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Ecogenomics Reveals Metals and Land-Use Pressures on Microbial Communities in the Waterways of a Megacity

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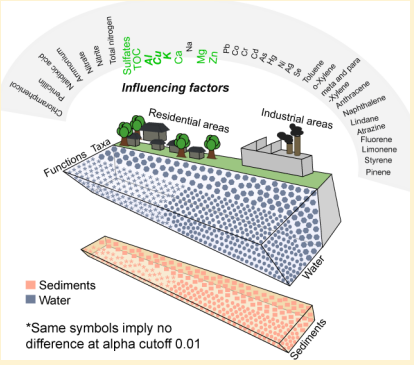
Supporting Information

ABSTRACT: Networks of engineered waterways are critical in meeting the growing water demands in megacities. To capture and treat rainwater in an energy-efficient manner, approaches can be developed for such networks that use ecological services from microbial communities. Traditionally, engineered waterways were regarded as homogeneous systems with little responsiveness of ecological communities and ensuing processes. This study provides ecogenomics-derived key information to explain the complexity of urban aquatic ecosystems in well-managed watersheds with densely interspersed land-use patterns. Overall, sedimentary microbial communities had higher richness and evenness compared to the suspended communities in water phase. On the basis of PERMANOVA analysis, variation in structure and functions of microbial communities over space within same land-use type was not significant. In contrast, this difference was significant between different land-use types, which had similar chemical profiles. Of the 36 environmental parameters from spatial analysis, only three metals, namely potassium, copper and aluminum significantly explained between 7% and 11% of the variation in taxa and functions, based on distance-based linear models (DistLM). The ecogenomics approach adopted here allows the identification of key drivers of microbial communities and their functions at watershed-scale. These findings can be used to enhance microbial services, which are critical to develop ecologically friendly waterways in rapidly urbanizing environments.



INTRODUCTION

Rapid urbanization and land-use changes in the past few decades have highlighted the necessity to ensure water security across many parts of the world,[1] especially in emerging megacities of developing countries.[2,3] Engineered waterways, including urban-hydro systems, have been designed to capture and retain more water in areas where demand is high.[2,4] Unlike natural river systems, these waterways are specifically designed to increase the hydraulic capacity in response to urbanization.[5] Transformations of urban land-surface, such as increased imperviousness of watershed surfaces and use of land for diverse purposes,[6,2] affect waterways ecosystems in number of ways. The result of increased imperviousness is high peak discharge and short duration of turbulence in waterways following rain events.[6,7] Shifts in these hydrodynamics result in the erosion of the top layer of soil, heterogeneous settlement of sediments and the release of chemical constituents in a land

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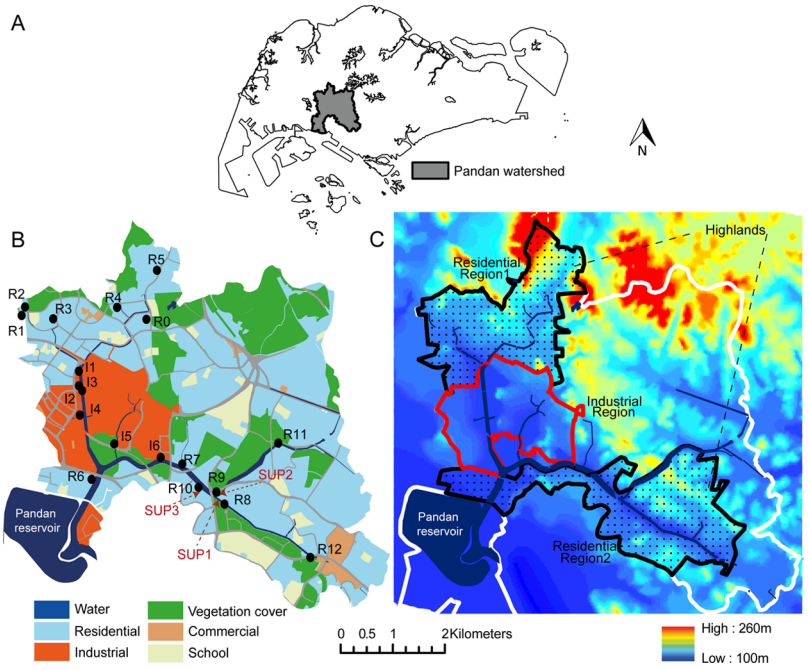

Figure 1. Landscape distribution in Ulu-Pandan watershed. (A) The watershed lies in the southwestern region of Singapore and spreads across 25 km^2. (B) Sediment and water samples collection points from 19 locations distributed nonuniformly across the drains and canal network, which serves different land-use types, mainly residential and industrial (see color legends). Three locations in residential region 2, SUP-1, 2, and 3, for the temporal monitoring of environmental parameters. (C) Ulu-Pandan canal network is shown in context of natural elevation confounded with land-use patterns. The sample collection in the watershed is broadly divided into two residential and one industrial region based on their geography.

use-specific manner.[8−10] As both sediments and chemical constituents vary according to land-use types, these factors can adversely impact the freshwater ecosystem of these waterways.[2] Unlike natural freshwater ecosystems,[11] it is not clear to what extent the pulsed changes in physicochemical properties[7] in urban environments impact the composition and function of microbial communities.

