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Extracellular Hydrolytic Enzyme Activities of the Heterotrophic Microbial Communities of the Rouge River: An Approach to Evaluate Ecosystem Response to Urbanization

S. M. Tiquia

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Abstract The potential effects of urbanization on the bioavailability of dissolved organic carbon (DOC) were tested by determining the extracellular enzyme activities of the heterotrophic microbial communities of the Rouge River. The activities of 19 enzymes were monitored across two water samples (river water and groundwater) at different spatial and temporal scales. High phosphatase, esterase, and aminopeptidase activities was observed in site 9 (site most exposed to anthropogenic sources) showed higher concentrations of DOC compared to sites 1 and 8 (sites exposed to less anthropogenic sources), where moderate activities of diverse range of enzymes were observed. High relative contributions of phosphatase, esterase, and aminopeptidase activities to the overall enzyme activity as observed in site 9 stressed the increased importance of peptides as C source for heterotrophic communities and high in-stream carbon processing, which account for high nonspecific extracellular enzyme activities. In contrast, high contribution of glycosyl hydrolases occurred consistently across all sites, which highlights the significance of microbial detrital and plant biomass as carbon sources. Majority of the enzymes showed evidence of activity at various extents during spring and summer. However, higher activities of leucine aminopeptidase, valine aminopeptidase, β-glucosidase, and α-mannosidase were observed in the summer; and alkaline phosphatase and α-glucosidase in the spring. The results presented here suggest a shift in organic carbon bioavailability across all sites of contrasting urbanization, despite similarities in DOC concentrations. Hence, API ZYM technique can be used as an effective indicator of river water and groundwater system health across an urban gradient.

S. M. Tiquia (✉)
Department of Natural Sciences, University of Michigan,
115F Science Building,
Dearborn, MI 48128, USA
e-mail: smtiquia@umd.umich.edu

Introduction

Rivers play a role in both human life and ecological balance. While they are used in transportation and as drinking water source for humans, they are also the main link between terrestrial and aquatic habitats as part of the hydrological and nutrient cycles. The main difference of river ecosystems compared to the other hydrological ecosystems systems (i.e., oceans, lakes, groundwater sources) is the continuous movement of the running water. As a result of this continuous movement, rivers are temporarily storage and transport ways of various materials. The Rouge River located in southeastern Michigan was once part of a healthy and diverse ecosystem. The main pollution sources include atmospheric deposition, hazardous wastes from manufacturing facilities and abandoned dumps, sewage from combined sewer overflows (CSOs), dredge materials, and storm water runoff [23, 33]. Degraded water quality remains one of the most important impediments to the overall health of the Rouge River. Conditions generally decline from upstream to downstream. The lower branch of the river has the worst water quality and contains significant levels of a wide range of both organic and inorganic constituents [23, 33], which can potentially percolate through the soil and contaminate the nearby shallow groundwater system. Groundwater is considered the major component of flow for small streams throughout the year [18]. Groundwater and rivers are very closely connected [8, 18], since they depend on each other for organic matter and nutrients. Groundwater ecosystems

depend on energy from the surface water in the form of dissolved and particulate organic matter [17, 30] and on the other hand, many streams receive considerable inputs of nutrients from groundwater [13, 15].

Microbial communities respond rapidly to environmental changes [2, 43] and their response to these alterations can make them useful biological indicators. For instance, their activities can assist in understanding the level of disturbance in microbial communities, which can also offer a cost and time effective method for the management of the river water and the adjacent groundwater system. Heterotrophic communities, particularly the heterotrophic bacteria, play a critical role in the cycling of dissolved organic carbon (DOC) [3]. They produce a range of extracellular enzymes that hydrolyze large organic compounds into smaller ones, so that the carbon previously unavailable for uptake can be assimilated [14, 25, 40]. This process includes biotransformation of organic compounds from autochthonous and allochthonous origins [32]. Because most of the organic matter in the aquatic systems is polymeric in nature, it cannot be assimilated by heterotrophic bacteria because of the insufficient permeability of their membranes [21]. Therefore, they synthesize various extracellular hydrolytic enzymes to depolymerize macromolecular compounds to mono- or oligomers that they can actively assimilate [31]. The action of extracellular hydrolytic enzymes usually marks the first step in the microbial degradation of organic compounds [11]. Extracellular hydrolytic enzymes are substrate specific, and hence measurements of their activities can also provide insights into specific functional profile of the microbial communities of the Rouge River.

The ability of heterotrophic communities to utilize different organic sources (substrates) is dependent on the enzyme they possess. Depending on the availability and variety of substrates in the environment, some microorganisms may use many substrates, while others may use a few. The main goal of this study is to determine the extracellular enzyme activities of the river water and the groundwater heterotrophic communities on temporal and spatial scales and determine the implications of the enzymatic fingerprints on the composition and bioavailability of DOC of the Rouge River. Three sampling sites (Lotz, Lilley, and Ford Field) were chosen in order to reflect spatial differences. Samples were collected during spring and summer seasons to reflect seasonal patterns. The extracellular enzyme profiles from selected samples were used to determine functional diversity of the heterotrophic microbial communities using API ZYM assay. API ZYM is a semi-quantitative micro-method that permits the analysis of a wide spectrum of extracellular hydrolytic enzymes belonging to phosphatases, esterases, aminopeptidases, proteases, and glycosyl hydrolases. Overall, this study tested the potential of API ZYM™ assay to determine the relative abundance of extracellular enzymes resolving the functional diversity of heterotrophic communities in Rouge River; and assessed its potential as an effective ecological indicator of changes in stream function attributable to perturbation. As composition of the DOC changes in aquatic environments associated with perturbations, the production of enzymes by bacteria should change in response to changes in DOC compounds [48]. Hence, patterns of bacterial enzyme activity should provide biologically relevant approach in assessing the health of the river water and nearby ground water system across an urban gradient.

