BRAZILIAN JOURNAL OF MICROBIOLOGY

http://www.bjmicrobiol.com.br/

Environmental Microbiology

# Comparative metagenome of a stream impacted by the urbanization phenomenon

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ARTICLE INFO

*Article history:*
Received 25 March 2015
Accepted 2 February 2016
Available online 4 July 2016
Associate Editor: Valeria Maia de Oliveira

*Keywords:*
Aquatic environment
Urbanization
Microbial community
Metagenomic

ABSTRACT

Rivers and streams are important reservoirs of freshwater for human consumption. These ecosystems are threatened by increasing urbanization, because raw sewage discharged into them alters their nutrient content and may affect the composition of their microbial community. In the present study, we investigate the taxonomic and functional profile of the microbial community in an urban lotic environment. Samples of running water were collected at two points in the São Pedro stream: an upstream preserved and non-urbanized area, and a polluted urbanized area with discharged sewage. The metagenomic DNA was sequenced by pyrosequencing. Differences were observed in the community composition at the two sites. The non-urbanized area was overrepresented by genera of ubiquitous microbes that act in the maintenance of environments. In contrast, the urbanized metagenome was rich in genera pathogenic to humans. The functional profile indicated that the microbes act on the metabolism of methane, nitrogen and sulfur, especially in the urbanized area. It was also found that virulence/defense (antibiotic resistance and metal resistance) and stress response-related genes were disseminated in the urbanized environment. The structure of the microbial community was altered by uncontrolled anthropic interference, highlighting the selective pressure imposed by high loads of urban sewage discharged into freshwater environments.



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http://dx.doi.org/10.1016/j.bjm.2016.06.011

## Introduction

Rivers and streams contain approximately 0.006% of the freshwater available on Earth,[1] but this resource is becoming limited. More than 30% of the renewable freshwater available for consumption is used for agricultural, industrial and domestic purposes. The main consequence of these activities, and of urbanization, is the injection of large quantities of waste into the water, contaminating it with xenobiotic compounds.[2] Pollution can modify the structure and composition of rivers and streams by altering their geomorphology, temperature, pH, nutrients, and biotic community.[3] The chemical pollution of natural waters can render environments dangerous for life. This problem occurs not only developing countries, which lack suitable waste management, but has already become a major public concern in most of the world.[2,4]

Numerous studies have sought to demonstrate the impacts of urbanization on freshwater ecosystems. Some of these studies, which focus on physicochemical parameters such as nutrients, show that urban environments contain increased levels of phosphorus, nitrogen, nitrate, ammonia, and potassium.[3,5–8] Metal and pesticide contaminants have also been identified in urban areas.[9] However, this type of analysis yields limited information when the objective is to understand the complexity of ecosystems, and, in this case biological components must be taken into account. Planktonic microorganisms (Bacteria, Archaea, members of Eukarya, and viruses) dominate these ecosystems in terms of abundance and biomass. They represent a large and diverse pool of species responsible for sustaining metabolic activities, including biogeochemical processes, and organic matter and nutrient recycling.[10–12] The microbial community of aquatic ecosystems is extremely important for the maintenance and sustainability of these environments since microbes are highly sensitive to anthropogenic stress.[13] However, only a few studies have analyzed the effect of urbanization on the microbial community.[12,14,15]

A previous study by our group focusing on an urban stream showed a higher concentration of dissolved nutrients in the urbanized waters. Using culture-independent methods such as Fluorescence *in situ* hybridization and PCR, we observed that urbanization alters the density of Nitrosomonadaceae, Nitrospiraceae, and *Nitrobacter*, microbes involved in the nitrogen cycle, and increases the occurrence of *Enterococcus*, *Streptococcus*, *Bacteroides/Prevotella/Porphyromonas*, *Salmonella*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and the diarrheagenic strains of *Escherichia coli*, which are considered potentially pathogenic to humans.[8] However, to date, no in-depth and comprehensive description is available about the taxonomy and functionality of microbes in urban freshwater ecosystems. Therefore, metagenomic comparisons of preserved and polluted areas of a stream may contribute significantly to a better understanding of the real anthropogenic impacts on aquatic environments.

Metagenomic is an important tool for understanding microbial ecosystems, given its ability to provide information about the diversity and distribution of the different members of a community and their metabolic potential.[16] This methodology has increased the knowledge about diverse microbiomes, such as oceans,[17] the human body,[18–20] and soil,[21–23] especially due to high-throughput sequencing technologies. In this context, the aim of this study was to make a comprehensive description of the taxonomic and functional profile of the microbial community in an urban stream, comparing a polluted and a preserved area. This was achieved by means of a metagenomic approach using 454-pyrosequencing.

## Material and methods

### *Sample collection and DNA extraction*

Approximately 6 and 12 L of samples were collected from the subsurface water of the urbanized and non-urbanized sites, respectively, of the São Pedro Stream located in Juiz de Fora, Brazil, in December 2010. The water samples were stored separately in 15 L bottles that had been previously rinsed three times with sample from each site. The sites were characterized in a previous study,[8] as follows: the urbanized site (661799E/7591070N), which is surrounded by houses, is polluted with sewage release. The system at this point is considered eutrophic, since it has extremely high contents of ammonia, nitrite, nitrate, total organic nitrogen, and total phosphorus. The non-urbanized site (668307E/7591772N), located in a farming region, is upstream from the urban area and has a low concentration of dissolved nutrients, characterizing it as a preserved system.

