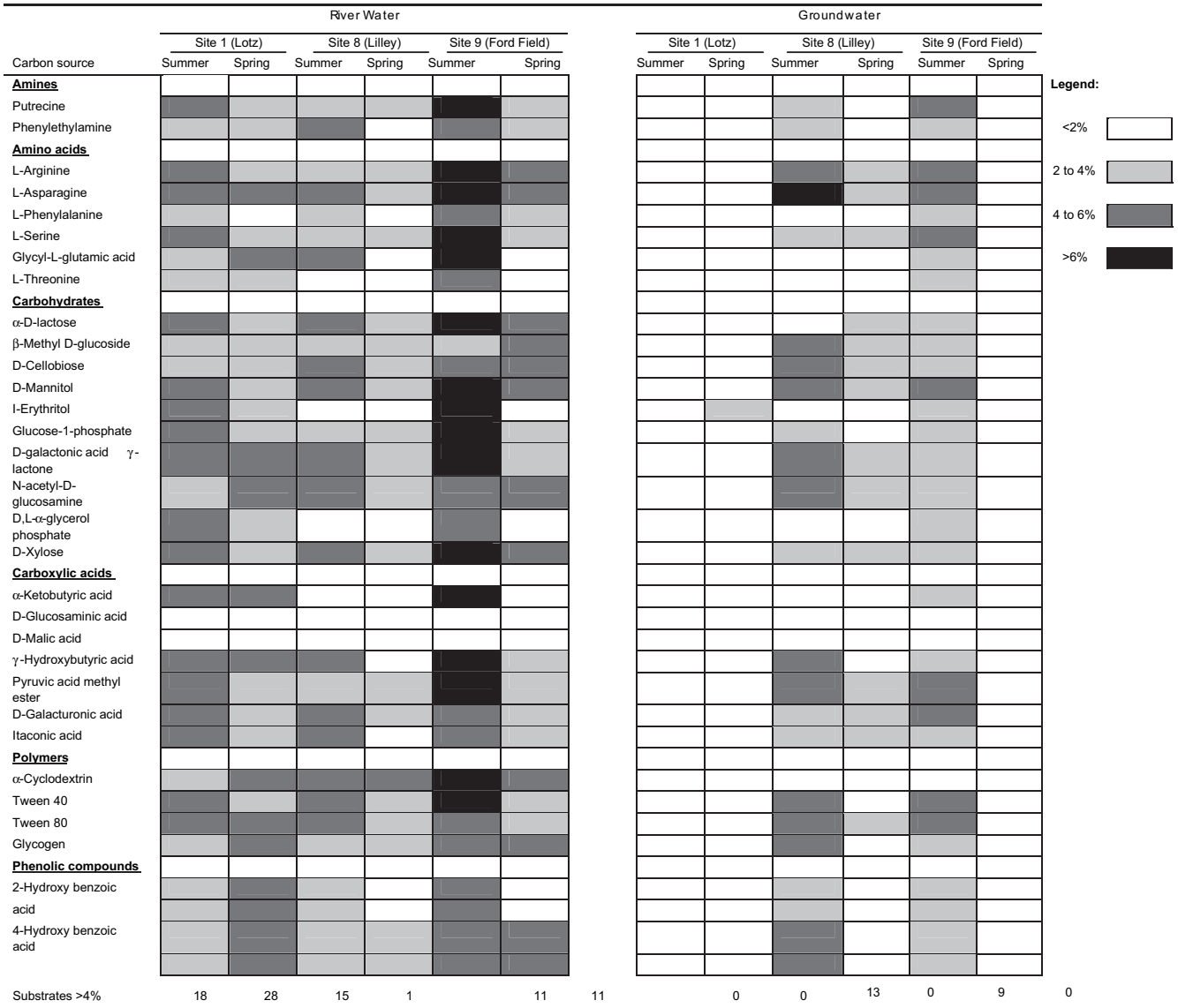

Fig. 1. Pattern of utilization (based on mean AWCD) of the 31 carbon substrates for the groundwater and river water samples taken during spring and summer from sites 1 (Lots), 8 (Lilley) and 9 (Ford Field). Shading in the boxes indicates the range of percentage absorbance of the total absorbance of the plate. Values are as follows: White, <2%; light grey, 2–4%; dark grey, 4–6%; black, >6%. The number of substrates with >4% absorbance for each sampling site is indicated below each column.