Materials and Methods

Sites and Sample Collection

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 Field) 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 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. Water samples were taken four times during periods of high flows (spring; May–June) and four times during low flows (summer; July–August). The average air temperature in the spring sampling was 16°C whereas it was 20°C in the summer [43]. Average precipitation in spring was twice higher (0.4 cm) than summer (0.2 cm). Wind speed (10 km h^{-1}) and barometric pressure (1,013 kPa) averages were similar in both seasons. 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°C to 17.6°C in the summer. Dissolved oxygen concentrations were higher in the spring (6.22± 2.68 mg l^{-1}) than the summer (3.62±1.01 mg l^{-1}) with pH values were relatively close between seasons (6.34±0.29 in spring; 7.31±0.36 in summer) [43].

Groundwater samples were collected at a depth of 1.9 to 2.1 m using a low–low purging pump (Geo Scientific, Ltd.,

Vancouver, British Columbia), while surface water samples were collected manually by lowering a sterile glass bottle at a depth of 1 m. At each sampling location, 1-h composite of surface water samples were collected. The hourly composite samples were collected by combining four 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. All the samples were analyzed within 8 h of sampling.

Dissolved Organic Carbon, Chlorophyll Content, and Prokaryotic Cell Density

Water samples for DOC were filtered through 47-mm diameter Whatman GF/C filters and acidified to pH 3. 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 [20, 38].

API ZYM Assay

API ZYM™ strips (BioMerieux, Marcy l'Etoile, France) consist of 20 microcupules containing dehydrated chromogenic substrates of 19 different enzymes (Table 1) and a control (a microcupule that does not contain any enzyme substrate). These enzymes include three phosphatases, three esterases, three aminopeptidases, two proteases, and eight glycosyl hydrolases. The enzyme substrates in the system are shown in Table 1. The kit has been successfully used for the study of enzyme activities of microorganisms and cell suspensions [5, 16, 26], manure compost extracts [42, 45], landfill refuse extracts [35], municipal solid waste extracts [36], and industrial wastewaters [49]. Groundwater and river water samples were inoculated into the API ZYM™ strips within 1 day of sample collection. An aliquot (65 μl) of the water samples was dispensed into each of the microcupules. API ZYM™ strips were then covered at 15°C for 24 h. The reaction was terminated by addition of 30 μl of reagent ZYM A (containing 10% sodium dodecyl sulfate; BioMerieux), and color was developed by addition of 30 μl of reagent ZYM B (containing

Table 1 Substrate composition, pH, and expected results from the test

Enzyme assayed for	Enzyme group	Substrate	pH	Result	
				Positive	Negative
Control	–	–	–	Pale yellow	Pale yellow
Alkaline phosphatase	Phosphatase	2 Naphthyl-phosphate	8.5	Violet	Pale yellow
Acid phosphatase	Phosphatase	2 Naphthyl-phosphate	5.4	Violet	Pale yellow
Phosphohydrolase	Phosphatase	Naphthyl AS-BI-phosphate	8.5	Blue	Pale yellow
Lipase	Esterase	2 Naphthyl-myristate	7.5	Violet	Pale yellow
Lipase esterase	Esterase	2 Naphthyl-caprylate	7.5	Violet	Pale yellow
Esterase	Esterase	2 Naphthyl-butyrate	6.5	Violet	Pale yellow
Leucine aminopeptidase	Aminopeptidase	L-Leucyl-2-naphthylamide	7.5	Orange	Pale yellow
Valine aminopeptidase	Aminopeptidase	L-Valyl-2-naphthylamide	7.5	Orange	Pale yellow
Cystine aminopeptidase	Aminopeptidase	L-Cystyl-2-naphthylamide	7.5	Orange	Pale yellow
Chymotrypsin	Protease	*N*-glutaryl-phenylalanine-2-naphthylamine	7.5	Orange	Pale yellow
Trypsin	Protease	*N*-benzol-DL-arginine-2-napthylamide	8.5	Orange	Pale yellow
α-Galactosidase	Glycosyl hydrolase	6-Br-2-naphthyl-α-D-galactopyranoside	5.4	Violet	Pale yellow
β-Glucosidase	Glycosyl hydrolase	6-Bromo-2-naphthol-α-D-galactopyranoside	5.4	Violet	Pale yellow
N-acetyl-β-glucosaminidase	Glycosyl hydrolase	1 Naphthyl-*N*-acetyl-βD-glucosaminide	5.4	Brown	Pale yellow
α-Glucosidase	Glycosyl hydrolase	2 Naphthyl-2-D-glucopyranoside	5.4	Violet	Pale yellow
β-Galactosidase	Glycosyl hydrolase	2 Naphthyl-βD-galactopyranoside	5.4	Violet	Pale yellow
β-Glucuronidase	Glycosyl hydrolase	Naphthyl AS-BI-βD-glucuronide	5.4	Blue	Pale yellow
α-Mannosidase	Glycosyl hydrolase	6-Bromo-2-naphthyl-2-D-mannopyranoside	5.4	Violet	Pale yellow
α-Fucosidase	Glycosyl hydrolase	2 Naphthyl-αL-fucopyranoside	5.4	Violet	Pale yellow

0.35% Fast Blue BB; BioMerieux) to each microcupules. Color development was scored as positive and no color development as negative. For microcupule that showed positive result, a numerical value of 1–5 (1=5 nM; 2=10 nM; 3=20 nM; 4=30 nM; 5=40 nM) was assigned according to the color chart provided by the manufacturer. For the purpose of this study, the results were reported as reactions of low intensity [1], moderate intensity [2–3], and high intensity [4–5].

