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Impact of pollution on the microbial diversity of a tropical river in an urbanized region of northeastern Brazil

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Summary. Rivers are important ecosystems that are integrated into biogeochemical cycles and constitute an essential resource for numerous human uses. However, the assessment of the biological diversity and composition of microbial communities found in rivers remains incomplete, partly due to methodological constraints which are only recently being resolved with the advent of next generation sequencing (NGS) techniques. Using 454-pyrosequencing of the 16S gene, the present study analyzed the microbial diversity of the planktonic and sediment populations in a tropical river in northeastern Brazil that is exposed to severe pollution. Six water and six sediment samples were analysed. The dominant bacterial phyla in both sediment and water were the Proteobacteria, followed by Bacteroidetes and Actinobacteria in the water column and by Chloroflexi and Acidobacteria in the sediment. Biological diversity appeared to be greatly decreased by environmental pollution, whereas the microbial community structure was variable across the analyzed transect. Moreover, a narrow relationship between industrial and urban sources of contamination and the bacterial genera detected at these sites has been observed. A variety of potentially pathogenic bacteria was detected, including *Klebsiella*, *Treponema*, *Faecalibacterium* and *Enterococcus*, indicating that the river might pose a substantial risk to public health. [**Int Microbiol** 20(1): 11-24 (2017)]

Keywords: environmental pollution · river plankton microbiota · biodiversity

Introduction

Rivers and other freshwater habitats play an important role in biogeochemical processes, in which numerous microorganisms, such as bacteria and protists, are integrated into complex physiological networks. These networks participate in carbon, nitrogen and other biogeochemical cycles, as well as driving primary production through photosynthetic activity, in particular. Lotic and other freshwater systems also provide important resources to human populations in the form of water for consumption and crop irrigation, as well as transportation routes and areas for recreational activity. Ongoing urbanization and industrialization is often well in advance of environmental protection measures, which leads to formerly

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pristine freshwater systems becoming polluted by industrial on point sources and agricultural and domestic nonpoint sources [2,51]. The contamination of rivers and streams can have a dramatic impact on ecological functioning and poses a serious threat to the health of the population in the drainage area of the disturbed fluvial ecosystems.

As of today, ecological surveys are still needed to assess the structure and composition of microbial populations in lotic ecosystems and to identify composition patterns on a global scale, as well as the possible drivers behind adaptive responses in the microbiota to changing environmental conditions, such as anthropogenic pollution. Previous attempts to assess the community structure and composition of river ecosystems were limited due to the selective bias introduced by the available methodology (culture based techniques) or the limited coverage offered by molecular techniques, such as DGGE (Denaturant Gradient Gel Electrophoresis) or gene library construction, followed by Sanger sequencing [4,13,17,30,45,50,54]. The recent development of economically accessible high-throughput sequencing techniques has provided a far more complete inventory of the microbial communities present in lotic ecosystems, with sampling depths orders of magnitudes higher than those used in previous studies.

By using these new techniques, an emerging set of studies of geographically distinct river ecosystems has expanded our knowledge of lotic [7,9,22,42] and sediment [23] communities, as well as their physiological functions. The microbial communities of rivers in temperate and tropical climates show underlying similarities and differential characteristics. In most studies, Proteobacteria, Actinobacteria and Bacteroidetes are among the most abundant phylogenetic groups encountered, while others, such as Firmicutes and Cyanobacteria, are present in a variable abundance range and are apparently dependent on certain environmental and physicochemical parameters.

The Northeast of Brazil possesses a vast network of freshwater habitats, including many rivers that provide essential resources for domestic, agricultural and recreational purposes. These ecosystems are often put under environmental stress by the introduction of untreated industrial and domestic wastewater and sludge. The Jaboatão River in the vicinity of Recife (state capital of Pernambuco) is an example of an aquatic ecosystem that is exposed to ongoing contamination by industrial and domestic sources in a watershed that is continuously being urbanized and industrialized. The river flows through several municipalities where the population uses its water for household purposes, consumption and bathing. The high degree of pollution in the Jaboatão River has direct implications on the public health, given the alarmingly high numbers of hospitalizations caused by waterborne pathogens in the area [19]. Along the river's course, two paper mills discharge their effluents containing a large number of highly toxic compounds, many of which do not occur naturally [44].

The aim of the present study was to increase knowledge about lotic biodiversity by studying the microbiota of both the sediment and the water column of a tropical river in a progressively urbanized region in Brazil. The survey was performed along a transect from its headwaters in a rural location to areas of dense human population, distributed over six sampling points. This research also sought to determine possible alterations to the biological diversity and community structure resulting from the massive contamination caused by domestic and industrial activities along the river's course.

Materials and methods

Sampling sites. The Jaboatão River is located in the state of Pernambuco in northeastern Brazil. It has a watershed area of 413 km^2 and receives input from six communities: Vitória de Santo Antão; Cabo de Santo Agostinho; Moreno; São Lourenço da Mata; Jaboatão dos Guararapes and Recife. The 75 km long river has its source in the vicinity of Vitoria de Santo Antão and flows into the Atlantic Ocean at Barra de Jangada beach, near Recife.