The water samples were sonicated on ice three times for 60 s, at an amplitude of 90%, using a Vibra-Cell VCX 130 PB ultrasonic processor (Sonics & Materials, USA). The samples were filtered twice, first through a paper filter (3M, USA) and then through a GF/F filter (Whatman Ltd, UK).[8] The filtered water was centrifuged at 8000 rpm for 15 min in 500 mL bottles. The microbial DNA was extracted using a PowerMax Soil DNA Isolation Kit (MoBio, USA). DNA integrity was checked by agarose gel electrophoresis and quantified spectrophotometrically in a NanoDrop ND 1000 instrument (Thermo Scientific, USA).

### *Sequencing and analysis*

Five micrograms of DNA were used for sequencing in the 454 Sequencing GS FLX Titanium platform at the National Laboratory for Scientific Computation (LNCC) (Petrópolis, Rio de Janeiro, Brazil). The DNA from each of the two areas constituted one-quarter of the plate, without replicates. The obtained reads were quality-trimmed to remove short sequences (fewer than 180 bp) or sequences with Phred quality ≤20, using LUCY software.[24] To eliminate artificially replicated sequences, 454 Replicates[25] were used. The resulting sequences were uploaded to the Metagenomics RAST server (MG-RAST)[26] and made publicly accessible under code numbers 4464295.3 and 4464296.3 for non-urbanized and urbanized metagenomes, respectively. The NCBI access number for the sequences is SRA051287.

### Taxonomic profile

The taxonomic profile was determined first by BLASTN and BLASTX of all the reads against the NCBI-NT and NCBI-NR databases, respectively, using a cut-off E-value of 1e−5. The results were visualized on MEGAN v 4.0 (MetaGenome Analyzer software)[27] with the LCA algorithm (max. number of matches per read: 5, min. support: 5, min. score: 35, top percent: 10). The 16S rDNA reads were also used for taxonomic classification. These sequences were extracted from each dataset using Meta-RNA software.[28] The 16S rDNA sequence was classified using RDP Classifier v 2.5 software[29] with a confidence threshold of 50% against the RDPII database. Analysis was also performed using the fully automated MG-RAST. The system conducts BLASTX searches against the SEED database using a max. E-value cut-off of 1e−5, and min.% identity cut-off of 60.

### Functional profile

Functional classification was performed using BLASTX (cut-off E-value of 1e−5) against NCBI-NR. Annotation results were loaded into MEGAN v 4.0, and classification was realized using KEGGs and SEED identifiers. BLASTX against SEED was also performed using MG-RAST.

Sequences assigned to DNA, RNA and protein metabolism, virulence, stress response, nitrogen, sulfur and methane metabolism were extracted from the dataset, using MEGAN v 4.0, in order to identify the taxonomic groups that contribute these genes to the environments. The extracted sequences were compared to NCBI-NR using BLASTX (cut-off E-value of 1e−5) and further taxonomically classified using MEGAN v 4.0, as previously described.[30]

### Statistical analysis

Statistically significant differences between the two water samples were determined using the Statistical Analysis of Metagenomic Profiles (STAMP)[31] software package. The analysis was performed using two-tailed Fisher's exact test, while the confidence intervals were calculated using the Newcombe–Wilson method. The Benjamin–Hochberg FDR method was used for multiple-test corrections.

## Results

### Taxonomic profile of freshwater metagenome

A total of 620,443 reads were obtained from pyrosequencing of the urbanized and non-urbanized water samples. After trimming, the valid sequences for each metagenome were 242,356 (average size 300 bp, 54 ± 10% GC content) from the urbanized and 220,441 (average size 329 bp, 56 ± 9% GC content) from the non-urbanized areas. Considering the annotated reads, both metagenomes were dominated by bacteria (99.2% and 96.3% in urbanized and non-urbanized, respectively), followed by a smaller fraction of archaea (0.3% and 2.9%). The percentages of viruses and eukaryotes were quite low.

A total of 681 partial sequences of 16S rDNA were obtained in the dataset, 475 from the urbanized metagenome and 206 from the non-urbanized. The taxonomic classification obtained from 16S rDNA sequences and from all the reads was similar in the urbanized metagenome (Fig. 1). However, due to the large number of unclassified bacteria (33.3%) found in the 16S rDNA classification for the non-urbanized metagenome, the comparison of the two datasets was somewhat divergent (Fig. 1). Considering the complete data classified for the domain Bacteria, the most prevalent phylum in both metagenomes was Proteobacteria (77.1% in the urbanized and 70.6% in the non-urbanized), followed by Bacteroidetes (13.2%), Firmicutes (4.5%), and Actinobacteria (3.1%) in the urbanized and Firmicutes (5%), Acidobacteria (5%), and Verrucomicrobia (4.5%) in the non-urbanized metagenome (Fig. 1). Considering only the most prevalent phylum, Proteobacteria, a higher incidence was observed in the beta division, followed by gamma in the urbanized metagenome and alpha in the non-urbanized. It should be noted that the taxonomic affiliations between phyla in the non-urbanized metagenome were distributed more equally (Fig. 1).