Statistical Analyses

Resulting data were transformed by natural logarithm to achieve normal distribution. Paired *t* test was used to analyze differences between two samples (river water and groundwater) and two seasons (spring and summer). Pearson-product moment correlation was used to calculate general correlation between enzymes and biological parameters (bacterial density, chlorophyll content, and DOC). Analysis of variance was calculated to determine differences between three sites (sites 1, 8, and 9). When ANOVA showed significant difference between sites ($P \leq 0.05$), means were separated using Bonferroni's *t* test. Multivariate analysis can summarize the variability of a complex data set and present it in a more interpretative form. For this reason, Principal components analysis (PCA) also computed to compare enzyme profiles of the water samples at different sites. To compare the enzyme profiles, water samples were compared with the 19 variables (enzyme substrates). This was accomplished by projecting the sample loadings onto new axes, or PCs [28]. 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. Statistical analyses were calculated using SYSTAT statistical computing package (SYSTAT Version 9.0, SPSS Inc., Chicago, IL, USA).

Results

Bacterial Numbers, DOC, and Chlorophyll Content

Bacterial densities were significantly higher (P=0.019) in the summer (15.98±5.83×10^5 ml^{-1}) than in spring (^{1}0.92±1.73×10^5 ml^{-1}; Fig. 1a). DOC concentrations (spring=5.47±2.48 mg l^{-1}; summer=4.74±2.77 mg l^{-1}; Fig. 1b) and chlorophyll contents chlorophyll contents (spring=1.13±1.71 μg l^{-1}; summer=1.58±2.61 μg l^{-1}; Fig. 1c) were relatively close between seasons. The river water samples had significantly higher bacterial density (P=0.044), DOC (P=0.0001), and chlorophyll content (P=0.0001) than the groundwater samples. Groundwater samples, on the other hand, has been reported to have significantly higher EC than the river water samples (groundwater=2,190±844.60 μS cm^{-1}; river water=1,598±157.99 μS cm^{-1}), but