The involvement of microbial communities in a wide range of biogeochemical processes, such as nutrient cycling and primary and secondary productivity, makes them indispensable to any freshwater ecosystem.[12−16] These microbial communities, however, are influenced by environmental factors both at local and regional scales.[17] Especially in urban environments where land-use patterns may be highly interspersed with respect to the environmental parameters[18,19] to introduce spatial heterogeneity within the watershed. In such heterogeneous environments the composition and function of microbial communities are expected to be variable. Understanding of the associations of these microbial communities with their environment is essential to the development of ecologically sustainable waterways, as well as to improve their overall function.[20,21] In this approach, a key goal is to facilitate the optimum microbial capacity for inherent self-purification of the waterway. Since the principles governing microbial functioning in pristine aquatic ecosystems cannot be directly applied to the urban waterways systems, a much improved understanding of the composition and functions of microbial communities in urban systems and their key environmental regulators is necessary.

On the basis of the unique nature of urban waterways, we designed a field study to understand the influence of an urbanized watershed with mixed and interspersed land-use patterns on the composition and functions of sedimentary and suspended microbial communities in these waterways. As a model system, we adopted a subnetwork of a well-managed watershed with different land-use patterns in the megacity of Singapore. The efficiency of the waterway network within the watershed is indicated by its ability to transport water rapidly and efficiently to the respective reservoirs.[22] We adopted widely used ecogenomics approaches[23−27] to analyze the composition and potential functions of microbial communities in combination with environmental metadata. This approach has provided us ecogenomics-derived information to identify the key trends and associations between microbial communities and their functions. Our study is driven by two broad hypotheses; first, that land-use types in well-managed and densely interspersed watersheds influence the composition and functions of sedimentary and suspended microbial communities. Second, the environmental pressures influencing the composition and functions of microbial communities are associated with a set of few parameters. Here, we report analyses of 48 environmental parameters measured over a period of one year combined with spatial analysis of microbial communities and their functions to explore their associations. From the measured environmental parameters, we identified parameters and watershed characteristics associated with microbial communities and their functions. This study provides key information about potential drivers of microbial communities in highly urbanized environments, which is necessary to develop ecologically sound engineering approaches in rapidly urbanizing environments in Asia and other parts of the world.

MATERIALS AND METHODS

Watershed Map. ArcMap 10.1 module of ArcGIS from ESRI (USA) was used to create the Ulu-Pandan catchment map of Singapore. Shape files were obtained from Public Utilities Board (PUB), Singapore.

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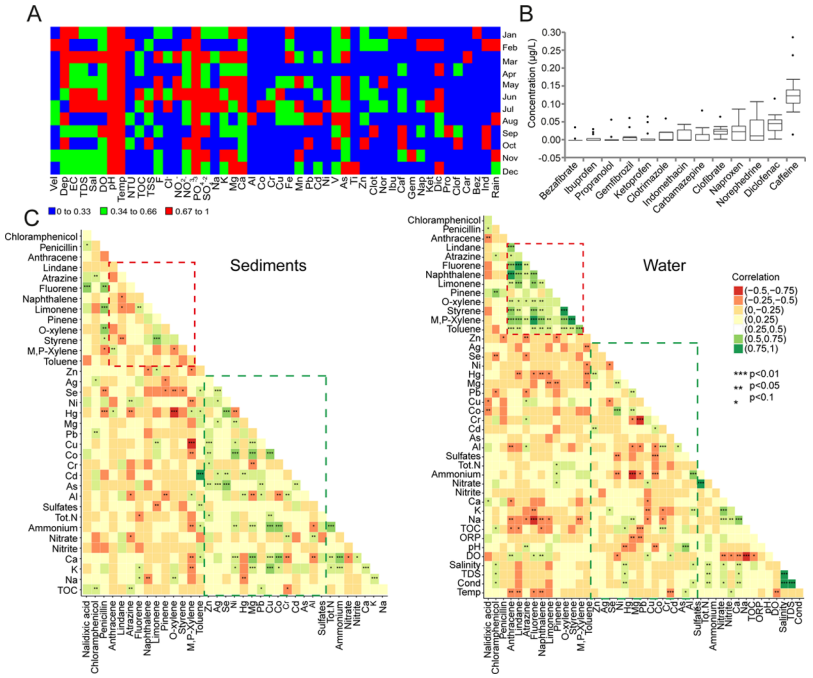

Figure 2. Spatial and temporal relationships of environmental parameters. (A) Heat map showing the scaled levels of 48 environmental parameters measured over a period of one year. (B) Box plot of the selected pharmaceuticals average concentrations in water samples from SUP1, SUP2, and SUP3 locations of residential region 2 along the Sungei Ulu Pandan waterway. Dots depict values outside the upper and lower quartile which are signified by the box. Short line in the box represents the median. Average values of all the parameters with maximum and minimum values detected during the one year period can be referred from Supporting Information Table S2, 3 and 4. (C) Spatial analysis of physicochemical properties showing Kendal Tau correlations among 36 parameters for sediments and 43 parameters for water samples. Stars depict the Benjamini Hochberg (FDR) corrected *p*-values. Boxes represent broad groupings of properties that show different correlation patches (red, organics; green, metals and nutrients) in sediment and water samples. Refer to Supporting Information Table S1 for full list of abbreviations.

Sample Collection. For temporal analysis of water quality, three sites, SUP1, 2 and 3 were selected from a residential region (Figure 1B). Water samples were collected and analyzed monthly for 48 parameters (see Figure 2A; Supporting Information Table S1), over a period of one year from January to December, 2009. Sampling procedure of water samples was same as described for spatial analysis in the following paragraphs.