The taxonomic affiliation of the sequences in bacterial genera showed a significant statistical difference between the two environments (Fig. 2). The most prevalent genus in the urbanized metagenome was *Burkholderia*, accounting for 7.93% of the proportional differences between the sequences observed in the metagenomes. This was followed by *Escherichia* (7.86%), *Shigella* (4.56%), *Bacteroides* (3.49%), *Acidovorax* (2.64%), *Salmonella* (2.04%), *Acinetobacter* (1.83%), *Polynucleobacter* (1.54%), *Vibrio* (1.48%), *Yersinia* (1.4%), *Pseudomonas* (1.28%), and *Albidiferax* (1.13%) (Fig. 2). Some of the genera enriched in this metagenome are considered potentially pathogenic to humans and other animals. In the non-urbanized metagenome, the highest occurrences were of *Candidatus Solibacter* (2.32%), *Geobacter* (2.01%), *Bradyrhizobium* (1.7%), *Magnetospirillum* (1.51%), *Rhodopseudomonas* (1.42%), *Opitutus* (1.28%), and *Anaeromyxobacter* (1.28%) (Fig. 2).

### Functional profile of freshwater metagenome

Of the total urbanized and non-urbanized metagenomic sequences, 72.7% and 52.9%, respectively, contained predicted proteins with known functions, according to MG-RAST. Thus, a total of 26.5% of the urbanized metagenomic sequences were of unknown function, and a higher value 46.2%, was observed in the non-urbanized metagenome. The sequences with known functions were classified into subsystems. The five most abundant subsystems were Protein metabolism (13% and 12.2% for urbanized and non-urbanized metagenomes, respectively), carbohydrates (11.4% and 11.5%); amino acids and derivatives (10.6% and 10.2%); cofactors, vitamins, prosthetic groups, and Pigments (8.2% and 8.3%); and RNA metabolism (6.6% and 6.1%). It is important to note that the proportion of sequences for all the subsystems showed only slight variations between the metagenomes, but there was much more abundance of hits in the urbanized one.

Considering the results obtained from KEGG and SEED databases, we decided to concentrate further on the functional categories that we considered relevant for the urbanization process, and we classified then as energy metabolism

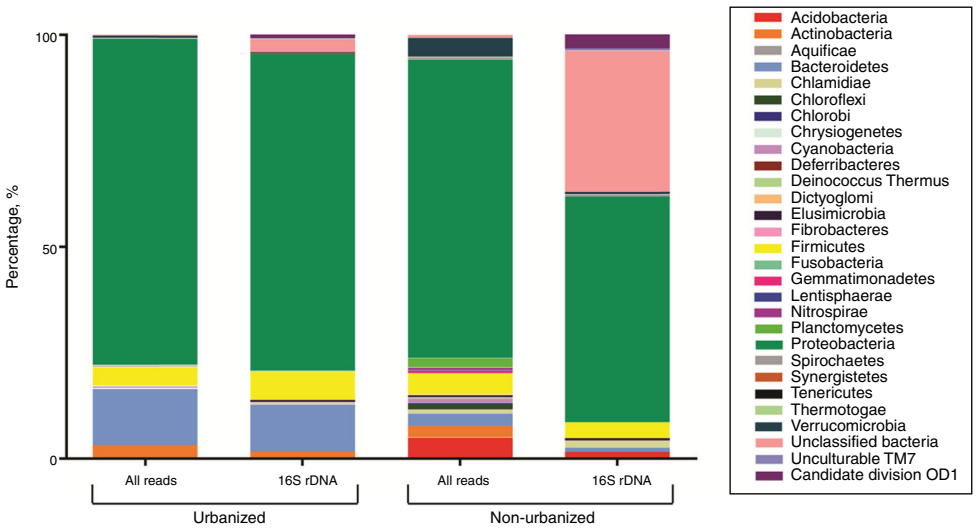


**Fig. 1 – Phylogenetic profile of the freshwater urbanized and non-urbanized metagenome using the 16S rDNA sequences and all the shotgun reads.**

(methane, nitrogen, and sulfur), housekeeping pathways (metabolism of DNA, RNA, and protein), and virulence/defense and stress responses.

**Energy metabolism** – In methane metabolism, the biosynthesis of methane from formaldehyde and the conversion of formaldehyde into C2 and C3 compounds was represented by annotated sequences in both metagenomes. However, there were a higher number of sequences coding for enzymes involved with the oxidation of formaldehyde, which originates from methane, into a $CO_2$, as an energy source, in the urbanized metagenome (Fig. 3). The steps involved in nitrogen metabolism were observed in both metagenomes, but a larger number of sequences affiliated with the core processes of the nitrogen cycle (ammonification, nitrification, and denitrification) were present in the urbanized metagenome (Fig. 3). As for sulfur metabolism, the annotation of sequences relevant to this metabolism revealed the presence of genes involved in the conversion of sulfate into adenylylsulfate and to the further generation of hydrogen sulfide ($H_2S$) from sulfite. The conversion of $H_2S$ into acetate was also represented. The urbanized metagenome contained a larger number of sequences in the latter processes (Fig. 3).