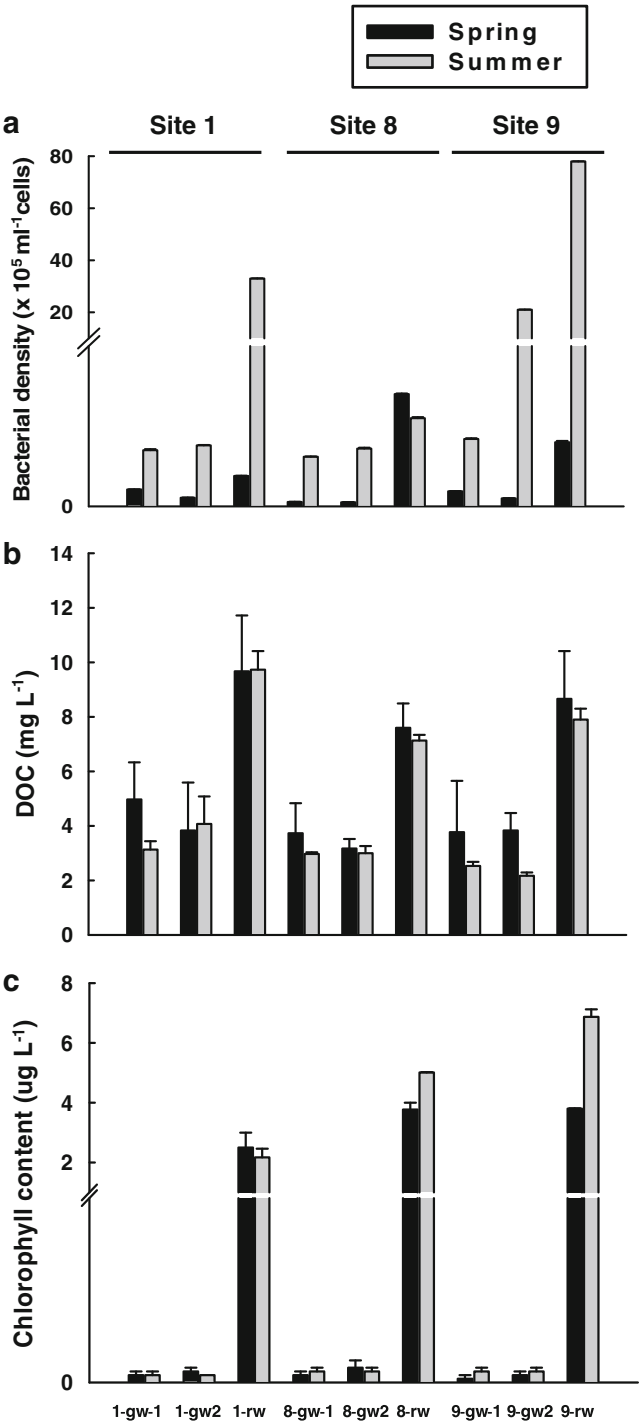

Figure 1 Spatial variation of (**a**) bacterial density, (**b**) DOC, and (**c**) chlorophyll content of the groundwater and river water samples at two different seasons. The river water samples are referred to as *rw* whereas the groundwater samples are referred to as *gw1* (north side) and *gw2* taken (south side). Samples were collected from site 1 (*1-gw1*, *1-gw2*, *1-rw*), site 8 (*8-gw1*, *8-gw2*, and *8-rw*) and site 9 (*9-gw1*, *9-gw2*, and *9-rw*). *Vertical bars* represent the standard deviation of three replicates

their pH values were similar (groundwater=6.68±0.37; river water=6.79±0.17) [43]. Seasonal variation (spring and summer) did not significantly change the properties of the water samples with the exception of bacterial density, which showed statistically higher value (P=0.019) in the summer than in spring. Similarity between groundwater samples from the north (gw1) and south (gw2) sides of the river was also evident (Fig. 1).

Enzymatic Hydrolysis Patterns

Enzyme profiles from different water samples showed that the measured activities were typically different among enzymes, and revealed differences and similarities among water samples. Of the 19 enzymes, only 7–12 showed evidence of activity (Fig. 2). These enzymes were detected in river and groundwater samples collected at different sites and seasons. Five enzymes including, phosphatase, cystine aminopeptidase, chymotrypsin, trypsin, and α-fucosidase showed no evidence of activity. Enzymes that produced consistently low activities were probably present in a few members of the microbial communities in the sample. Nonetheless, all water samples supported at least some extracellular enzyme activity. Alkaline phosphatase, acid phosphatase, lipase, lipase esterase, esterase, α-galactosidase, and β-glucosidase activities were observed in varying levels on all water samples collected.

Investigation of the extracellular enzyme profiles also revealed some differences between the groundwater and river water samples (Fig. 2). In general, fewer numbers of enzymes were found active from the groundwater samples (seven to nine enzymes) than the river water samples (eight to 12 enzymes). Overall, the river water samples showed higher enzyme activity, while the groundwater samples displayed moderate activity with only two to five enzymes exhibiting high enzyme activities (Fig. 2). Paired t test results demonstrated significant difference between the two water samples. That is, the river water samples contained significantly high activities of seven different enzymes compared to groundwater samples (Table 2). These enzymes include one phosphatase (acid phosphatase), two esterases (lipase and esterase), and four glycosyl hydrolases (α-galactosidase, N-acetyl-β-glucosaminidase, α-glucosidase, and β-glucuronidase; Table 2).

Seasonal and Spatial Differences in Extracellular Enzyme Activity

To determine the seasonal effects on enzyme activities, water samples were collected four times during the periods