For spatial analysis, sediment and water samples were collected (one time) from residential and industrial land-use types after 2−3 h of a rain-event in the last week of April, 2009. Residential regions constituted 13 locations with 6 locations in region 1 and 7 locations in region 2 (Figure 1B and C). Industrial land-use type had only one region with 6 sampling locations. Only 1 industrial region exists in this watershed, which prevented spatial replication of this land-use type at the regional level, while 2 residential regions exist in this watershed. These residential regions are spatially interspersed with the industrial region (Figure 1B and C), preventing spatial confounding. The sampling design and subsequent statistical analyses of data adopted in this study is derived from designs advocated for their robustness in environmental impact assessments, in particular "Beyond BACI" (Before-After/Control-Impact) designs.[28−30] Environmental impacts (e.g., an oil-spill) usually are not replicated in space (i.e., an oil-spill typically happens in one area, not in several, spatially distant areas simultaneously); thus, replicate samples can only be collected in that area alone. Samples from this unique 'impacted' area must, however, be compared to replicate samples collected in multiple nonimpacted or "control" areas to allow determining whether a true impact has occurred, rather than observed differences being simply due to spatial variability.[29] Such designs are intrinsically unbalanced and asymmetrical.[28,29] Similarly, in our study, only one industrial area is available, but because two residential areas exist, samples from both areas can be compared to industrial samples using multivariate asymmetrical statistical analyses that allow disentangling the effects of land-use from simple spatial variation.[31] Sediment and water samples were collected from the previously described locations for microbial and environmental parameters analysis. Each sediment sample was prepared by pooling fresh sediments from top ∼5 cm layer of 5 random spots within 5m diameter. The samples were collected in clean nuclease free Falcon 50 mL Conical Centrifuge Tubes (Thermo Fisher Scientific) using an autoclaved spatula. About 5 mL of overflowing water was added on the top of each sediment sample. The samples were immediately transferred to the icebox and transported to the laboratory for storage in −80 °C till further analysis. The DNA was extracted from fresh samples. The time elapsed from the collection of the first sample to the beginning of DNA extraction was <2.5 h. About 1.2 L of water was collected for DNA extraction in autoclaved glass bottles. For metals and nutrient analysis, 50 mL of water was collected in separate clean Falcon 50 mL Conical Centrifuge Tubes (Thermo Fisher Scientific). For organics analysis, 1 L of water sample was collected in amber glass bottles. While, only water samples were tested in situ using YSI 556 MPS (YSI Inc.) probe for physicochemical properties, both sediment and water samples from all the locations were analyzed for other physicochemical properties (Figure 2C and D).

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A subset of the samples, collected for spatial analysis, was chosen randomly to represent the industrial and the two residential locations. These samples were analyzed for structure and functional potential of microbial communities; however, the presence of inhibitory substances caused issues with DNA cleanup and therefore, some samples, particularly from the sediment in residential region 1, failed to amplify. Thus, two water and three sediment samples from the industrial region, four water and one sediment samples from residential region 1 and three water and four sediment samples from residential region 2 were analyzed for functional gene abundances. For taxa profiles, four water and four sediment samples from industrial region, four water and one sediment samples from residential region 1 and six water and four sediment samples from residential region 2, were analyzed.

GeoChip and PhyloChip Microarray Hybridization. Genomic DNA was extracted from the about 5 g (wet weight) of sediment and 1.2 L of water samples immediately after the samples were brought to the lab. Sediments were dewatered and DNA was extracted by a combination of mechanical, chemical and thermal lysis and chloroform-isoamyl alcohol purification using protocol described elsewhere.[32] Water samples were filtered through sterile Millipore 0.22 μm membrane filter (Thermo Fisher Scientific Inc.) to collect the biomass, followed by the DNA extraction protocol similar to the DNA extraction protocol for sediment samples. Humic substances were removed using OneStep PCR Inhibitor Removal Kit from Zymo Research Corporation (USA). The DNA quality and quantity was measured using Quant-iT PicoGreen dsDNA Assay Kit (Life technologies) before loading onto microarrays. Functional gene array (FGA), GeoChip 3.0 was used to detect the functional gene abundance of microbial communities in the watershed. The information on probe and covered sequences is available elsewhere.[33] Labeling of genomic DNA sequences, hybridization of labeled sequences and array image scanning were performed as described previously.[34] A cutoff of Signal to Noise (SNR) = (signal mean − background mean)/(background standard deviation) > 2 was set as signal threshold.[34] The resulting functional genes abundance data matrix was then analyzed using square-root and presence/absence transformation. PhyloChip (G3) was used to analyze the microbial community composition in the watershed following the previously described procedures for PhyloChip assay design and assay analysis to obtain hybridization scores and criteria for presence/absence calls.[35]

Analysis of Physicochemical Properties. Physicochemical properties, such as metals, nutrients, pharmaceutical drugs, organics and water-quality parameters were measured according to standard protocols. The details of these protocols are provided as Supporting Information.