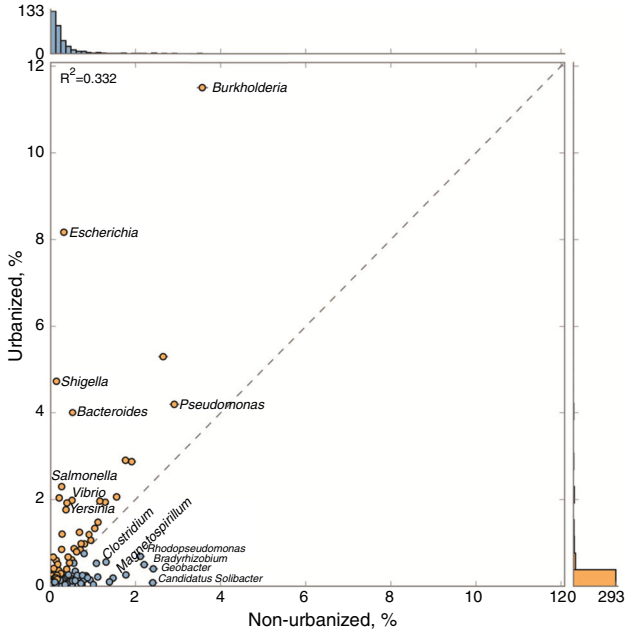


**Fig. 2 – Statistically significant differences between genera observed on the freshwater urbanized and non-urbanized metagenome.**

**Housekeeping pathway** – The protein metabolism represented the major number/proportion of sequences in both metagenomes (Fig. 4A). This subsystem was divided into protein biosynthesis (bacterial ribosomal SSU, tRNA-aminoacylation), processing and modification of proteins. The urbanized metagenome had a higher number of sequences codifying those functions (Fig. 4A). Regarding RNA metabolism, the majority number/proportion of sequences were classified into RNA processing and modification (RNA methylation, tRNA processing) followed by transcription (bacterial RNA polymerase, transcription factors), and a higher number of sequences were again present in the urbanized metagenome (Fig. 4B). For DNA metabolism, there were also the majority of sequences annotated for replication, DNA repair, and DNA structural proteins in the urbanized metagenome (Fig. 4C).

**Virulence/defense and stress responses** – There was an augmented number of sequences classified into resistance to antibiotics and toxic compounds in the urbanized metagenome (Fig. 4D) and there was also an increased number of sequences that codify proteins such as phosphate acetyltransferase, cobalt-zinc-cadmium resistance protein, cation efflux system, and RND efflux system in this metagenome (Fig. S1). Regarding stress response, there was a higher number of sequences classified into heat shock, periplasmatic, and acid stress in the urbanized metagenome (Fig. 4E), together with an overrepresentation of peroxidase, chaperona, catalase, RNA polymerase sigma factor, and arginine decarboxylase (Fig. S2).

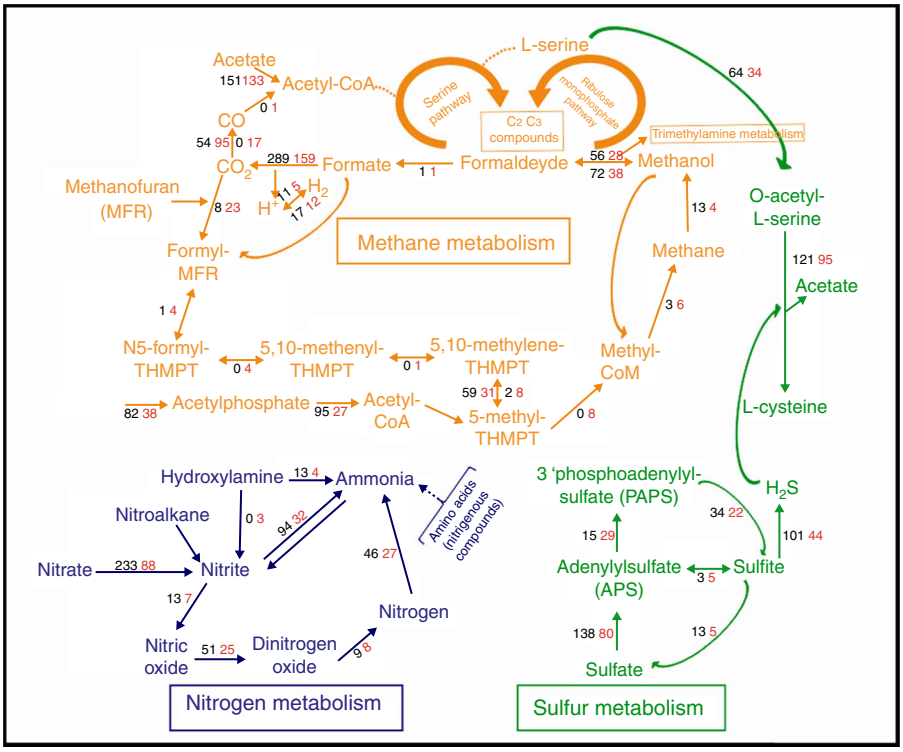


**Fig. 3 – Metabolic pathways of methane, nitrogen and sulfur from the freshwater metagenomes. The numbers label in black indicate the number of sequences from urbanized metagenome affiliated with the KEGG function and the number label in red indicate the non-urbanized metagenome. Data obtained through MEGAN.**