profiles. The three sites were statistically different ($P \leq 0.05$) in terms of the number of all six functional substrates (Fig. 3B). Groundwater samples from sites 8 and 9 (sites exposed to more anthropogenic sources), showed higher carbon utilization than site 1 (site exposed to less anthropogenic sources). None of carbon sources was utilized from the groundwater samples from site 1 except for carbohydrates (N-acetly-D-glucosamine, D-galactonic acid γ-lactone, and α-D-lactose) (Fig. 3A).

3.6. Multivariate analysis

HCA was performed on the transformed Biolog data collected from the Rouge River to group the data by similarity. The data were separated into two main clusters (Fig. 4). Sample type (river water and groundwater) was the most obvious difference between the two clusters. The upper leaves (cluster 1) were all river samples, while the lower leaves (cluster 2) were mostly groundwater samples (12 out of 14). There was no clear separation between seasons. The most dissimilar samples (cluster 1) were river water samples collected from sites 8 and 9 (sites more affected by human activities) and apparently showed the greatest number of substrates utilized in this study (Figs. 3 and 4). The groundwater samples were more closely associated with each other with less distance between them, indicating a less diverse community than the river water samples (Fig. 4).

PCA was carried out on 54 transformed Biolog data sets from groundwater and river water samples for both spring and summer (Fig. 5). The two principal components (PCs) accounted for 81.6% of the total variance. PC1 separated the groundwater samples from site 1 (the site less affected by anthropogenic sources) from the rest of the samples (Fig. 5). The groundwater samples from site 1 showed the lowest number of substrates utilized both in the spring and summer trials (Fig. 1). PC2 separated the groundwater samples from the river water samples (Fig. 5). To relate the utilization of individual carbon substrates to the differences in the sole carbon source utilization patterns, the correlation between the substrate variables and the PCs were examined (Table 3). The higher the correlation, the more important the substrate was for