Sediment (S) and water (W) samples were taken on the 29th of May 2013 from six sampling points along the course of the Jaboatão River (Fig. 1). The accumulative precipitation on that day, measured in the town of Jaboatão, was 87 mm [1], being representative of a week with rainfall (the values for the days preceding the sampling date were 120.5 mm, 8.4 mm, 60.3 mm and 51.2 mm). The Jaboatão River is subject to a significant level of anthropogenic contamination throughout its course. In the year 2001 it was exposed to a potential pollutant load of 6679, 25,435 and 35,226 kg of Biochemical Oxygen Demand (BOD) per day by means of industrial, agricultural and domestic activities, respectively [12]. The first three locations are subject to diffuse source agricultural pollution while the other three sites receive untreated wastewater from both domestic and industrial sources. Samples from site S1/W1 were taken near the river's source. Samples from site S2/W2 were taken from a water collection dam in an area used for the cultivation of sugar cane. Site S3/W3 was located upstream of the community of Moreno in a forested area. S4/W4 samples were taken from Moreno, a densely populated area where the river is polluted by the discharge of untreated domestic and industrial wastewater. A paper mill is located in this city. The S5/W5 site was situated near the biggest settlement along the river, Jaboatão dos Guararapes, downstream from another paper factory. Site S6/W6 was situated in the area of the Jaboatão municipality, close to and upstream of a major cereal processing food company. The sediment samples were retrieved at a depth between 4 and 8 cm, avoiding the upper layer of the sediment that is in contact with the water column. The water samples were retrieved at a depth of approxi-

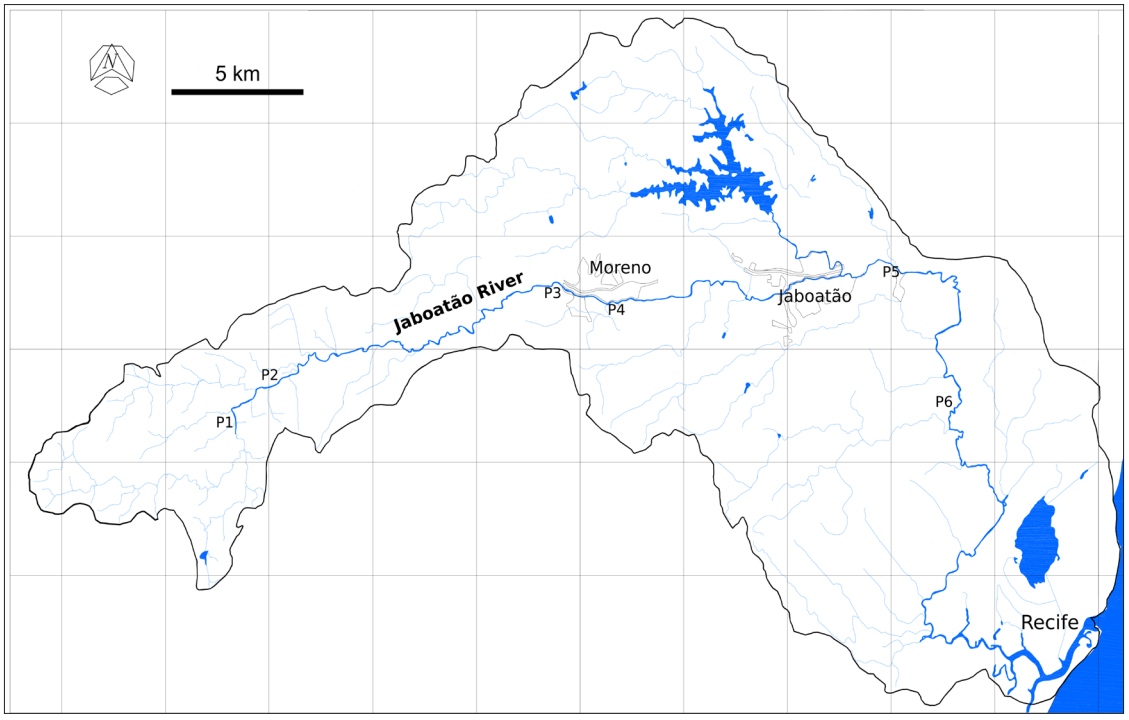

Fig. 1. Area map of the Jaboatão River's watershed, showing the sampling points (P).

mately 10 cm from the water surface. The values for the river depth at the sampling points were approximately 10 cm (S1/W1), 100 cm (S2/W2, S3/W3, S6/W6) and 40–50 cm (S4/W4, S5/W5). All samples were taken at distance of 40–50 cm from the river bank. To minimize sampling errors, three sub-samples were collected for each sampling point (both water and sediment) and analyzed as a composite sample.

Physicochemical and bacteriological analyses. The water hardness, salinity and chemical oxygen demand (COD) were measured using standard methods [24]. The water temperature, pH, dissolved oxygen (DO), electrical conductivity (EC) and oxidation reduction potential (ORP) were measured on-site with a Hach HQ40d multi-probemeter (Hach, Loveland, CO, United States). The ORP was also measured in the sediment immediately prior to sampling. Therefore the probe was inserted approximately 6 cm into the sediment. For the other analyses, the samples were stored in sterile airtight containers on ice and processed upon arrival in the laboratory. The total organic carbon (TOC) and inorganic carbon (IC) content were analyzed using a Shimadzu TOC-V CSH analyzer (Shimadzu, Kyoto, Japan). Ion concentrations were determined with a Dionex ICS-1100 (cations) or an ICS-2100 (anions) chromatograph (Thermo Scientific, Sunnyvale, CA, United States). The *Escherichia coli* plate counts of the water samples were determined by filtering different dilutions of the water samples and incubating the filters on Chromocult coliform agar (Cat.# 1.10426, Merck, Darmstadt, Germany) at 37 °C for 24 h. Colony forming units (cfu) of *E. coli* were determined according to the manufacturer's instructions.

DNA extraction and PCR. 300 ml of each water sample, or 0.5 g of each well-mixed sediment sample, were subjected to the extraction method with the Powersoil DNA extraction kit (MO BIO, Carlsbad, CA, USA). The water samples were filtered onto a 0.22 µm pore size cellulose ester membrane (Fmaia, São Paulo, Brazil) prior to extraction, while the sediment samples were processed directly, following the manufacturer's instructions.

The primer set 926F [28] / 1392R [29] was used for the pyrosequencing analysis, partially amplifying the small subunit ribosomal RNA in bacteria and archaea (16S rRNA). PCR was performed with 80 ng of template DNA, 1.25 u Invitrogen Platinum Taq DNA polymerase (Life Technologies, São Paulo, Brazil), 1x reaction buffer, 2 mM Mg^{2+}, 0.2 mM dNTPs (New England Biolabs, Ipswich, MA, USA) and 0.5 µm of each primer, in a reaction volume of 50 µl. Five min at 94 °C initial denaturation were followed by 28 cycles of 30 s at 94 °C, annealing for 30 s at 55 °C, and 30 s at 68 °C of template extension. A final extension step of 10 min at 68 °C ended the program.