### *Taxonomic classification of functional subsystems*

To determine which organisms contributed to the main functional subsystems that are influenced by urbanization, we analyzed the taxonomic classification of the sequences annotated for housekeeping pathways (DNA, RNA, and Protein metabolism), energy metabolism (methane, nitrogen, and sulfur), virulence/defense and stress responses (Table S1).

In the urbanized metagenome, 19 taxonomic groups were affiliated with the selected subsystems. The groups Betaproteobacteria, Gammaproteobacteria, and Bacteroidetes were the most widely represented in all the analyzed subsystems except for the stress response, which was represented by Betaproteobacteria followed by Gamma and Alphaproteobacteria (Table S1). The results also indicated that the highest proportion of sequences in the virulence/defense subsystems belonged to the groups Gammaproteobacteria and Bacteroidetes. The housekeeping pathway and virulence/defense subsystems were represented in the majority of the taxonomic groups and were distributed uniformly among them. The phyla Chlorobi, Spirochaetes, Viridiplantae, Planctomycetes, Acidobacteria, Chlamydiae, and Chloroflexi were represented by only a few sequences in some subsystems (Table S1).

As for the non-urbanized metagenome, 24 taxonomic groups that contributed to the selected subsystems were identified (Table S1). The classes Beta, Alpha, and Deltaproteobacteria represented the largest number of sequences annotated for RNA, protein, nitrogen metabolism and for the virulence/defense and stress–response subsystems. The subsystems DNA and sulfur metabolism were represented by Beta, Alpha, and Gammaproteobacteria. In methane metabolism, the sequences were represented mostly by the groups Beta, Alphaproteobacteria, and Euryarchaeota. Unlike the urbanized metagenome, the phylum Euryarcheota had an important representation in the subsystems, especially in housekeeping pathways and methane metabolism. The housekeeping pathway and virulence/defense subsystems were represented in most of the taxonomic groups, as they were in the urbanized metagenome. The groups represented by a only few sequences in the non-urbanized metagenome were Opisthokonta, Deinococcus-Thermus, Viridiplantae, Epsilonproteobacteria, Chlorobi, Spirochaetes, Gemmatimonadetes, and Thaumarchaeota, while Opisthokonta, Deinococcus-Thermus, Gemmatimonadetes, Thaumarchaeota, and Nitrospirae were present exclusively in the urbanized metagenome (Table S1).

## Discussion

Urbanization, particularly in under-developed countries, leads to pollution of freshwater ecosystems, posing a major threat to this resource and severely limiting its use.[4] Earlier studies have sought to demonstrate the impact of urbanization on freshwater ecosystems. However, they focused only on one or a few aspects of urbanization, e.g., chemical pollution,[3,9] nutrient modification,[5,32] microbial density,[14,15] and pathogen contamination,[33–35] and therefore only pinpointed the consequences of this process. Our previous study[8] showed that