Quantitative-PCR Analysis. Sediment samples were collected from two residential sites (R8 and R10) and two industrial sites (I2 and I3). Samples were collected 1 h before rain, 4 h after rain, and two samples were collected at the interval of 24 h after rain in triplicate. This resulted into 48 samples from four time-points (prerain and postrain 1, 2, and 3). Samples were transported on ice to the lab, and DNA was extracted and cleaned using the protocols described above. Genomic DNA was serially diluted and adjusted to 25 ng/μL to be used as template. SYBR Green PCR Master Mix from Life Technologies, Thermo Fisher Scientific Inc. was used to prepare the reaction mixture. The composition of reaction mixture is as follows: SYBR Green PCR Master Mix (2×), 10 μL; forward and reverse primers, 1 μL (200 nM, final concentration); template DNA, 2 μL (25 ng/μL); and water: 6 μL. The conditions for Q-PCR cycles are as follows: holding stage, 95 °C (10 min); cycling stage (40×), 95 °C (15 s), 62 °C (30 s), 72 °C (30 s). The Q-PCR products were subjected to melt-curve analysis to check the specificity of amplification. The melt-curve was performed from 60 to 95 °C with a step increase of 0.5 °C. As the objective of this experiment was to validate the trend of functional genes abundance observed in GeoChip between the two land-use types, analysis was done for relative abundance rather than absolute quantification. The amplification was monitored by ABI Prism 7500 (Applied Biosystems, Life Technologies, Thermo Fisher Scientific Inc.). C_t values were compared to confirm the trend observed in functional gene abundance from GeoChip data. The primers, specific to the target genes, are provided in Supporting Information Table S9.

Statistical Analysis. Microbial community and function data were compared between land-use types and phase using permutational multivariate analyses of variance[31] with PERMANOVA add-on (version 1.0.3) in PRIMER v6 (version 6.1.13) from PRIMER-E Ltd. (USA). Cluster analysis and correlations were performed using R 3.0.2.[36] Please refer to the Supporting Information for details.

RESULTS

Characteristics of Sungei Ulu Pandan Watershed. Sungei Ulu Pandan watershed, which lies in the Southwest region of Singapore (Figure 1A) and drains water to the Pandan reservoir, was used as a model area for this study. It has two major land-use types, residential (10.01 km^2) and industrial (4.1 km^2) (Figure 1B, Supporting Information Table S5). The two broad land-use types are confounded with other smaller land-uses, such as managed or untouched vegetation cover, commercial areas and educational buildings. The regions of different land-use types in the watershed are divided by a combination of elevation topology, urban engineering and land-use types. For example, regions 1 and 2 of residential land-use types are fragmented by elevation and space (Figure 1C). The industrial region is at a lower altitude with industries such as warehouses, industrial storage, and water treatment plant. Water from the residential region 1 enters the western arm of Sungei Ulu Pandan and flows into the industrial region. Water in residential region 2 is collected by two subcanals and drains into the eastern arm of Sungei Ulu Pandan canal. It can be clearly seen that the two main arms of Sungei Ulu Pandan canal collect water from the two sides of highlands. These highlands, therefore, seem to be fragment the watershed into eastern and western regions with limited mixing.

Spatiotemporal Relationships of Environmental Parameters. To study the temporal changes in physicochemical properties of a model watershed, which consisted of two spatially separated land-use types, three sites, SUP1, 2, and 3 were selected from a residential region, which represented a confluence of two large canals (Figure 1B and 1C). Water samples were tested for 48 parameters (Figure 2A, Supporting Information Table S1) on monthly basis for a period of one year from January to December, 2009. Temporal variations in the physicochemical properties were detected as low as 6% (pH and temperature) and as high as 178% for metals such as cadmium and aluminum. However, they remained below the permissible limits (see Supporting Information Tables S2, S3, and S4). Water levels subsided within 4−5 h after a typical rain

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event that ranged between 27 and 64 mm/h.[37] This supports the effectiveness of the dense canal network (see Supporting Information Table S5) for water drainage. However, there were major variations in the bulk water flow velocity, with ~2.6 cm/s at basal flow and ~40 cm/s at peak flow (see Supporting Information Table S2), measured during a dry period and after a rain event respectively at SUP1. Among metals and organics, while metals remained in parts per billion range (see Supporting Information Table S3), common pharmaceuticals, such as caffeine and diclofenac, were in the detectable range but lower than their reported levels of minimum inhibitory concentrations (MIC)[38,39] by 5−6 orders of magnitude (Figure 2B, Supporting Information Table S4). Interestingly, caffeine was observed throughout the year and at concentrations higher than the other pharmaceutical compounds, consistent with its widespread usage among urban population.

To identify the spatial relationships among environmental variables, Kendal Tau, nonparametric correlation coefficient, was calculated for 36 parameters in sediments and 43 parameters in water samples from 19 locations. Two clusters of relationships emerged in the correlation matrix obtained (Figure 2C; boxed areas), showing different trends in sediment and water samples. In the first block (red box), ten organic compounds including solvents and pesticides displayed significantly positive correlations with each other in the water phase but not in the sediment phase. The second block (green box) of relationship was that of metals and nutrient ions, where sediments showed positive and water samples showed negative correlations. Within this block, positive correlations were observed between ammonium and metals/sulfates in water and negative correlations (except aluminum) were detected in sediment samples. Levels of organic compounds and antibiotics were either very low or below detection limit of GC-MS, both in sediment and water phase (see Supporting Information Table S6a and S6b).

Sediments Harbor More Diverse Microbial Communities than Water Column. To understand the distribution of microbial communities in the sediment and water phases, we studied taxa profiles in 9 sediment and 14 water samples across the watershed in the two land-use types. The microbial communities were composed of 2083 taxa groups at the subfamily level. Secondary-producers such as *Proteobacteria*, *Firmicutes*, and *Bacteriodetes* were the topmost of the 35 phyla, which accounted for nearly 58% of the community composition (see Supporting Information Table S7). Hence, the dominant phyla in this watershed seem to be similar to freshwater systems, studied earlier, such as mesotrophic lakes and glacier fed streams.[40,41] *Proteobacteria* was the most dominant phylum, which accounted for 29% of total subfamilies detected. However, the *Cyanobacteria*, which are one of the primary-producers, were found to be low in abundance. Other commonly reported aquatic microbial taxa, such as; *Planctomycetes*, *Actinobacteria*, and *Acidobacteria* were also represented in the range of 4 to 6% of total microbial community composition (see Supporting Information Table S7).