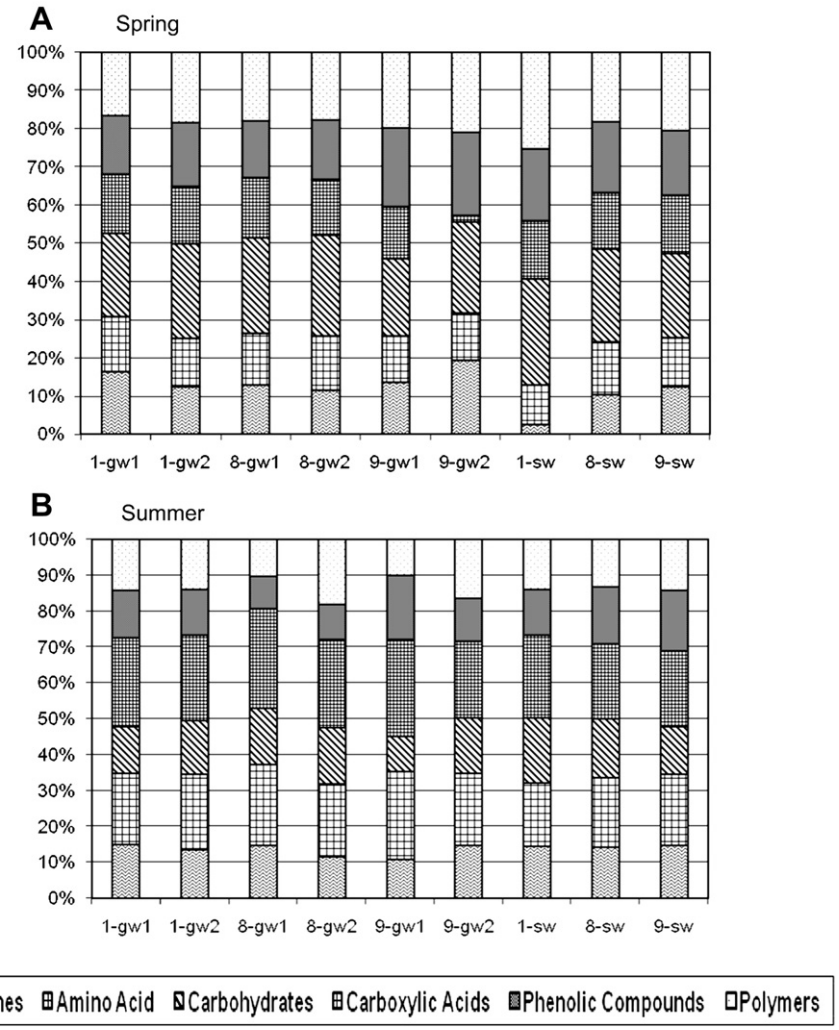

Fig. 2. Relative distribution of the substrates used separated into substrate categories for each sampling site. The river water samples are referred to as sw whereas the groundwater samples are referred to as gw1 (north side) and gw2 taken (south side). Samples were collected from site 1 (1-gw1, 1-gw2, 1-sw), site 8 (8-gw1, 8-gw2, and 8-sw) and site 9 (9-gw1, 9-gw2, and 9-sw). Mean values of the different sampling times were used to calculate the relative distribution of the substrates from each site.

differentiating between samples. A high correlation does not imply that the carbon source was well utilized. A high correlation means that the difference in the utilization of the carbon source was highest between samples. For PC1, the variability was explained a contrast among utilization of amines, amino acids, carbohydrates, carboxylic acids, polymers and phenolic compounds. Variability in PC2 was explained principally by responses of amines, carboxylic acids and phenolic compounds (Table 3).

4. Discussion

Rivers are an important link between terrestrial environments, lakes and the ocean, and represent dynamic systems with highly variable conditions; on both temporal and spatial scales (Leff, 1994). They are frequently subjected to anthropogenic impact. Organic carbon utilization by heterotrophic bacterial strongly influences internal nutrient cycling in this system and impacts eutrophication process (Sinsabaugh et al., 1997). More specifically, the sediments release nutrients into the water column in greater quantities, which are then utilized by the indigenous bacteria in the river water and adjacent groundwater. The study presented here attempted to monitor metabolic diversity in river water and the adjacent groundwater using, a phenotypical approach (Biolog EcoPlates), and the potential of this technique to link pollution among sites of contrasting urbanization. The CLPP data generated from the EcoPlates represents the microbial community functional potential. By considering the limitations of EcoPlates (i.e. inoculum density, incubation temperature) and modifying our protocol to avoid these limitations, the results can be robustly interpreted.

Functional diversity, understood in this study as the utilization of carbon sources in Biolog EcoPlates, showed distinct differences between groundwater and river water samples. PCA analysis based on carbon substrate utilization indicated that carbohydrates, carboxylic acids, polymers and amino acids contributed to significant variation in increased and/or decreased substrate utilization between two water samples. In general, river water samples were characterized by a higher utilization of these four functional substrates with AWCD values significantly higher than the groundwater samples (Fig. 1). The higher functional diversity implicated in the river water was due to its physico-chemical and biological properties. It generally had higher concentrations of DO, DOC and chlorophyll, and bacterial density (Table 1). The river water was exposed to light and therefore, had higher primary production (higher chlorophyll content). Due to the lack of light, no primary production took place in the groundwater; hence the chlorophyll content was negligible (Table 1). As a result, the groundwater food