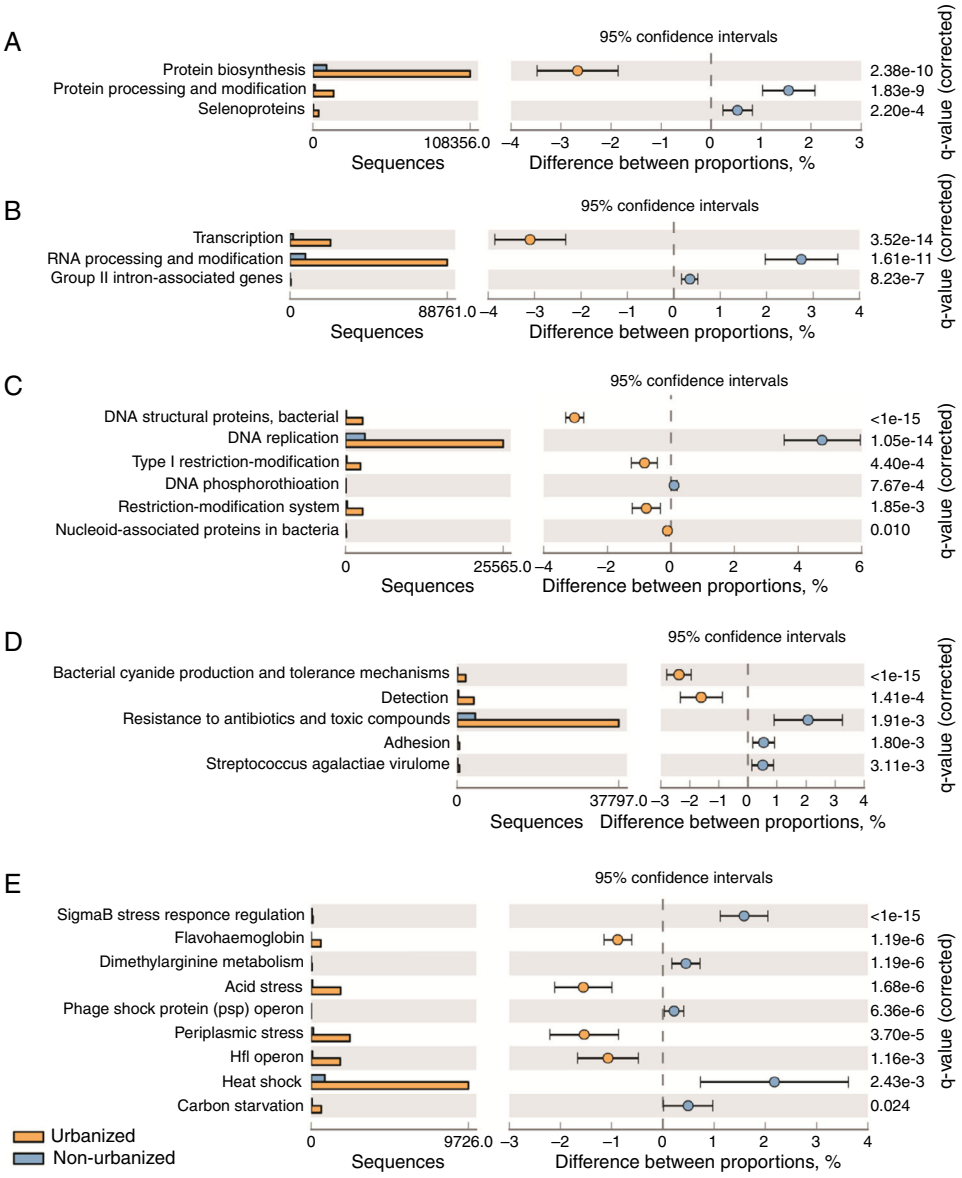


**Fig. 4 – Functional profile of the freshwater urbanized and non-urbanized metagenome. (A) Percent of genes in the "Protein Metabolism" subsystem. (B) Percent of genes in the "RNA Metabolism" subsystem. (C) Percent of genes in the "DNA Metabolism" subsystem. (D) Genes assigned to the "Virulence, Disease and Defense" subsystem. (E) Percent of genes in the subsystems "Stress Response". Data obtained through MG-RAST.**

the release of xenobiotic compounds, which is one of the main environmental consequences of urbanization, alters the biological characteristics of those environments, leading to modifications of the microbial community. However, a study offering a global description of the effects of urbanization on the microbial community was unavailable to date. To fill this gap, based on a metagenomic approach, our work presents a detailed taxonomic and functional profile of microbes in a polluted stretch and a preserved stretch of an urban stream.

Aquatic ecosystems are dominated by microorganisms, considering biomass and biodiversity.[12,36] Bacteria are the most abundant and widely studied prokaryotes of these environments. Our analysis of both datasets (all reads and 16S rDNA) of the two metagenomes found that, as expected, the dominant group in the Bacteria domain was Proteobacteria. The phylum Proteobacteria is well known and considered one of the most successful microbial groups on the planet.[37,38] However, the other dominant taxa in the urbanized and non-urbanized stretches of the stream differed. In the urbanized metagenome, the classes Betaproteobacteria and Gammaproteobacteria had a high number of annotated sequences such as the phyla Bacteroidetes, Firmicutes, and Actinobacteria. These taxonomic groups have typical aquatic bacteria as the phyla Acidobacteria and Verrucomicrobia, which were prevalent in the non-urbanized metagenome (Fig. 1). These microbes have been found in rivers,[39,40] lakes,[41,42] saline waters,[43–45] and aquifers.[46,47] The difference between the two freshwater metagenomes of this study can be explained

by the urbanization process. It is a well known, the urbanization leads to the discharge of high loads of untreated sewage containing human and animal feces and xenobiotic compounds into water bodies.[3] All the overrepresented groups of the urbanized metagenome (Betaproteobacteria, Gammaproteobacteria, Bacteroidetes, and Firmicutes) are usual members of the microbiota of humans and other animals, which are easily found in stools.[20] Thus, the selective pressure resulting from the high loads of sewage discharged into the stream may be responsible for the presence of these microbes (Betaproteobacteria, Gammaproteobacteria, Bacteroidetes, and Firmicutes) in the urbanized environment of this study. Another finding that indicates the action of selective forces on the urbanized environments is the specificity of this metagenome. A larger number of sequences were annotated for a few specialized phyla in the urbanized metagenome, unlike the non-urbanized one, which showed a more balanced distribution of almost all the existing phyla (except for the high prevalence of Proteobacteria).