Three-replicate PCR products were pooled and purified with the Invitrogen Purelink system (Life Technologies, São Paulo, Brazil). DNA concentrations were measured on a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and 454 pyrosequencing was performed by Macrogen (Seoul, South Korea) on a GS-FLX system (454 Life Sciences/Roche, Branford, CT, USA).

The present study was registered with the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) under the BioProject identifier PRJNA276740. The data set containing the sequence reads was deposited in the BioSamples database, accessible under the ID numbers SAMN03380194 and SAMN03460198 through SAMN03460208.

Phylogenetic and statistical data analysis. The pyrosequencing raw data were prepared for analysis using SFF Tools v2.6 (Roche 454) and homopolymer errors were corrected with Acacia v1.52 [6]. OTU clustering at a similarity threshold of 97% was performed using UPARSE v6.1.544 [14], excluding singleton OTUs, due to the probability that they were artifacts caused by sequencing errors in homopolymeric stretches. Chimeric sequences were detected and removed using UCHIME v4.2.52 [15]. Assignment of

Table 1. Physico-chemical measurements of samples taken from the water column, with exception of *ORP, sed (redox potential, sediment sample). All values in mg/l if not indicated otherwise

	W1	W2	W3	W4	W5	W6
T (°C)	24.9	25.6	26.2	28.1	28.3	27.9
pH	7.3	7.2	6.9	7.3	7.3	6.8
EC (µS/cm]	123.0	107.1	108.9	174.2	250.0	293.0
Salinity [PSU]	0.06	0.05	0.05	0.08	0.11	0.13
DO	8.1	8.0	6.2	6.7	5.9	0.7
ORP, water [mV]	242.0	221.9	105.9	77.7	42.8	84.8
ORP, sed.* [mV]	255.6	–230.0	–116.6	–110.0	–284.1	–206.6
IC	4.0	5.2	6.6	10.8	15.5	22.9
TOC	5.0	5.3	7.3	14.9	11.4	7.6
Nitrate	2.6	1.4	0.8	1.5	3.4	0.5
Chloride	15.4	13.6	13.5	21.5	24.3	25.9
Sulfate	14.5	7.3	4.4	8.1	21.0	26.4
Sodium	13.8	12.5	13.6	19.3	22.0	18.7
Potassium	3.6	3.1	2.5	3.8	5.9	6.4
Magnesium	2.5	2.1	2.2	2.9	3.3	3.8
Calcium	3.7	3.1	2.8	7.3	14.0	22.7
Hardness [mg/l $CaCO_3$]	17.8	14.9	14.7	28.3	46.2	69.7
[COD [mg/l O_2]	13.4	14.1	19.6	39.9	30.4	20.4]
E. coli (cfu/100 ml)	16,375±7421	1512±778	4180±2574	60,875±18,268	48,067±8833	19,200±3506

the taxonomic affiliation of the high-quality reads was then performed in Qiime v1.7 [8] applying the RDP classifier v2.6 [49]. To analyze the most abundant bacterial genera in the data set, another OTU matrix was created by assigning all the OTUs that could be determined at genus level (confidence level of ≥ 50 % via the RDP classifier) to their respective genera and obtaining total abundance values.

The calculation of biological diversity indices was performed using the R software environment v3.0 [39], including the vegan v2.0 and GUniFrac v1.0 libraries [11,35]. To allow for the comparison of samples of different size, the OTU matrix was first rarefied to the lowest common sequencing depth obtained in the present study (sample W1, 19791 reads). Several diversity indices were then computed and presented as effective numbers of species (ENS), which is a normalization of diversity measures that permits the quantitative comparison of results between samples and different surveys [27]. The diversity indices used here differ in their sensitivity towards distinctly abundant groups of OTUs. The Shannon-Wiener index takes into consideration even the presence of rare species to contribute to biodiversity, the Berger-Parker metric calculates diversity exclusively based on the proportion of the most abundant OTU and the Simpson index gives a somewhat balanced view, albeit with more weight to the common or more dominant species/ OTUs.

Results

Physicochemical and bacteriological analyses.

All of the water samples were turbid with a pH ranging from slightly acidic to slightly basic (Table 1). The total organic carbon load reached a peak at sampling point 4, which was the first sampling site in an urbanized area along the river's course with confirmed industrial pollution. ORP values for the sediment samples were all negative, with the exception of the first sampling point near the river's origin. The water redox potential was positive in all cases, although it was lower in the last three samples, where higher domestic and industrial contamination was recorded. The DO concentration in the water column was by far the lowest in the last sample, with a value close to zero, thus qualifying it as an anaerobic environment, while the other stations ranged from 6 to 8 mg O_2/l. Ion concentrations, as well as EC and salinity, correspond with normal values found in freshwaters. They are an index of the quality of the Jaboatão's waters. The values of IC and hardness run in parallel, increasing from sampling site W3 to W6, showing an increase in the inorganic carbon dissolved. TOC, as well as COD, serve as an index of the contamination the river's water is exposed to by human activities, showing a peak at site W4 (an urbanized and industrialized area). Concentrations of *E. coli* covered a wide range from 1500 to 61,000 CFU/100 ml. The highest reads were detected in the last three samples, with a sharp peak at site W4 in the community of Moreno. These values dramatically exceeded the