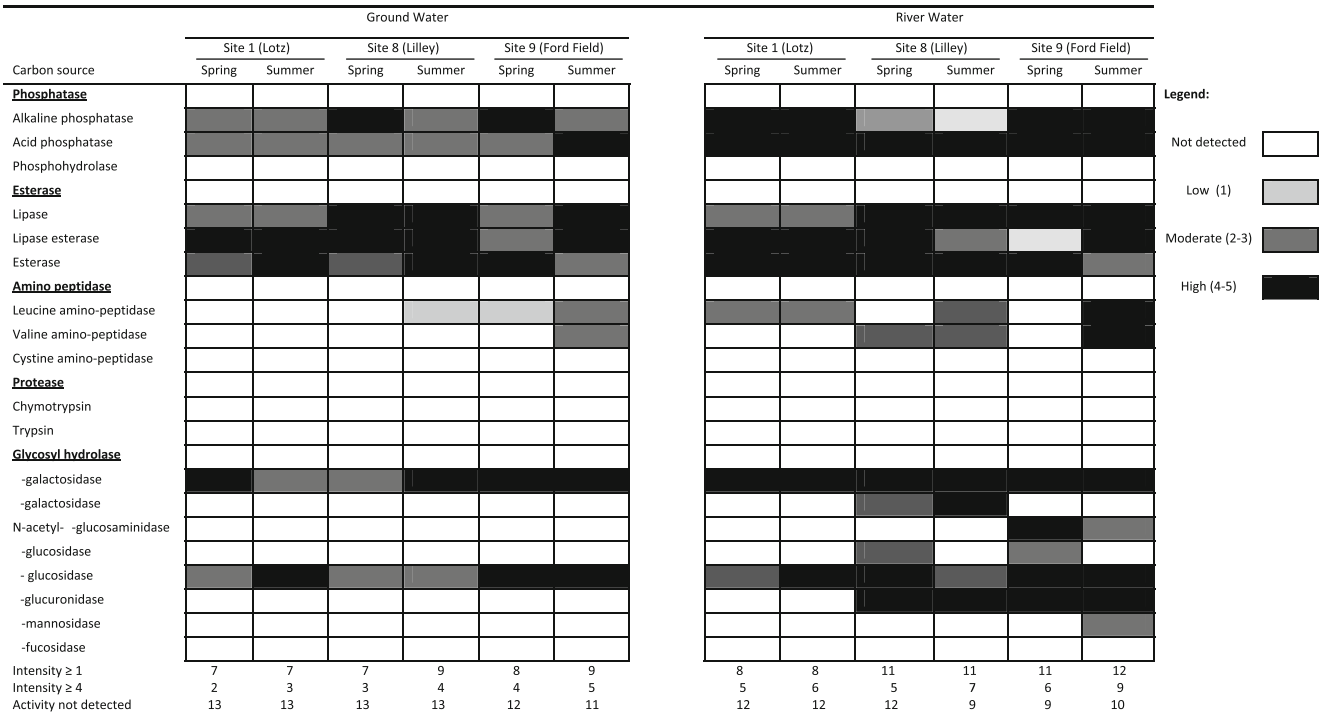

Figure 2 Profiles of the 19 extracellular enzymes from the groundwater and river water samples taken during spring and summer from sites 1 (Lots), 8 (Lilley), and 9 (Ford Field). *Shading in the boxes* indicates the relative abundance of the enzyme. Values are as follows: *White*, not detected; *light gray*, reactions of low intensity (value of 1); *dark gray*, reactions of moderate intensity (values of 2–3); *black*, reactions of high intensity (value of 5). The number of enzymes showing reactions ≥1, >4, and the total number of enzyme that did not display activity are indicated below each column

Table 2 Results of *t* test showing the effect of season (spring versus summer) and type of water sample (river water versus ground water) on the 19 enzymes

Enzyme	Spring versus summer		River water versus ground water	
	P values	Significance	*P* values	Significance
Phosphatase				
Alkaline phosphatase	0.045	*	0.543	ns
Acid phosphatase	0.363	ns	0.004	**
Phosphohydrolase	–	–	–	–
Esterase				
Lipase	0.235	ns	0.046	*
Lipase esterase	0.332	ns	0.444	ns
Esterase	0.041	*	0.004	**
Amino peptidase				
Leucine aminopeptidase	0.042	*	0.801	ns
Valine aminopeptidase	0.363	ns	0.765	ns
Cystine aminopeptidase	–	–	–	
Protease				
Chymotrypsin	–	–	–	
Trypsin	–	–	–	
Glycosyl hydrolase				
α-Galactosidase	0.465	ns	0.041	*
β-Galactosidase	0.363	ns	0.275	ns
N-acetyl-β-glucosaminidase	0.809	ns	0.040	*
α-Glucosidase	0.999	ns	0.045	*
β-Glucosidase	0.679	ns	0.394	ns
β-Glucuronidase	0.363	ns	0.038	*
α-Mannosidase	0.363	ns	0.363	ns
α-Fucosidase	–	–	–	–

ns not significant

t test with *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$

of high flows (spring; May–June), and four times during low flows (summer; July–August). Paired *t* test results only showed significant difference for three enzymes: alkaline phosphatase, esterase, and leucine aminopeptidase (Table 2). Although there were no significant trends between spring and summer seasons for most enzymes assayed, more enzymes exhibited high activities during summer (three to nine enzymes) than in spring (two to six enzymes; Fig. 2). Another key difference included higher activities of leucine aminopeptidase, valine aminopeptidase, β-glucosidase, and α-mannosidase in the summer; and alkaline phosphatase and α-glucosidase in the spring.

Frequency distribution of extracellular enzyme activity based on substrate categories of the river water and groundwater samples showed some differences among the three sites (Fig. 3). The river water samples from the three sites differed significantly ($P=0.05$) with respect to the percent contribution of each enzyme to the total enzyme activity (Fig. 3a). Site 9 showed significantly higher phosphatase ($P=0.018$) and esterases ($P=0.033$) than sites 1 and 8. The microbial communities in site 9 tend to use more substrates specific to alkaline phosphatase, acid phosphatase, lipase, lipase esterase, and esterase while the microbial communities in sites 1 and 8 utilized substrates specific to α-galactosidase and β-glucosidase were the most active enzymes. Frequency distribution of the groundwater samples from sites 1 and 8 were similar also (Fig. 3b), and showed significantly lower phosphatases ($P=0.049$), esterases ($P=0.039$) and glycosyl hydrolase ($P=0.046$) activities. Utilization of substrates specific to lipase esterase, esterase, and α-galactosidase were more frequent in groundwater samples from sites 1 and 8, whereas for site 9 specific substrates for α-galactosidase and β-glucosidase were frequently used.

Biological Parameters and Enzyme Activities

The relationships between biological parameters (bacterial cell count, DOC, and chlorophyll content) and water samples (river water and groundwater) were explored (Table 3). Correlations were derived from all water data collected at different sites (sites 1, 8, and 9) and seasons