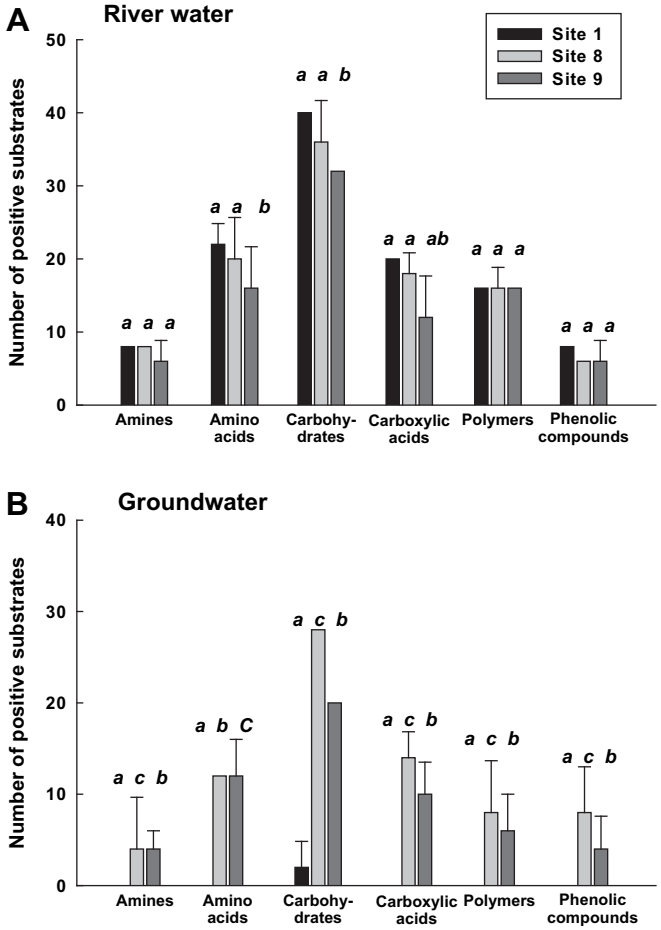

Fig. 3. Number of positive substrates separated into substrate categories for samples from different sites. For each site, at each substrate category, values represented by histograms with the same letter are not significantly different ($P = 0.05$).

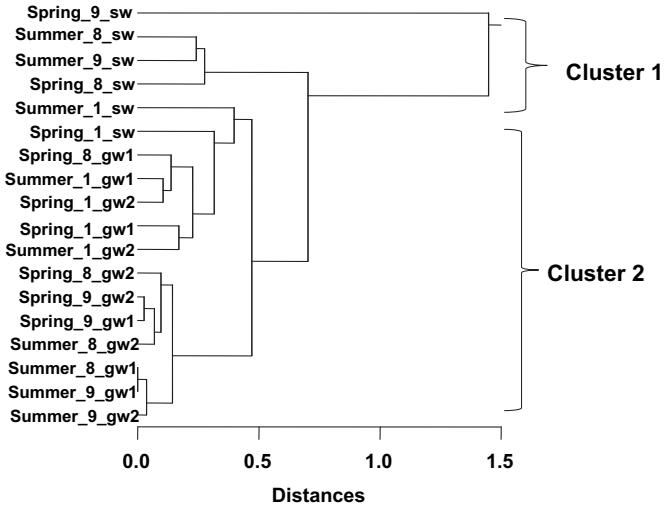

Fig. 4. A hierarchical cluster analysis of the mean AWCD used to detect differences among the patterns of substrate utilization at the 3 sites sampled between spring and summer. B. Matrix showing the 31 different carbon sources utilized at each sampling site. The river water samples are referred to as sw whereas the groundwater samples are referred to as gw1 and gw2 taken at site 1 (1-gw1, 1-gw2, 1-sw), site 8 (8-gw1, 8-gw2, and 8-sw) and site 9 (9-gw1, 9-gw2, and 9-sw).

web was almost heterotrophic and contained more recalcitrant organic matter. Groundwater bacteria have much lower density and are much less capable of responding to nutrient input due to greater environmental stress (Hazen et al., 1991). This was supported by the lower frequency of the groundwater samples to assimilate Biolog Ecoplate compounds, hence the lower metabolic diversity.

No clear seasonal patterns of carbon source utilization were found, since very few carbon substrates showed significant difference between seasons (Table 1). Nonetheless, more substrates were utilized in summer than in spring (Fig. 1). This trend corresponded to the higher bacterial density and lower DO of the water samples in the summer. The mean seasonal values shown in Fig. 4 indicated that samples clustered based on sample type rather than seasons or sampling site. Most substrates in the Biolog EcoPlates were used at various extents during both seasons; however, carbohydrates, polymers, and phenolic compounds were utilized more frequently by the microbial populations in the spring, and carboxylic acids and amino acids in the summer (Fig. 4). During the onset of the spring, characterized by depletion of dissolved oxygen, the bacterial assemblages preferably used carbohydrates over carboxylic acids and amino acids. As summer progressed, indicated by warm temperature and replenishment of dissolved oxygen, the substrate preference shifted to carboxylic acids and amino acids. One possible explanation for the pattern of high carbohydrate utilization is that the bacterial populations that are opportunistic and capable of using carbohydrates increase when the supply of carbohydrates is increased. Much of the dissolved organic matter that is released in the river is fixed carbon, mostly carbohydrates (Biddanda and Benner, 1997; Grover and Chrzanowski, 2000). Nonetheless, the preferred catabolic pathway of most aerobic and facultative heterotrophs is the one that involves oxidation of carbohydrates with oxygen being the terminal electron acceptor (Rosenstock and Simon, 2003). Other causes could be due to algal bloom collapses which cause autolysis and grazing, both which release carbohydrates into water (Grover and Chrzanowski, 2000). Lower carbohydrate utilization in the summer season is possibly attributed to depletion of carbohydrates.