However, considering the taxonomic profile of the freshwater metagenomes, significant differences in genera were found between the two environments. In the non-urbanized metagenome, the most annotated genera were *Geobacter*, *Candidatus Solibacter*, *Bradyrhizobium*, *Rhodopseudomonas*, *Magnetospirillum*, and *Clostridium* (Fig. 2). All these genera are members of the groups Acidobacteria, Deltaproteobacteria, Alphaproteobacteria, and Firmicutes, and they are characterized as free-living microbes widely distributed in soils and aquatic sediments, acting in the maintenance of ecosystems.[48–51] It should be noted that the non-urbanized area of the stream is located near its headwaters, where water is shallow and constantly mixed with sediment. This is consistent with the finding that the microbes identified in this area are typically present in soils and sediments.

Furthermore, our results revealed an enrichment of genera that harbor potential pathogens in the urbanized environment (Fig. 2). The genera *Escherichia*, *Salmonella*, and *Shigella* are members of the gastrointestinal microbiota of mammals and other animals but also can be found in water and soil.[52,53] This finding has been confirmed in studies using different methodologies, which have shown the occurrence of these potential pathogens in freshwater environments impacted by different levels of urbanization.[33,54–58] In addition, based on earlier PCR assays, the presence of these microbes was reported in this area of study.[8] Another member of the human microbiota that was abundant in the urban stretch of the stream was *Bacteroides* (Fig. 2). Their incidence in high levels in freshwater is attributed to the enrichment of this environment with intestinal wastes of humans. Moreover, given their anaerobic nature, the presence of these bacteria in the aquatic ecosystem may indicate recent contamination.[59,60]

Other potential pathogens enriched in the urbanized environment were *Burkholderia*, *Pseudomonas*, and *Vibrio* (Fig. 2). These bacteria can be found in different ecological niches (e.g., coral, oceans, freshwater, plant-association).[61–63] However, when in contact with potential hosts and expressing virulence factors, they can cause severe disease in humans.[64–66] Thus, the findings of this study indicate that the urbanized area is rich in potential pathogenic microbes, which may enter this environment through urban runoff, and which have a strong impact on human health. According to UNESCO,[4] urban areas without waste management can became dangerous environments for life, posing a threat to public health, since such environments can act as reservoirs of pathogens. Water related diseases account for more than 5 million deaths each year, 50% of which are caused by bacterial intestinal infections.[67,68] It is noteworthy that many of these bacterial pathogens have been associated with resistance to multiple antimicrobial drugs.[65,69–72]

The annotation of numerous sequences related to microbial virulence and defense provides important data associated with the potential pathogenicity of the microbes identified by metagenomic analysis of the urbanized environment (Fig. 4D). These sequences, as well as the sequences of pathogenic bacteria, were affiliated particularly to Betaproteobacteria, Gammaproteobacteria, and Bacteroidetes. Additionally, among the microbial virulence/defense sequences, there were hits for genes related with antimicrobial resistance, such as beta-lactamase, the RNA efflux system, Acriflavin resistance protein, and toxic-compound resistance (Fig. S1). The beta-lactamase proteins involve important mechanisms described for resistance to penicillin and other β-lactam antibiotics. These enzymes are able to cleave the β-lactam ring, leading to their inactivation.[73] Acriflavin resistance proteins are drug efflux systems belonging to the RND superfamily. These systems are associated with resistance to multiple antibiotics in Gram-negative pathogenic bacteria such as *Campylobacter jejuni*, *E. coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa* and *Salmonella enterica*.[74] Therefore, these results suggest that the potential pathogenic microbes found in the urbanized environments carry genes related to virulence/defense. Again, the data obtained in this study emphasize the public health risk posed by urban runoff water contamination.

Metal resistance sequences were also annotated in the virulence/defense subsystem of the urbanized metagenome, including cobalt, zinc, and cadmium, as well as cation efflux (Fig. S1) usually associated with the copper/silver efflux system.[75] Several studies suggest that metal contamination of natural environments may play an important role in the maintenance and proliferation of protein encoding genes related to antibiotic resistance through the phenomena of co-resistance (different resistance genes located near each other in the genome, forming the so-called resistance islands) or cross resistance (the same gene responsible for resistance to metals and antibiotics).[76,77] Thus, the selective pressure exerted by antibiotics does not necessarily have to occur in the environment in order to spread resistance genes. Our findings therefore indicate that the public health risks resulting from urbanization may be aggravated.

The discharge of raw sewage and xenobiotic compounds can be considered the cause of a stress related condition in the urbanized environment of the stream. As shown earlier,[8] the concentration of nutrients at this site underwent changes that led to a disturbance of the local microbiota when compared to those in the non-urbanized area. Selective pressure can lead to the maintenance of genes whose products are required for bacteria to survive stress.[78] Our findings corroborate this statement, showing an increase in sequences annotated as peroxidase, chaperone, catalase, and sigma

factor in the urbanized metagenome (Fig. S2). Sigma factors are key regulators of response to any type of stress in *E. coli* and other Gammaproteobacteria.[79,80] Catalase and peroxidase are associated with oxidative stress[81] and chaperone with thermal stress, as well as with changes in osmotic pressure, salinity, and organic acids.[82,83] The stress related genes were affiliated with the taxonomic groups Betaproteobacteria, Gammaproteobacteria, Alphaproteobacteria, and Bacteroidetes (Table S1), which predominated in this urban environment.