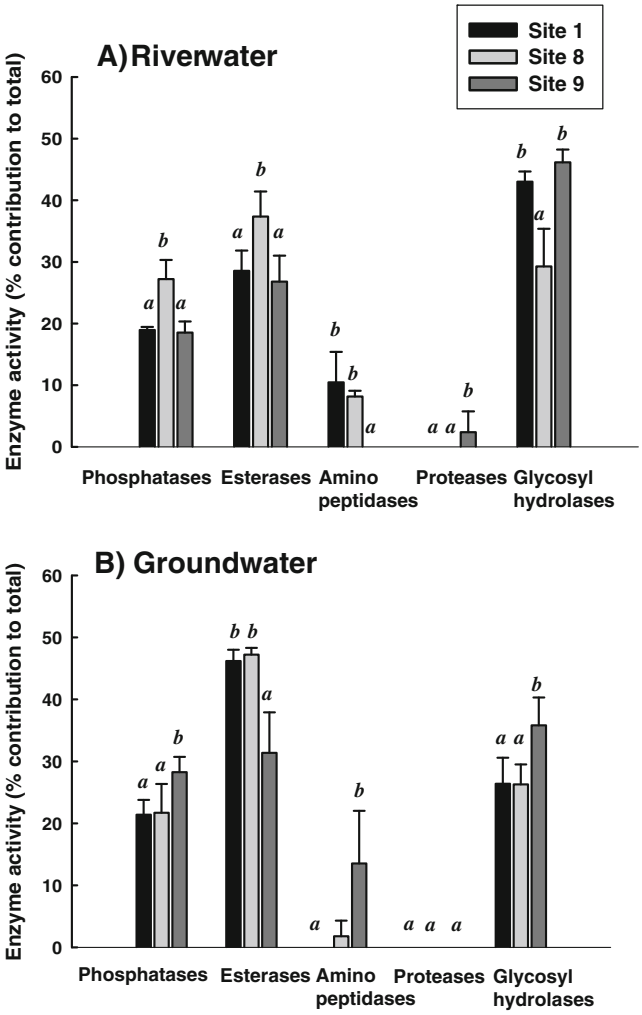

Figure 3 Percentage contributions of the extracellular enzyme activities to the total enzyme activity in **a** river water and **b** ground water samples. For each site, at each substrate category, values represented by histograms with the same letter are not significantly different (P=0.05)

(spring and summer). In the river water samples, seven significant correlations occurred for bacterial cell counts. These included one phosphatase, three esterases, two aminopeptidases, and one glycosyl hydrolases. These enzymes exhibited higher activities as the bacterial cell count increased. Seven significant correlations also occurred for DOC content. These included two esterases, one aminopeptidase, and four glycosyl hydrolases. For the chlorophyll content, three positive correlations occurred but the correlations were all negative: one esterase and two glycosyl hydrolases (Table 3). Fewer enzymes correlated with the biological parameters in the groundwater samples. Esterase, α-glucosidase, and α-mannosidase positively correlated with bacterial numbers; alkaline and acid phosphatases positively correlated with DOC content; and β-glucuronidase negatively correlated with chlorophyll content (Table 3).

Multivariate Statistical Analysis

PCA was carried out on 54 transformed API ZYM data sets from river water and groundwater samples for both spring and summer. Only utilizations that had greater than 50% fit (i.e., significant loadings) onto the two axes collectively were included for interpretation. Additionally, only the first two PCA axes were retained for interpretation as the other axes did not account for a significant percentage of the total variance as determined by application of the broken-stick model [29, 37]. The first two principal components (PCs) accounted for 75% of the total variance (Fig. 4). The two PCs separated site 9 (river water) from sites 1 and 8 (river water). Groundwater samples from sites 1 and 8 also clustered together and were separated from groundwater samples collected from site 9 (Fig. 4). These results correlated with the frequency distribution pattern reported in Fig. 3. To relate the activity of individual enzymes to the differences in total enzyme activities, and which enzyme accounted for the differences between sites (sites 1, 8, and 9) and samples (groundwater versus river water), the correlation between enzyme variables was examined (Table 4). For PC1, significant loadings were observed for seven of the 19 extracellular enzymes, all strongly positively associated (Table 4). These loadings included two phosphatases (alkaline and acid phosphatase and acid phosphatase), three esterases (lipase, lipase esterase, and esterase), and two glycosyl hydrolases (α-galactosidase and β-glucuronidase). Variances in PC2 were explained principally by activities of three aminopeptidases (leucine aminopeptidase, valine aminopeptidase, and cystine aminopeptidase), and two glycosyl hydrolases (β-galactosidase and *N*-acetyl-β-glucosaminidase). Hence, phosphatases, esterases, aminopeptidases, and glycosyl hydrolases contributed the highest relative activity by the microbial communities, and contributed to differences between sites and samples.

Discussion

Previous investigations on microbiological properties of the river waters and groundwater wells along the Rouge River have focused on the enumeration of indicator bacteria that impact surface water quality [34]; isolation and characterization of bacterial isolates [46, 47] for biotechnological applications [44]; and carbon utilization patterns [43]. DNA sequence analysis of the isolates indicates the dominance of many heterotrophic populations belonging to α- and γ-*Proteobacteria* and *Firmicutes* [46, 47], some of which are capable of degrading toxic organic compounds such as trichloroethylene, 2-chlorobiphenyl, and cyclohexane [44]. Assessment of the heterotrophic microbial communities in

Table 3 Pearson-product moment correlation coefficients between biological parameters and enzymes among across 48 samples for the river water samples and 96 samples for the groundwater samples