Carboxylic acid utilization was significantly higher in the summer possibly due to longer photoperiods during the summer, which would increase the amount of organic acid rich dissolved organic compounds in the water, selecting for organic acid utilizing bacteria (Christian and Lind, 2007). Carboxylic acids are a very diverse group of substrates both molecularly as well as their chemical configurations. There is very little known about them in

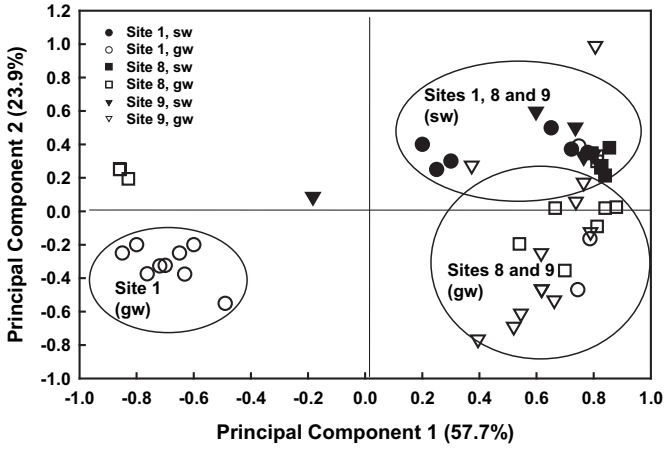

Fig. 5. Ordination diagrams of CLPPs from principal component analyses of carbon source utilization profiles of river water and groundwater samples collected during spring and summer. gw = groundwater; sw, river water; site 1 (Lotz); site 2 (Lilley); site 3 (Ford Field).

Table 3
Correlation coefficients for scored of the first two principal components (PC1 and PC2) from the water samples collected at three different sites. All substrates with an r value > 0.5 are sample are shown ($P < 0.001$); r = Pearson correlation coefficient.

Carbon source	PC1	PC2
	r	
Amines		
Putrecine		−0.521
Phenylethylamine	0.793	
Amino acids		
L-Arginine	0.744	
L-Asparagine	0.825	
L-Phenylalanine	−0.763	
L-Serine	0.789	
Glycyl-L-glutamic acid	0.632	
L-Threonine	0.699	
Carbohydrates		
α-D-lactose	0.665	
β-Methyl D-glucoside	0.812	
D-Cellobiose	0.722	
D-Mannitol	0.793	
I-Erythritol	0.700	
Glucose-1-phosphate	0.736	
D-galactonic acid γ-lactone	0.616	
N-acetyl-D-glucosamine	0.813	
D,L-α-glycerol phosphate	0.779	
D-Xylose	0.651	
Carboxylic acids		
α-Ketobutyric acid	0.540	−0.551
D-Glucosaminic acid	0.618	
D-Malic acid		0.502
γ-Hydroxybutyric acid		
Pyruvic acid methyl ester		−0.511
D-Galacturonic acid		
Itaconic acid		
Polymers		
α-Cyclodextrin	0.719	
Tween 40	0.662	
Tween 80	0.618	
Glycogen	0.828	
Phenolic compounds		
2-Hydroxy benzoic acid	0.598	−0.597
4-Hydroxy benzoic acid		−0.532

aquatic and sediment systems (Christian and Lind, 2007). Carboxylic acids are an important carbon source for bacterioplankton in the Mediterranean Sea and other aquatic environments (Obernosterer et al., 1999; Pullin et al., 2004; Sala et al., 2006a) and are considered part of the liable pool of organic matter.