The distribution pattern of unique taxa was different within phase (sediment and water) and within the two land-use types (residential and industrial) (Figure 3A). On an average, sediment contained slightly higher unique taxa groups than the water phase (59 and 40, respectively). However, for land-use types, the distribution of unique taxa group (averaged over samples) was skewed in favor of residential sites, which had twice as many of them as compared to industrial sites (29 in

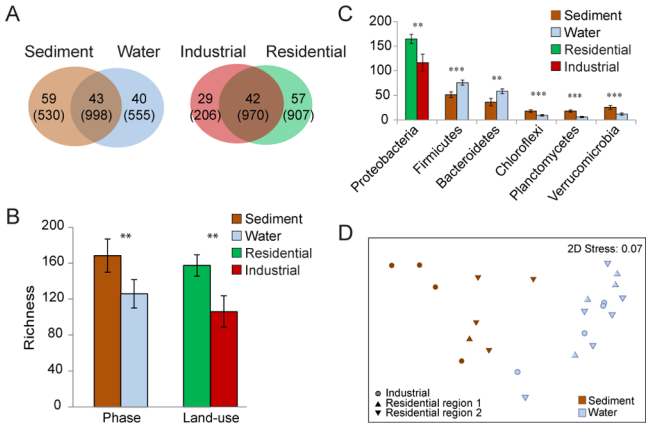

Figure 3. Microbial community composition in the sediment and water phase of urban waterways. (A) Distribution of unique and common taxa at subfamily level, averaged over the number of samples in respective categories between land-use and phases using presence/absence data. Values in brackets indicate total number of unique and common taxonomic units (B) Specie richness calculated on number of taxa detected in phylum for each sample. Average species richness in both, phases and land-use types, is plotted. Error-bars indicate the standard error from mean. Significance for differences were tested using *t* test (C) Phyla (no. taxa detected in phylum) showing significant difference between both the land-use types and phases are shown. Error-bars indicate the standard error from mean. (D) NMDS plot of taxonomic abundance data from PhyloChip using Bray−Curtis as distance matrix. $^{**}p < 0.05$, $^{***}p < 0.01$

industrial and 57 in residential). Similarly, the pattern of microbial community composition richness was different within phase and within land-use types. The sedimentary microbial communities had higher species richness than the suspended microbial communities ($p < 0.05$) (Figure 3B). However, the high richness was largely attributed to lesser dominant phyla such as *Chloroflexi*, *Planctomycetes*, and *Verrucomicrobia*, which were significantly higher in sedimentary microbial communities ($p < 0.01$) (Figure 3C). For the same reason, sedimentary microbial communities had a higher evenness compared to suspended communities, whose composition was largely dominated by two phyla: *Firmicutes* and *Bacteriodetes*. Interestingly, *Cyanobacteria*, which are one of the primary producers, were similar between the two phases. In contrast, within land-use types, residential microbial communities showed higher species richness than industrial communities ($p < 0.05$) (Figure 3B). This was mainly due to *Proteobacteria* (Figure 3C and Supporting Information Table S7), which was the most dominant phyla among all.

Sedimentary and suspended microbial communities were clearly separated based on their composition as visualized by nonmetric multi-dimensional scaling (NMDS) plot (Figure 3D). In comparison, such clear separation was not obvious between microbial communities from residential and industrial land-use types. For instance, three samples from industrial sites were present in the cluster representing residential samples, while two samples from residential sites fell within the cluster of samples from industrial sites. These qualitative observations were further substantiated by PERMANOVA results, which showed significant differences between the structure ($p = 0.039$) and compositions of microbial communities ($p = 0.02$, Table 1A) from the two land-use types. The differences were more pronounced between the communities from two phases ($p < 0.01$, Table 1A) than between land-use types (components

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Table 1. PERMANOVA Results for Structure and Functions of Microbial Communities Based on the Bray−Curtis Similarity for Abundance and Jaccard Similarity Measure for Composition (Presence/Absence) Data between Phases and Land-Use Types[a]

	A				B			
source	df	MS	pseudo-F	*p*(perm)	df	MS	pseudo-F	*p*(perm)
LU	1	97 (3261)	15.60 (1.46)	**0.039 (0.02)**	1	2865 (2719)	2.79 (1.57)	**<0.001 (0.047)**
Ph	1	744 (6452)	2.39 (2.88)	**0.001 (<0.01)**	1	1963 (2785)	1.91 (1.61)	**0.041 (0.049)**
Lo(LU)	1	46 (2451)	pooled		1	1181 (1763)	pooled	
LUxPh	1	79 (2817)	1.58 (1.26)	0.145 (0.07)	1	1615 (2222)	1.57 (1.28)	0.087 (0.133)
PhxLo(LU)	1	47 (2264)	pooled		1	843 (1552)	pooled	
Res	17	52 (2229)			11	1018 (1730)		