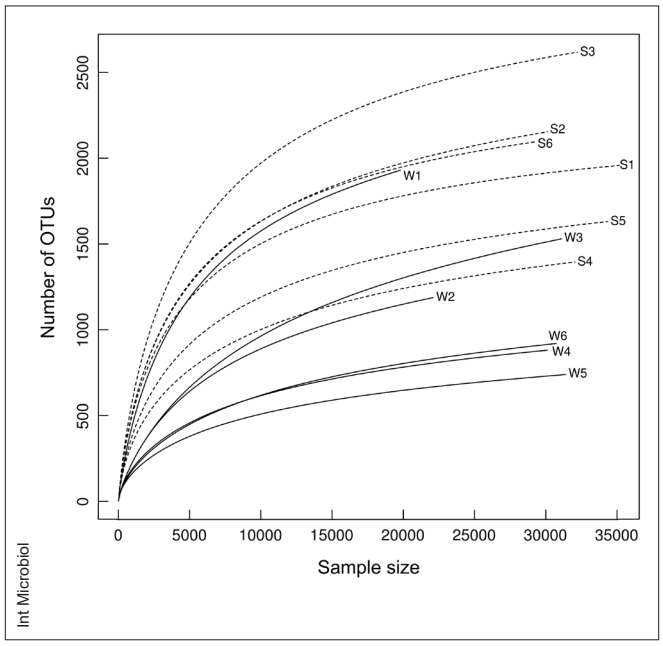

Fig. 2. Rarefaction curves for the OTUs of the sediment and water samples.

limit (statistical threshold value) established by the US Environmental Protection Agency, for partial and full body contact, of 410 CFU/ 100 ml [16].

Microbial community structure. After quality filtering and chimera removal, a total of 358,355 high quality sequences remained, which represented 60.2% of the raw data reads. These were then classified into 19,033 OTUs. Table 2 displays the distribution of the bacterial reads and OTUs across the individual samples. While the number of reads obtained for the water samples was similar to those of the sediment, the number of OTUs detected decreased drastically in the last three water samples (W4, W5 and W6).

The OTU coverage of the communities by the sequencing approach was assessed via the Chao 1 estimator. When compared with the numbers of different OTUs detected, the Chao 1 metric indicated a more complete census for the sediment samples (82−90%) than for the water samples (72−79%). However, the rarefaction analysis shows an asymptotic curve shape at higher sample sizes in all cases (Fig. 2), indicating a valid sampling effort.

The biological diversity of the samples was assessed by a series of descriptors (Table 2). In the present data set, the sediment samples appeared to be distinctly more diverse than the water samples, with values by an order of magnitude higher, when considering the Shannon-Wiener, Simpson and Berger-Parker indices, transformed into ENS [27]. These indices take into account both the OTU richness and the equality of the distribution of individuals throughout the OTUs (evenness). The reciprocal Berger-Parker index, a simple measure based on the single most abundant OTU of the community, confirmed a more dominant structure for the water and a more even structure for the sediment-derived communities.

The log-abundance plots of the sediment and water samples (Fig. 3) showed two trends in the structures of the corresponding communities. Firstly, the histograms of the sediment communities are approaching a bell shape, which indicates a more uniform distribution of species abundance where relatively few OTUs are very rare, a large number of OTUs are moderately abundant and only a small number of OTUs are very abundant. In contrast, in the water-derived samples, the communities include a higher proportion of rare OTUs and fewer OTUs that contain many members. This indicates a more stable and diversified community structure in the sediment samples, corroborating the observations of the rarefaction analysis and the values of the diversity metrics, in particular the Pielou and Berger-Parker indices. Secondly, the histograms of the water samples W4, W5 and W6 exhibited a flat and tailing shape, when compared with samples W1, W2 and W3, indicating a decrease in rare OTUs and the presence of a small number of highly dominant OTUs with many representatives (sequences).

Microbial community composition. A total of 28 different officially described bacterial phyla, and four candidate divisions, were detected in the sequence set in both the sediment and water samples.

Proteobacteria are commonly found in surveys of river and freshwater microbial communities and were the most dominant phylum in both sediment and water samples in the present study (Fig. 4). While they covered between 52% and 82% of the total number of reads in the water communities, the sediment samples generally exhibited lower abundance values for this group (35−40%) and were more evenly populated by a higher number of different phyla.

While less abundant in the river sediment, members of the class Betaproteobacteria clearly dominated the water-derived communities (35−72% of the sequences) and were by far the most dominant phylogenetic group, followed by the alpha- and gamma- classes. Betaproteobacteria affiliated reads were mainly composed of members of the orders Burkholderiales

Table 2. Descriptors of OTU richness, coverage and alpha-diversity of the sediment and water samples. Shannon-, Simpson- and Berger-Parker indices are transformed to effective numbers of OTUs

	Number of reads	Number of OTUs	Chao1	Shannon-Wiener [exp(H')]	Simpson (1/D)	Berger-Parker (1/d)	Pielou evenness (H'/ln S)
W1	19791	1928	2447	424	100	20.6	0.80
W2	22110	1186	1659	32	4.3	2.1	0.49
W3	31107	1531	2125	73	19.9	8.4	0.60
W4	30101	880	1171	66	22.1	8.4	0.63
W5	31398	738	996	54	18.0	6.2	0.62
W6	30737	920	1230	54	14.3	4.6	0.60
S1	35150	1958	2145	661	256	33.5	0.87
S2	30115	2154	2497	715	273	46.6	0.87
S3	32220	2618	2911	1021	468	75.0	0.89
S4	32040	1395	1698	260	86	16.6	0.78
S5	34355	1630	1917	327	104	20.3	0.80
S6	29241	2095	2347	714	271	40.3	0.87

and Rhodocyclales. *Curvibacter* and *Polynucleobacter* were the two most abundant betaproteobacterial genera detected in this survey.

The most common proteobacterial representatives in sediment samples were Beta- (*Sphaerotilus*, *Azonexus* genera) and Deltaproteobacteria. The sediments exhibited lower redox potential than the water column. All sediment samples were reductive except S1. These conditions allow a variety of anaerobic bacteria to grow, of which the most abundant phylogenetic group was the Deltaproteobacteria. Within this class, the most common orders were Myxococcales, Syntrophobacterales, Desulfuromonadales and Desulfobacterales.