River water		Groundwater	
Bacterial cell count		Bacterial cell count	
Enzyme	Correlation coefficient (r^2)	Enzyme	Correlation coefficient (r^2)
Acid phosphatase	0.987***	Esterase	0.918***
Lipase	0.691*	α-Glucosidase	0.918***
Lipase esterase	0.683*	α-Mannosidase	0.655*
Esterase	0.888**		
Leucine aminopeptidase	0.965***		
Valine aminopeptidase	0.987***		
α-Galactosidase	0.672*		
DOC content		DOC content	
Enzyme	Correlation coefficient (r^2)	Enzyme	Correlation coefficient (r^2)
Lipase	0.770**	Alkaline phosphatase	0.678*
Esterase	0.676*	Acid phosphatase	0.641*
Leucine aminopeptidase	0.910***		
N-acetyl-β-glucosaminidase	0.615*		
β-Glucosidase	0.910***		
β-Glucuronidase	0.770**		
α-Mannosidase	0.676*		
Chlorophyll content		Chlorophyll content	
Enzyme	Correlation coefficient (r^2)	Enzyme	Correlation coefficient (r^2)
Esterase	−0.756**	β-Glucuronidase	−0.642*
α-Glucosidase	−0.663*		
α-Mannosidase	−0.856**		

Only significant correlations are shown. Data are inclusive of all samples (river water and groundwater) collected at different sites and seasons
*P values<0.05, **P values<0.01, ***P values < and 0.001

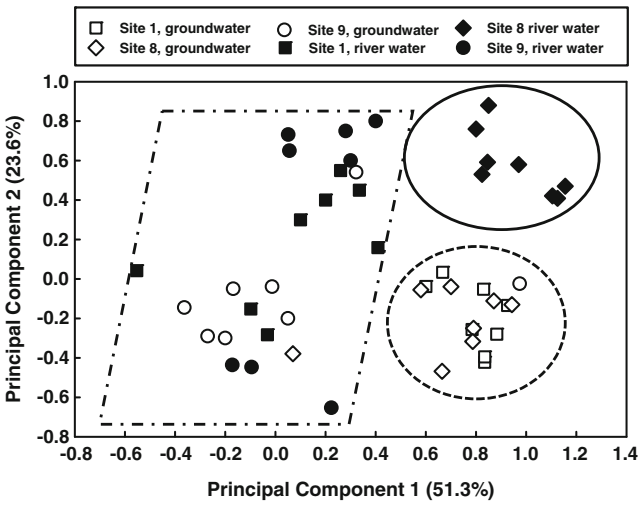

Figure 4 Ordination diagrams of API ZYM profiles from principal component analyses (PCA) of enzyme activities of river water and groundwater samples collected during spring and summer. *Site 1* (Lotz), *site 8* (Lilley), *site 9* (Ford Field). The variance explained by each PC is given in *parentheses*

the water samples indicated utilization of high carbon substrates including carbohydrates, carboxylic acids, polymers, and amino acids [43]. This finding suggests that the heterotrophic microbial communities at the site possess high number of metabolic pathways (high metabolic diversity) that allow them to exploit many different carbon sources. The present study attempted to monitor the extracellular enzyme activity in river water and adjacent groundwater, using a phenotypical approach (API ZYM), and the potential of this technique as an effective ecological indicator of changes in stream function attributable to perturbation in the Rouge River. A wide range if hydrolytic enzymes were found associated with the microbial communities in the river water and groundwater samples (Fig. 2). The presence of phosphatases, esterases, aminopeptidases, and glycosyl hydrolases confirms previous finding from other rivers [6, 10, 24] and groundwater systems [9, 27]. However, the occurrence of esterases (lipase, lipase esterase, and esterase) has not yet been previously reported and broadens the spectrum of the

Table 4 Correlation between principal components (PC1 and PC2) and single variables for the PCAs described in the text

Enzyme	PC 1	PC 1
Phosphatase		
Alkaline phosphatase	0.702	
Acid phosphatase	0.839	–
Esterase		
Lipase	0.722	–
Lipase esterase	0.541	–
Esterase	0.582	–
Amino peptidase		
Leucine aminopeptidase	–	0.564
Valine aminopeptidase	–	0.817
Cystine aminopeptidase	–	−0.647
Protease		
Trypsin	–	−0.747
Glycosyl hydrolase		
α-galactosidase	0.761	–
β-galactosidase	–	0.582
N-acetyl-β-glucosaminidase	–	−0.657
β-glucuronidase	0.786	–

All substrates with an r value>0.5 are samples shown with $P<0.001$; r = Pearson correlation coefficient

metabolic capabilities of the microbial communities in the Rouge River.

The metabolic diversity, understood in this study as the hydrolysis of substrates in API ZYM strips, showed distinct differences between the river water and the groundwater samples. PCA analysis based on enzyme activity indicated that phosphatases, esterases, and glycosyl hydrolases contributed to significant variation in extracellular enzyme activities between the two water samples. In general, the river water samples were characterized by higher utilization of these four classes of enzymes, with color development values significantly higher than the groundwater (Table 2). The higher functional diversity in the river water was due to higher bacterial density, DOC, and chlorophyll content (Fig. 1). It is interesting to note that the number of enzymes that showed evidence of activity was similar for both water samples, although the relative abundance was higher for the river water samples than the groundwater samples. Chapelle [9] noted that the groundwater food web is almost heterotrophic but contained lower bacterial density that makes it less capable of responding to nutrient input. Groundwater samples are also exposed to greater environmental stress [19]. This observation was supported by the lower frequency of the groundwater samples to assimilate Biolog EcoPlate compounds in previous study [43].