The metagenomic data presented here show that the microbial community in the urbanized environment under study is also adapted to the geochemical conditions imposed by urbanization, as indicated by the abundance of reads related to nutrient metabolism (methane, nitrogen, and sulfur) (Fig. 3). Methane metabolism is part of the global carbon cycle and involves mainly methanotrophic and methanogenic microorganisms. Methanogenic organisms, which produce methane as their metabolic by-product, are a group of obligate anaerobes belonging to the phylum Euryarchaeota of the domain *Archaea*. These microbes are usually found in freshwater sediments.[84,85] In this study, some sequences of the phylum Euryarchaeota were found to contribute to methane metabolism (Table S1), suggesting that archaea play an effective role in this nutrient cycle, which is consistent with previous studies.[86–88] Methanogenesis, which was found to occur in both metagenomes, is facilitated in the presence of increased dissolved organic matter (as in urban environments).[85] Methane is the only carbon source for methanotrophic microbes, and its oxidation can occur via the anaerobic or aerobic route. Aerobic bacteria are members of the phyla Gammaproteobacteria, Alphaproteobacteria, and Verrucomicrobia.[85,89] Once again, our findings were consistent with those of previous studies, since the urbanized metagenome showed an abundance of sequences encoding enzymes involved in the oxidation of formaldehyde originating from methane to $CO_2$. Thus, it can be inferred that some microbes use this nutrient (abundant as a result of urbanization) as an energy source. In both environments, it was also found that sequences related to methane metabolism belong to the above mentioned phyla (Gammaproteobacteria, Alphaproteobacteria, and Verrucomicrobia) (Table S1).

With regard to nitrogen metabolism, many sequences related to different steps of the cycle of this element were annotated in both metagenomes. However, the main processes of ammonification, nitrification, and denitrification were enriched in the urbanized metagenome. These metagenomic data are in agreement with a previous study performed in this area, in which PCR revealed an accumulation of molecular markers for nitrogen cycle in the urbanized environment.[8]

Another biogeochemical cycle represented in the metagenomes under study was the sulfur cycle, which is closely related to the carbon and nitrogen cycles. Sulfate-reducing microorganisms play an important role in sulfur transformation, since sulfate is taken up as a nutrient and reduced to sulfide, which is incorporated into enzymes and sulfur-containing amino acids.[90,91] Our results showed sequences that had hits with genes involved in the conversion of sulfate into adenylylsulfate and to the further generation of hydrogen sulfide from sulfite. The latter processes showed a larger number of sequences in the urbanized metagenome (Fig. 3). The high content of organic matter in this environment may explain the enrichment of this step of the sulfur cycle in the urbanized metagenome. Our data also showed that some bacterial phyla contributed with sequences related to these processes, but no sequences were classified in the metabolism of sulfur originating from microorganisms of the *Archaea* domain (Table S1).

Previous studies have shown that microbes must synthesize virulence or stress response-related factors or even metabolize excess nutrients in the environment in order to survive harsh conditions imposed on ecosystems.[92,93] This means they must increase protein synthesis, which in turn increases RNA and DNA synthesis.[94–96] The functional profile of the urbanized metagenome obtained here is in agreement with the above cited studies, since this metagenome contained more abundant housekeeping genes than the non-urbanized one (Fig. 4A–C). It is also worth pointing out that these genes were annotated particularly in the taxonomic groups with the highest prevalence in the urbanized metagenome (Betaproteobacteria, Gammaproteobacteria, and Bacteroidetes) (Table S1). However, housekeeping genes are constitutive and essential for the life of microorganisms;[97] therefore, this genetic cluster was represented in the other phyla of the urban metagenome (Table S1) and also in the non-urbanized (Table S1). Another aspect to keep in mind is the overrepresentation of DNA repair genes in the urbanized metagenome. This may be another mechanism that microbes use to diminish or eliminate the harmful effects of urbanization on the microbial community.

In conclusion, our study showed a detailed comparative analysis of the taxonomic and functional profile of the microbial community of a stream affected by anthropogenic impacts. It is important to consider that only one sample was sequenced from each site of the urban stream, without replications. The findings of this study represent the situation of the microbial community in the São Pedro Stream at the moment of sampling and must be considered with circumspection. Nevertheless, the differences observed in the structure and composition of the microbial community as a result of urbanization are real. Our findings brought to light the situation of a local stream but they reflect the conditions of many rivers and streams in Brazil and in other countries where the treatment of domestic sewage is inadequate or even absent. Our data are a cause for great concern, particularly from the standpoint of public health risk, given that sequences of pathogenic bacteria and virulence genes were found disseminated in the environment, underscoring the need to adopt measures aimed at reducing the impacts imposed by urbanization.

## Conflicts of interest

The authors declare no conflicts of interest.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjm.2016.06.011.

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