In the summer, groundwater and river water populations utilized more amino acids. Unlike carbohydrates and carboxylic acids, amino acids are rich in nitrogen (Madigan et al., 1997). Hence, the microbial communities would use the amino acids for nitrogen by incorporating the ammonium side chain in addition to using carbon. The ammonium is then made into organic molecules such as amino acids and proteins (Hollibaugh and Azam, 1983). This shift could be also be due to the same driving factor as before, increased supply of amino acids would favor opportunist classes of bacteria capable of utilizing amino acids. Seasonal variations of amino acid uptake suggest that amino acids originate from algal excretions (Hellebust, 1965).

This investigation also explored carbon utilization patterns at the different sites (Lotz, Lilley, and Ford Field). Very little difference was observed among the river water samples at three different sites (Fig. 3A). However the metabolic diversity between sites suggests that these microbial communities could have a higher number of metabolic pathways allowing them to exploit many different carbon sources. Sala et al. (2006a,b, 2008) reported on the changing of bacterial assemblages due to the shifts in organic matter. For instance, sewage outflows, runoff from urbanized surfaces as well as municipal and industrial discharges could increase the dissolve organic matter of the river, thereby changing the population and composition of the microbial communities of the river water. The most significant difference was observed from the groundwater samples at different sites, where carbon utilization patterns among microbial communities at different sites showed distinct variations (Fig. 3B).

Although the DOC concentrations were of the same order of magnitude, there was enough variation to detect a positive correlation between DOC content and river pollution. In the present study, DOC concentrations were higher from sites more impacted by anthropogenic sources (i.e. sites 8 and 9). Bank erosion, abandoned dumpsites, combined sewer overflows (CSO), contaminated ground, and storm water runoff accounted to a large extent for increases in DOC concentrations at these sites. Increases in DOC in this study might have favored the growth of bacterial assemblages with higher number of metabolic pathways that can exploit a wide variety of DOC molecules. Site 1 (less impacted by anthrophogenic sources), showed a markedly lower metabolic diversity, particularly the groundwater samples. The only substrates that were utilized in these water samples were carbohydrates. This result indicated that much of the dissolved organic matter that the groundwater is possibly fixed carbon, mostly carbohydrates (Ford and Naiman, 1989). The DOC and bacterial density at this site were also lower compared to that from sites 8 and 9 (Table 1).

Chlorophyll content showed correlation with metabolic diversity among river water samples from three different sites. If the number of substrates was used as an estimation of metabolic diversity, our results suggest a negative correlation. That is, the lower is the chlorophyll content; the higher is the metabolic diversity. At sites with higher metabolic diversity, chlorophyll contents were lower (site 8, sw = 4.38 ± 0.23 μg l^{-1}; site 9, sw = 5.37 ± 0.67 μg l^{-1}) than the site with lower metabolic diversity (site 1, rw = 2.34 ± 0.23 μg l^{-1}). This finding contradicts the results of Horner-Devine et al. (2003) who noted that primary activity has no effects on bacterial phylogenetic richness. Sala et al. (2006a) that show that a negative correlation between chlorophyll concentration and metabolic diversity. These results suggest that the microbial communities in the aquatic systems have a higher number of metabolic pathways to exploit a wide variety of DOC molecules present at low concentrations.

This study explored the metabolic capacities of microbial communities as a whole of the Rouge River, and our results suggest that this pattern can also be applied to other river ecosystems. Using Biolog EcoPlates, distinct shifts in microbial communities were observed between river water and groundwater samples at collected at different sites and season. Biolog EcoPlates are not capable of determining the species composition of a natural sample, but as has been demonstrated here, they are useful in differentiating between microbial communities, in the determining factors that most influence the separation of these communities and in identifying which substrates were most utilized by the communities. This information is also crucial to understanding the physiology of microbial communities in this environment. The Biolog Ecoplate showed a strong potential as an effective ecological indicator of changes in river function attributable to urbanization and pollution.

Acknowledgements

The author thanks Ziyad Elassal for his skillful technical assistance and data analysis. Support for this study was received from the Research and Sponsored Program, University of Michigan-Dearborn.

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