The microbial assemblage in Rouge River responded to seasonal shifts in substrate availability by changing the compositional extracellular enzymes released. Significantly higher esterase and leucine aminopeptidase and lower alkaline phosphatase activities were observed in the summer than in spring (Table 2). This trend corresponded with higher bacterial density (Fig. 1a) during summer. Majority of the enzymes showed evidence of activity at various extent during both seasons however no evidence of activity was observed for leucine aminopeptidase, esterase, valine aminopeptidase, β-glucosidase, and α-mannosidase in the summer; and alkaline phosphatase and α-glucosidase in the spring. The seasonal dynamics of specific extracellular enzyme activity was not only caused by variations in temperature [48] and bacterial abundance [43], but also the varying supply of major substrates. Previous investigation on the substrate utilization of the microbial communities at the site using Biolog Ecoplates noted that in the spring, the microbial populations utilize carbohydrates, polymers, and phenolic compounds more frequently than in the summer. On the other hand, carboxylic acids and amino acids were more often utilized in the summer than in spring [43]. During the onset of the spring, which is characterized by depletion of dissolved oxygen, the bacterial assemblages preferably used carbohydrates over carboxylic acids. As summer progressed, indicated by warm temperature and replenishment of dissolved oxygen, substrate preference shift to carboxylic acids and amino acids [43]. One possible reason for the dominance of leucine aminopeptidase, esterase valine aminopeptidase, β-glucosidase, and α-mannosidase in the summer was due to increased supply carbohydrate. The increased concentration of carboxylic acids and amino acids led to increase in esterase and alkaline phosphatase activities, respectively.

Extracellular enzyme activities at different sites (site 1, site 8, and site 9) were investigated. Very little difference was observed among the groundwater samples at three different sites. However, in the river water samples where differences were observed, differential responses of individual enzymes to DOC in sites least and most exposed to anthrophogenic sources indicated that specific extracellular enzyme activity varied with the level of urbanization. For instance, high phosphatase, esterase, and aminopeptidase activities in site 9 (site most exposed to anthrophogenic sources) showed higher amounts of bioavailable DOC compared to sites least exposed to anthrophogenic sources (sites 1 and 8). This result suggests that peptides are important C source for the heterotrophic microbial communities in highly urbanized site (site 9), possibly because of high in-stream processing (generation of organic carbon) of filamentous algae, which can often dominate organic matter biomass [22]. It is likely that the high bulk DOC concentrations in site 9 may induce high in-stream carbon processing, which would account for high nonspecific hydrolytic activity. Glycosyl hydrolase activities occurred

consistently across all three sites, which suggest that there was a steady supply of plant polysaccharides and bacterial detritus. The presence of these enzymes is consistent with high degradation of plant, animal, and microbial detritus [7, 14, 22]. The presence of α-galactosidase indicates the degradation of microbial detritus, whereas β-glucosidase activity indicates the presence of low-molecular-weight cellulose substances. The higher contribution of *N*-acetyl-β-glucosaminidase hydrolyses the amino sugars common in bacterial carbohydrates, such as chitin and murein, in the later stages of degradation [12, 14]. These results are supported by observations of more riparian vegetation, and hence greater litter input and habitat for stream biota, in less urbanized sites (i.e., site 1). The diversity of the 19 enzymes in less-urbanized site reflect the significance of microbial detrital material and plant-derived carbohydrates, as well as proteinaceous material as carbon source. Although the

Although DOC concentrations were of the same order of magnitude, there was enough variation to detect correlation between DOC content and river pollution. Increases in enzyme activities corresponded with increases in DOC content. In the present study, the DOC content was higher from sites 8 and 9 (more impacted by anthropogenic sources) than site 1 (less impacted by anthrophogenic sources). Bank erosion, abandoned dumped sites, CSO, contaminated ground and storm water runoff accounted to a large extent for increases in DOC concentrations [33, 43], which contributed to high extracellular enzyme activities at these sites. Extracellular enzyme activities were significantly correlated with bacterial abundance (Table 3), which is not surprising as bacteria are the main producers of extracellular enzymes. Extracellular enzymes have not much been considered as significant components of polluting discharges, although increases in enzyme activity, including aminopeptidase, due to sewage-works and fish-farm effluent, has been shown in English rivers [1, 10]. In the present study, the coupled increase in extracellular enzyme activities and bacterial abundance has shown to be a simple response to polluting discharges in the Rouge River.

Chlorophyll content also showed some correlation with enzyme activities (Table 3). Carbon produced from autochthonous primary production is commonly easier to assimilate and contains more biochemical energy per unit biomass [41], and it is immediately available for bacteria in the water column and also in sediments [1, 4, 39]. Hence, the extracellular enzyme activities were favored by algal abundance. Although this paper associates extracellular enzyme activities exclusively to the heterotrophic fraction of the microbial community, some such extracellular enzymes such phosphatases and leucine aminopeptidase have been measured in phytoplanktonic organisms. Hence, the development of such activities in the river water, where chlorophyll concentration was high, indicates active autotrophic biomass, may be related to autotrophic organisms [41].

This study explored the metabolic capacities of the microbial consortia as a whole of the Rouge River. Our results suggest that the API ZYM assay can also be applied to other river ecosystems. The assay showed strong potential as an effective ecological indicator of changes in river water and groundwater function attributable to urbanization. Our results suggest that the extracellular enzyme activities responded to urbanization, and thus may be used to assess changes in stream health. The assay used in this study is simple, rapid, and inexpensive compared to many existing methods, and it permits the analysis of an array of extracellular enzymes simultaneously.

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