Besides the predominating Proteobacteria, high proportions of Bacteroidetes were encountered in the Jaboatão River (up to 24% in the contaminated samples). The most abundant orders were Flavobacteriales, Bacteroidales, Sphingobacteriales and the *incertae sedis Prolixibacter*. Unsurprisingly, due to the metabolic traits of the classes involved, the most abundant genus affiliated to the Bacteroidetes was different in the sediment and water samples. In the sediment samples, *Meniscus*, a strictly anaerobic fermenter affiliated to the order Bacteroidales, was common in samples S3 through S6. The most abundant genus in the water column was *Flavobacterium*, a strict aerobe from the Flavobacteriales order, which was detected in all samples.

The phyla Chloroflexi and Acidobacteria were detected almost exclusively in the sediment samples. The most abundant orders of the phylum Chloroflexi were Anaerolineales, Caldilineales, Dehalococcoidales and Ktedonobacterales. The predominant genus encountered was *Bellilinea*, a filamentous anaerobic bacterium found in high numbers in all sediment samples. In the case of the Acidobacteria, many members of the Gp subdivision were found, especially Gp1, Gp3, Gp6 and *Bryobacter*, a recently discovered member of the Gp3 subgroup.

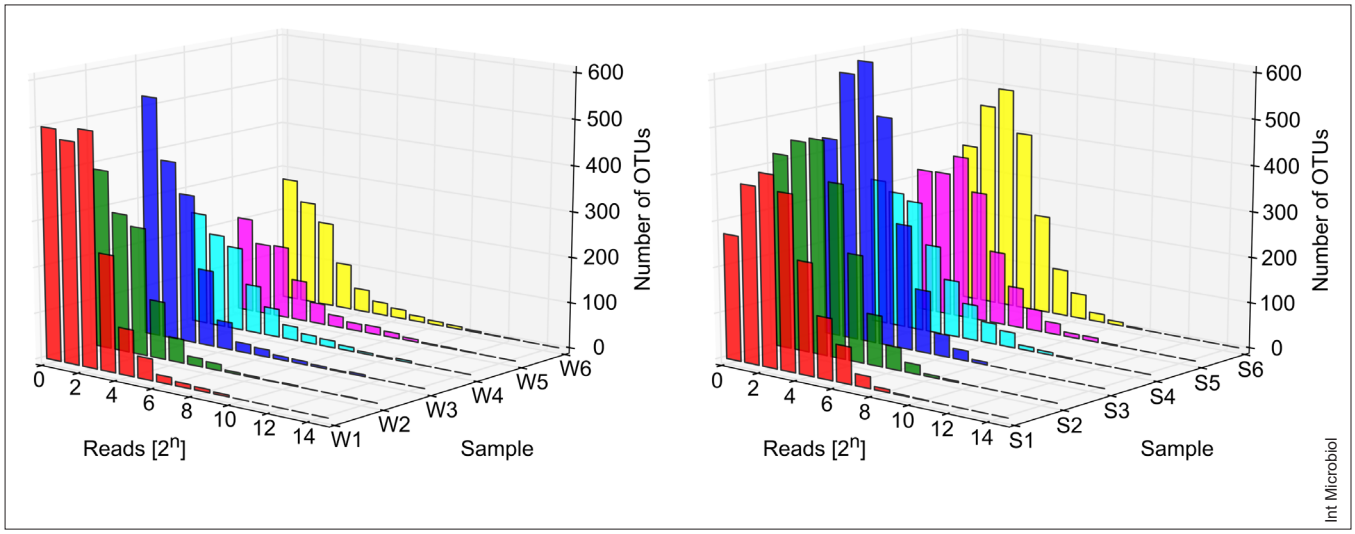

Fig. 3. Semilogarithmic Preston plots of the OTU abundance distribution of a) the sediment and b) the water samples.

Members of six of the Gp subgroups were encountered in the 40 most abundant genera of the sediment samples, suggesting their functional significance in this type of environment.

As indicators of bacterial phototrophic activity, the most abundant microorganisms were *Rhodobacter* (Alphaproteobacteria) and *Chlorophyta* (green algae). The diverse group of Cyanobacteria and chloroplasts contributed from 0.07% to 2% of the total reads in water and sediment samples.

A subset of 29 bacterial OTUs was present in all the water and sediment samples (data not shown). In terms of bacterial

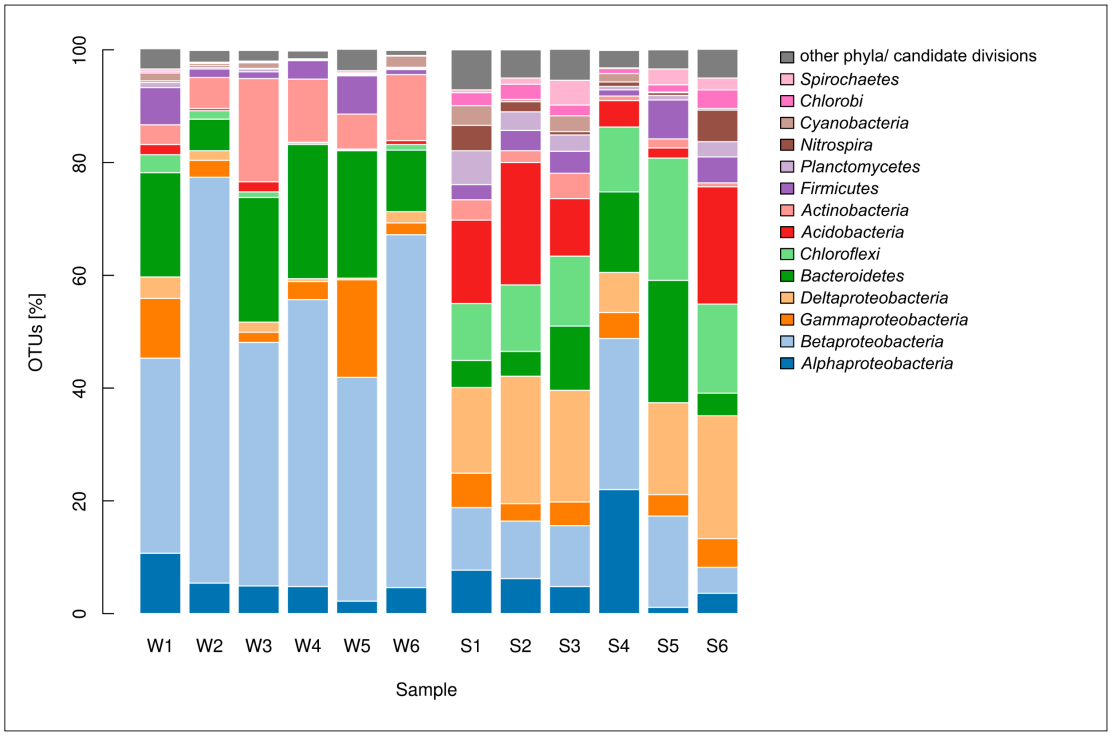

Fig. 4. Distribution of the relative numerical abundance values for the OTUs detected across bacterial phyla and classes of Proteobacteria.