[a](A) Results for square-root transformed taxonomic abundance data are shown here. Results for presence/absence data are shown in round brackets (B) Results for square-root transformed functional genes abundance data are shown in this panel. Results for presence/absence data are shown in round brackets. *p*-values were calculated using 9,999 permutations under a reduced model. LU: Land-use (residential and industrial); Ph: Phase (sediment and water); Lo: Regions (Residential and industrial regions).

of variation: phase > land-use; Supporting Information Table S8A and S8B). PERMDISP analysis, which tests the dispersion of intersample distances within clusters, showed no significant differences in dispersion between land-use types or phases (see Supporting Information Table S9). Therefore, the dissimilarities in the two clusters were largely due to differences among centroids (location) rather than multivariate dispersion. Centroid-based separation ruled out the possibility of one microbial community (for example from water phase) being a subset of another microbial community (for example sediment phase). It rather supported the observation that the microbial communities in these two phases are different with some overlapping taxa (Figure 3A).

Functional Genes Distinguish Microbial Communities of Land-Use Types. As indicated above, land-use types were distinguished based on structure (p = 0.039) and composition (p = 0.02) of microbial communities. However, this distinction was highly significant when based on relative abundances (p < 0.001) and to some degree, on the composition (p = 0.047) of functional gene profiles in the two land-use types (Table 1B). The composition and the structure of functional genes did not differ in terms of their dispersion between land-use types nor phases (see Supporting Information Table S9). Hence, functional potential of microbial communities, based on abundance, seem to be more sensitive to the local environment in the different land-use types than the taxa profiles. Conversely, within a given land-use type, namely residential regions, the functional potential of microbial communities were similar (Table 1B), despite being separated both by another land-use type and highlands (Figure 1B and C).

Microbial communities from residential and industrial land-use types formed different clusters in hierarchical clustering and NMDS plots based on functional gene abundances (Figure 4A and B). Within the cluster of each land-use type, the communities from the sedimentary and suspended phase formed different subclusters. The differences between profiles of functional gene abundances and composition from different land-use types (p < 0.001 and p = 0.047, respectively) and phases (p = 0.041 and p = 0.049, respectively) were also highly significant based on PERMANOVA analysis (Table 1B). However, the estimates of component of variations indicate that functional genes abundance differentiated the land-use types more than phase (see Supporting Information Table S8D). Similar to the microbial taxa profiles, these dissimilarities were largely due to differences among centroids (see Supporting Information Table S9), which indicated that the

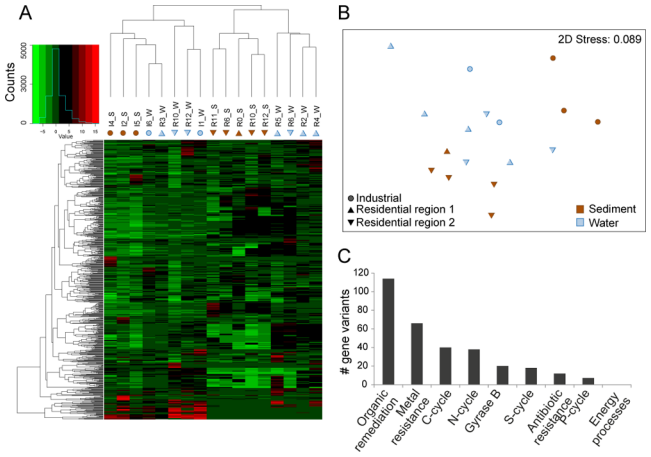

Figure 4. Distribution of functional genes abundance in different land-use patterns. (A) Hierarchical clustering heat-map of functional gene abundance. Spearman rank similarity matrix and complete-linkage method were used to generate clusters of the samples. The genes and samples were median-centered and scaled across rows (B) NMDS plot of functional gene abundance. The functional gene abundance was square root transformed before creating Bray−Curtis distance matrix for NMDS analysis. (C) Number of gene variants (counts) in different gene categories, across the watershed, contributing to the variation of functional gene abundances between the microbial communities from different land-use types.

functional gene profiles of microbial communities in the two land-use types were different with some overlap.

Functional potential of microbial communities of the watershed mainly belonged to nine functional gene categories with 11 848 gene variants (see Supporting Information Table S10). Antibiotic resistance, organic remediation and metal resistance genes were the most abundant in that order and represented 45−50% of the total number of gene variants on the GeoChip in their respective categories. PERMANOVA analysis showed that functional gene abundance profiles in antibiotic resistance and metal tolerance gene categories, tested independently, were significantly different in both land-use type and phase. However, gene abundance profiles of organic remediation gene category showed significant difference only between land-use types (see Supporting Information Table S11). Other gene categories, such as energy processes, as well as phosphorus, sulfur, nitrogen, and carbon cycling represented 30−40% of gene variants (see Supporting Information Table S10). As the land-use types were clearly differentiated by functions, we next asked which gene categories were associated

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with this differentiation. Our results showed that the difference among the land-use types based on functions was due to only ~3% (328) of total gene variants using *t* test (Bonferroni-corrected, unpaired *t* test: $p < 0.01$). The top two differentiating gene categories among this group were organic remediation and metal resistance, which respectively, had 35% and 21% of the total differentiating gene variants (Figure 4C and Supporting Information Table S10). In the most abundant set of organic remediation genes that differentiated the land-use types, both aromatic and aliphatic compounds degradation pathways of the organic remediation gene category were highly abundant in industrial region, in comparison only aromatic compounds degradation pathways were abundant in the residential regions (see Supporting Information Table S12). Among the differentiating metal resistance genes, copper and lead resistance genes were more abundant in industrial areas, while a set of cadmium efflux genes was more abundant in the residential areas. Among antibiotic resistance, SMR (small molecule resistance) and β-lactamase were widespread in both the land-use types. While, industrial regions had higher abundance of MFS family (major facilitator superfamily), residential regions showed higher abundance of specific antibiotic resistance such as Tet (tetracycline resistance) and Van (vancomycin resistance). Hence, among functional gene markers in the land-use types, the complexity of 11 848 gene variants was reduced to 36 gene variants from three gene categories (see Supporting Information Table S12).