richness, this core set represented only 0.4% of the OTUs detected. However, the number of reads that belonged to these 29 OTUs accounted for 16% of the entire 16S rDNA sequence library in the present study. All but two of the 29 core OTUs were affiliated to the Proteobacteria. The remaining two belonged to the Acidobacteria and Firmicutes. The most abundant members of the core population were affiliated to the order Burkholderiales (Betaproteobacteria, 21 to 88% of the core set), represented by the genera *Curvibacter*, *Limnohabitans*, *Sphaerotilus* and *Variovorax*. The second largest fraction of the core OTU set was affiliated to the order Rhodocyclales (Betaproteobacteria) and contributed between 5 and 34% of the core OTUs.

In order to identify the most abundant bacterial genera, the taxonomic assignments of the RDP classifier were filtered so that only OTUs that could be identified at a minimum confidence level of 50% remained. All OTUs pertaining to the same genus were joined and their read abundances were totaled to form a new genus-specific OTU. This new set of taxonomic units covered 53% of the original sequence database. Taking into consideration the 40 most abundant genus OTUs, the structure of the water microbial communities was skewed towards the class Betaproteobacteria (Fig. 5, lower part of the heatmap). In contrast, the most abundant OTUs in the sediment communities were more evenly distributed across the different phyla. Unsurprisingly, most of the sequences found in the water samples were phylogenetically affiliated to genera that are typically found in aquatic ecosystems, including fresh, polluted and wastewater habitats. From a metabolic point of view, approximately 80% of the 50 most abundant genera were chemoorganotrophs, with a strictly respiratory metabolism that uses oxygen as an electron acceptor, although there are a number of genera that can use nitrate as an alternative electron acceptor. The remaining 20% were strict or facultative anaerobes, the majority of which exhibited a fermentative metabolism, although certain genera use an anaerobic respiratory system.

Discussion

The present study shows how environmental pollution affects the biological diversity of microbial communities in a tropical freshwater ecosystem. Differences in community composition between distinct sampling sites were observed, as were possible functions of subgroups of the resident microbiota. The sediment samples, for example, contained many OTUs that were not present in the water column, while the latter shared most of its bacterial species with the sediment. A possible explanation for this distribution is that river sediment could act as a reservoir that is rich in bacterial species, releasing microorganisms into the water column via resuspension at the sediment-water interface [20,38].

When comparing the microbial communities in the different samples along the river's course, the choice of the descriptive metrics was important in terms of determining differences in alpha diversity. While the Pielou index, for example, showed relatively small differences in diversity between the different sets of samples, the Berger-Parker index detected strong variations between the water and the sediment communities. While both descriptors measure the evenness of a community, the Berger-Parker index was probably the more sensitive descriptor as, unlike the Pielou index, it is not constrained to values between zero and one. However, both metrics revealed that dominance increased and evenness decreased in the water-derived microbial communities when compared to the sediment samples. The evolution of the values for the Shannon-Wiener and the Simpson indices are similar along the river's course. The Shannon-Wiener index estimates the effective numbers of OTUs to be about three to four times higher than the Simpson index due to its inclusion of rare species. The Simpson metric does not consider these species to be contributing to biodiversity to the same degree. The sharp difference in the diversity metrics between sediment and water, with the exception of headwater-derived sediment, shows that, in contrast to the sediment, the water column is not a stable environment.

Due to its movement from the source to the sink of the river the water column at any given point is constantly changing its composition of bacterial species. In addition, factors like dilution by precipitation have a greater impact on the water communities than on those less exposed in the sediment.

The most basic of the diversity indices, the number of different OTUs, showed a marked decrease in the last three water samples (W4–W6). This decrease is also reflected by the values of the Shannon- and the Simpson metrics. This drop in species richness possibly indicates that the high degree of environmental pollution in the most urbanized and industrialized sites along the sampling transect had a profound impact on the microbial communities, exhibiting a strong drive for a smaller number of different species that are resilient enough to grow under more stressful conditions. The Chao1 richness

estimator predicts lower numbers of species/OTUs for these samples due to their flattened log-abundance curves (Fig. 3), which represent communities with a lower number of rare species. The samples with high numbers of rare OTUs represent the inverse case. Here further sampling will more likely result in the detection of new OTUs. According to the River Continuum Concept by Vannote et al. [48], which describes a river mostly as a longitudinal continuum, alpha diversity (see Table 2) would likely increase along the river's course, due to the increasing size of the microbial metacommunity, which continuously collects new species downstream. The opposite result was found in the present study for the water communities: diversity and evenness decreased sharply after the first sampling site at the headwaters of the river. The sediment communities were inconsistent in this regard and no pattern could be observed. The most probable explanation for the decline in biodiversity in the water samples might be a) the increase of selective pressure on the microorganisms through the extensive input of mainly organic contaminants and b) the influence of the river's layout as a dendritic network, which probably leads to the recruitment of new and readily-adapted species at the confluences with smaller streams. At these sites Vannote's model does not entirely apply, as the communities are modified by the inclusion of new species via mechanisms of resuspension from the sediment and transport along the lotic "conveyor belt" [5].

The overall relative abundance values for members of the Betaproteobacteria in the Jaboatão River were comparable to those detected in the Guyandotte River (USA) [42], the Changjiang River (China) [43], the Dongjiang River (China) [31] and several rivers in the Arctic [13]. The most abundantly present betaproteobacterial orders in the Jaboatão River were the Burkholderiales and the Rhodocyclales. Several members of the Burkholderiales are known for their capabilities in the degradation of even very recalcitrant organic compounds [37], suggesting a high degree of resilience against environmental pollution and a purposeful presence in contaminated freshwaters. They have also been previously encountered in river ecosystems [13,18,50], suggesting that Burkholderiales play a fundamental role in freshwater ecosystems. The Rhodocyclales contain many members that play an important role in the bioremediation of anthropogenic compounds and in biological reactor systems, such as wastewater treatment plants [34]. Among the most abundant representatives of the Rhodocyclales encountered in the present study were *Zoogloea* and *Dechloromonas*. These genera are capable of degrading a variety of complex organic pollutants [10,32].

Further bacterial phyla were detected at high relative abundance values, as in the case of Bacteroidetes-related OTUs, which coincides with findings from the studies of the Zenne River in Belgium [22] and the Athabasca River in Canada [53], both of which are highly contaminated with organic matter. Also, members of the phylum Actinobacteria are often found in lotic freshwater systems [22, 42,53]. In the present study, the highest abundance values for members of the other Gram-positive phylum, the Firmicutes, were observed at the most contaminated sites (S5, W4 and W5). The Firmicutes are well-known for having many members that are able to degrade even very recalcitrant organic compounds. Interestingly, Firmicutes-related OTUs were also detected to be abundant in the water and sediment samples W1 and S1 near the river's source, which apparently also suffered from fecal contamination, based on the *E. coli* counts and the presence of *Faecalibacterium*. The samples W4 and W5 exhibited the highest *E. coli* concentrations. The probable source of these peaks in fecal contamination was the discharge of untreated domestic wastewater from the towns of Moreno (W4) and Jaboatão (W5), whereas the pollution of the sampling site W1 was probably caused by small settlements and small-scale farming activities in the area. Possibly because of the proximity to the river's source the water at the S1/W1 site was more stationary and shallow (about 10 cm deep at a river width of 1.5 m). The combination of a relatively smaller water volume in comparison to the other sections of the river and a slow turnover of the water column might have contributed to the accumulation of fecal microorganisms at this site.

Members of the phototrophic Cyanobacteria exhibited low abundance values for the community composition of water and sediment samples. Higher proportions of these autotrophs were expected to be found in a fluvial system exposed to sunlight in a tropical region. The resuspension of sediment particles in the water column and the high input of allochthonous material through agricultural, domestic and industrial activities along the river's course might be the causes of the relatively low abundance of phototrophic bacteria in the present study. All of the sampling sites had a turbid river bed. The location directly downstream from the paper mill at S4 was also partially covered by foam. This correlates with the findings of a study of a lotic ecosystem in the USA [42], which compared a largely autochthonous stream with a smaller tributary river characterized by a higher proportion of allochthonous material and elevated turbidity. While the Ohio River