These trends of gene abundances were reproducible over time, both before and after rain, when quantified using a different platform of Q-PCR. Seven functional genes belonging to the top two gene categories, organic remediation and metal resistance were selected for validation studies (see Supporting Information Table S13). As the objective of this study was to validate the trend of functional genes abundance observed in GeoChip between the two land-use types, relative differences of gene abundance between land-use types were determined. The ratio (residential group/industrial group) of C_t values from Q-PCR data of all seven genes corroborated average gene abundance from GeoChip data (see Supporting Information Figure S1).

Metals Are Major Drivers of Microbial Communities. Environmental parameters did not vary between land-use ($p > 0.8$), indicating that the chemical markers were unable to differentiate land-use types. However, they were significantly different between phases ($p < 0.01$), based on PERMANOVA analysis (see Supporting Information Table S14). The major contributors of the differences between the phases were metals such as Ca, K, Cu, Mg, and Zn (see Supporting Information Figure S2). Among the variables that qualified for all samples (see Supporting Information Table S15), most of these (85%) independently explained 6−11% of the variation in microbial community composition, based on the marginal test of DistLM ($p < 0.05$) (see Supporting Information Table S16). The most parsimonious model indicated a subset of these variables (K, Cu, Ca, Mg, TOC, and Zn) as strong predictor variables of distribution of microbial community composition (Figure 5A), explaining 37% of the total variation. Of these, Cu and K significantly explained 7% and 11% of the total variation, respectively, in the microbial community composition (see Supporting Information Table S17).

In the final set of analyses we analyzed the role of environmental parameters on the functions of microbial communities, where again metals emerged as the major drivers.

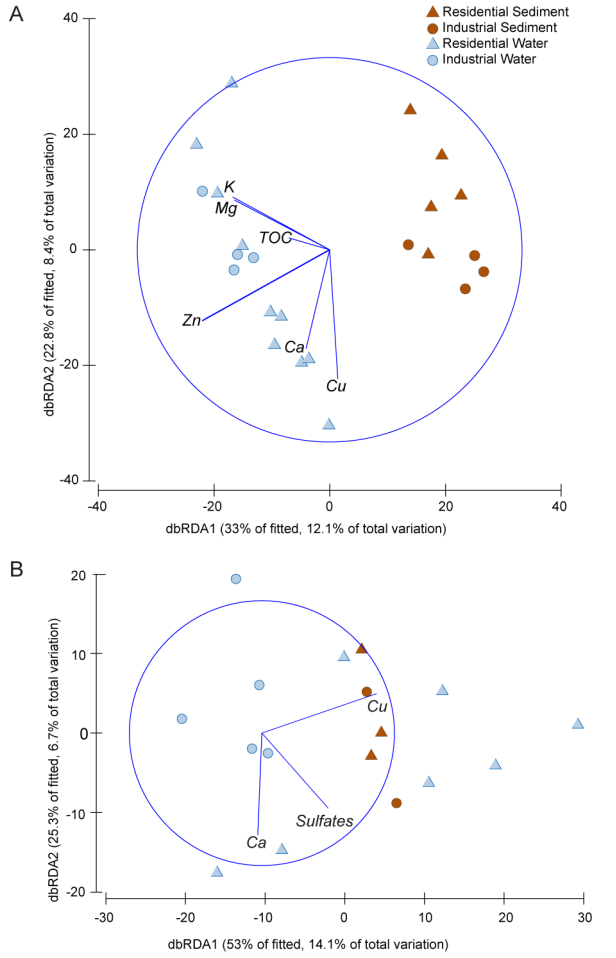

Figure 5. Distance-based linear models (DistLM) analysis with fitted model visualized using the distance-based redundancy analysis' constrained ordination (dbRDA) biplot of samples and environmental parameters data. Samples were plotted as dbRDA coordinate scores and environmental variables as eigenvectors. Most parsimonious model is fitted to the microbial community (A) composition and (B) functions. The circle depicts a correlation value of 1.

Cu, Ca, and SO_4^{-2} were identified as predictor variables of functional gene distribution (Figure 5B) and explained 27% of the total variation. Cu ($p < 0.05$) and Al ($p = 0.05$) individually explained about 11% and 10% variation of the functional composition of microbial communities in marginal tests (see Supporting Information Table S18), despite only Cu being significantly related to the variation in the functional composition of microbial communities (see Supporting Information Table S19).

DISCUSSION

Using a combination of phylogenetic and functional genes-based ecogenomics with meta-environmental data, two key relationships were uncovered between environmental parameters and land-use types, respectively, with highly complex microbial communities. These microbial communities are key factors, providing ecological services to the watershed ecosystem, such as elemental cycling, organic remediation, and nutrient removal. First, based on 36 parameters (both in sediment and water) studied, metals emerged as the top influencing environmental category for this watershed. Our approach led us to the identification of associations between

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different metals and microbial communities. While some metals related to both composition and functions of the microbial communities, others affected either one or the other trait. The presence of metals in the waterways could be attributed to their release as a result of turbulence and velocity variations in water.[42,43] While copper emerged as a potential key driver of both the composition and functions of microbial community, aluminum was most strongly related to community functions. Potassium was among the variables associated to microbial taxa. It is noteworthy that these metals can potentially have such strong influences on microbes even at levels below allowable limits. As these metals are at very low levels (ppm and ppb), it is possible that they affect the microbiome through their different roles in diverse metabolic functions, rather than through toxicity.[44−46]

Metals can influence microbial communities in complex ways. Copper has profound effects on the metabolism as it is one of the top three metals involved as cofactors in enzymes[47−49] and has been classified as a macro-nutrient required by living organisms. It is also known to affect viable but nonculturable property of bacteria.[45] Hence, it can have broad ranging effects on the microbiome. While the mode of action of aluminum on microbiome is not yet known, it is likely to be through the central metabolism, as studies in yeast show that changes in citrate metabolism affects aluminum tolerance.[50] Potassium could affect the assembly of microbial communities as it influences the functions of transporters by its ability to influence the cytosolic ionic strength.[51,52] In case of other metals, as they are taken up by overlapping set of transporters and can share the metabolic pathways,[46] it might not be possible to identify the individual effects of such metals.

The second relationship established was between the land-use types and their associated microbiomes. Our results showed that a physically separated land-use type, as exemplified by the two residential regions, does not lead to differentiation of microbial communities either in their composition or their functions. This similarity of microbiomes in physically separated regions of same land-use types, are perhaps the outcome of uniform management practices within the land-use type. Functional genes-based differentiation of land-use types more than taxa-based ones suggests that they can be developed into reliable tools for monitoring and assessing the health of aquatic ecosystem in response to change in local environments. Using a combination of ecogenomics and multivariate statistical approaches, the complexity of 11,848 gene variants has been reduced to 12 gene targets from a single gene category of metal resistance genes that can efficiently report on microbiome variation in different land-use types. As orthogonal data sets, microbial markers and environmental parameters converged on the same pathways of metal resistance. Such an integrative approach provides an additional level of confidence in identifying the drivers that influence processes and their outcomes in an urban watershed, rather than current practices that rely heavily on environmental parameters. For practitioners, a combination of markers from top influencing gene categories, such as ArsC (arsenic tolerance), TehB (tellurium tolerance) and CopA (copper tolerance) genes from metal resistance, could be useful in assessment approach.

Some common themes of community structure and functions that emerged from this study seem to be consistent with the other marine and natural fresh water systems.[53,54] The microbial communities of water and sediment phase are exposed to different pressures, which reflect their contribution to the ensuing processes and alter the physical and chemical properties of the waterways.[54] While water associated microbial communities have transient effect in canal networks, even at the basal flow-rates, the sedimentary microbial communities are more persistent and thus likely to have higher richness and contribute more to the functioning of the ecosystem.[55] In this study, suspended microbial communities in the waterways have strong influence of few dominant phyla, which are also present in the sedimentary microbial communities, but to a lesser extent. However, the sediment associated microbial communities have high richness due to the presence of less abundant phyla such as *Chloroflexi*, *Planctomycetes*, and *Verrucomicrobia*. These phyla belong to the group of secondary producers and have diverse metabolic capabilities[56−59] that have previously been reported to carry out utilization of a wide range of complex sugars, the reduction of nitrates and nitrites without denitrification, dehalogenation and degradation of halogenated organic compounds and phosphate removal.[56−59] Hence, the sedimentary microbial communities, which support high richness of these phyla, may provide them the advantage to survive in the low nutrient environment such as urban waterways. Relatively similar distribution of *Cyanobacteria* between phases, on the other hand, indicates that the differentiation in the microbial communities between sediment and water phase is mostly driven by heterotrophs that consume fixed carbon rather than the producers. The ability of the microbial communities to utilize diverse organic compounds suggests that the sedimentary microbial communities in urban waterways are equipped to survive in a limited nutrient environment, such as in the watershed under study.

On the basis of the above, molecular markers showed clear advantage in being more sensitive than environmental markers as they can differentiate between different land-use types under conditions present in this watershed. Within the molecular markers, differentiation of land-use types by functional assemblage of microbial communities more than taxa-based markers indicates function-based markers to be more sensitive to changes in land-use than taxa-based ones. This suggests that the unit of response of microbial communities to local environment seems to work at the level of functional adaptation rather than at the level of biological units of taxa. The observation of functions more than taxonomic-based microbial community assembly within a land-use type are consistent with other recent studies.[60−62] Microbial taxa composition, on the other hand, differ between phases but remain similar between land-use types in a given phase, indicating that composition of microbial communities have different preferences to colonize between phases rather than local environment offered by land-use.

Findings from this study will help the watershed managers to monitor the ecological health of waterways ecosystem with finer resolution using functional genes as markers rather than chemicals or taxonomic units. The strong trends in this study necessitate the need to replicate such studies in other urbanized environments so that a generalized ecogenomics-based framework for watershed management can be developed. Management interventions to control the local environments can then be perused to influence the functional potential of microbial communities in the waterways, enhancing critical ecological services to develop sustainable, self-cleaning waterways infrastructure.

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■ ASSOCIATED CONTENT

Supporting Information

Detailed protocols of physicochemical properties analysis, statistical analysis, Figures S1−S2, Tables S1−S19, and abbreviations. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE64286.

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Notes

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