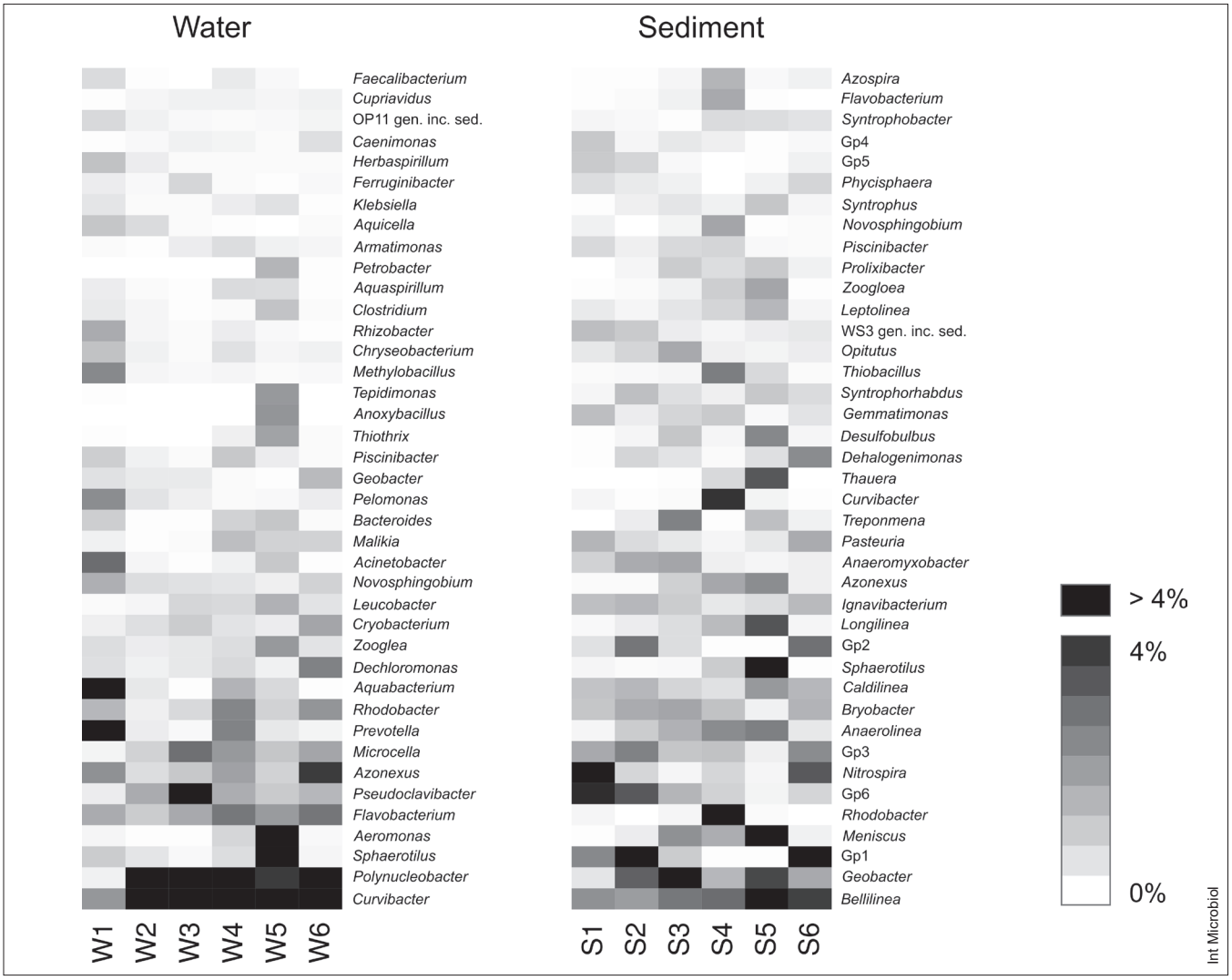

Fig. 5. Heatmap representing the 40 most abundant OTUs of the a) water column and b) sediment-derived samples and their affiliations at genus level. A new OTU set was generated for this analysis, including reads that could be identified with a minimum confidence of 50% by the RDP classifier.

was abundant in Cyanobacteria, related sequences accounted for less than 1% of the data set in the tributary Guyandotte River.

A variety of bacterial genera in the samples have been associated with the degradation and breakdown of xenobiotic compounds. *Dehalogenimonas* (phylum Chloroflexi) can reductively dehalogenate polychlorinated aliphatic alkanes and has been isolated from chlorinated solvent-contaminated groundwater. *Dechloromonas* (class Betaproteobacteria) exhibits a strictly respiratory metabolism, using chlorate, perchlorate and O_2 as electron acceptors and organic matter (acetate, propionate, butyrate, fumarate or succinate) as an electron donor. *Syntrophus* (Deltaproteobacteria) grows in syntrophic association with H_2-utilizing microorganisms and oxidizes substrates (crotonate, aromatic compounds, or fatty acids) to acetate. *Syntrophus*-affiliated sequences have been detected in anoxic sediments and sewage sludge. In the present study, members of this genus were most abundant in the sediment sample S5 (2.3% of all reads).The presence of these microorganisms is a possible indication that the Jaboatão River is exposed to a variety of xenobiotic compounds that are generated by industrial activity, including the two paper factories.

In the sediment-derived samples, the 50 most abundant genera ranged from 35−48% coverage of the sequences that were reliably assigned down to the genus level and were evenly distributed between aerobic and anaerobic microorganisms,

many of them isolated from sediments, water or anaerobic granular sludge. This fact seems to contradict the negative redox potential measured in the sediments (with the exception of S1). Since dissolved oxygen levels were relatively high throughout the river (from 6–8 mg/l, with the exception of W6: 0.7 mg/l), it can be hypothesized that, due to the heterogeneity of the river bed, there were microniches in the sediments in which aerobic bacteria could survive [40]. The high abundance of *Geobacter* and *Bellilinea* was common in the sediment samples, as well as several members of the Acidobacteria related uncultured Gp cluster. The low oxygen concentration at W6 is representative for this section of the river. Analyses performed in earlier campaigns in the years 2007 and 2008 showed a comparable mean value of 1.5 mg/l (data not shown). The low oxygen levels at this site were probably caused by the proximity to densely populated areas and an industrial park. Furthermore, an uncontrolled waste dump existed in the vicinity which continues to release its leachate to the surrounding area, including the Jaboatão River.

Nine out of the 50 most abundant genera detected in the present study were present in both water and sediment. However, with the exception of *Geobacter*, they were predominant in the water samples (Fig. 5). *Curvibacter*, *Sphaerotilus*, *Piscinibacter* and *Zoogloea* are Betaproteobacteria typically present in fresh or polluted waters. *Sphaerotilus* lives, attached to submerged plants or stones, in slowly running freshwater that is heavily contaminated with sewage or wastewater. Interestingly, strains of *S. natans* have previously been isolated from paper mill slime [36]. In the present study, *Sphaerotilus*-related OTUs exhibited a single peak in abundance in the samples S5 and W5, close to the outlet of a large paper factory, while the abundance values were relatively low in the other samples.

Geobacter was the dominant genus and one of the most abundant microorganisms in the sediment samples. It is known for its unique metabolic properties and ability to anaerobically degrade organic compounds, such as hydrocarbons. *Geobacter* oxidizes organic compounds, with Fe(III) serving as the sole electron acceptor.

The abundance and diversity of genera affiliated to the Chloroflexi in the sediments is striking.

Non-photosynthetic members of this phylum are usually found in anaerobic environments, both natural as well as engineered systems (such as anaerobic wastewater reactors in which they can be a co-dominant part of the population). For this reason, it has been suggested that they must play an important role in the degradation of organic matter in such environments [41,52]. Several genera affiliated to the Chloroflexi (e.g., *Bellilinea*, *Anaerolinea* and *Caldilinea*) are moderate thermophiles. *Leptolinea* was isolated from the granular sludge of a UASB reactor treating wastewater from a sugar-processing plant [52].

In our data set, a subgroup comprised of only 29 different OTUs, but with high abundance values, was present in all of the analyzed samples. This structural feature of the microbial communities could indicate how a very small fraction of the bacterial biodiversity could act as a ubiquitous backbone of the freshwater ecosystem of the Jaboatão River. The bacterial species of the core OTU set, most of which were affiliated to the Proteobacteria, might possibly be the most resilient towards the anthropogenic disturbances along the river's course and its varying physicochemical conditions.

OTUs affiliated to several possibly pathogenic bacterial species were detected. The highest abundance values for *Escherichia*/*Shigella*, *Enterococcus*, *Cloacibacillus*, *Klebsiella* and *Coprococcus* were found in the samples from W4 or W5, while *Faecalibacterium* was most abundant at W1. *Enterococcus* is a common part of the human intestinal biota and is a potentially disease-causing bacterium. Strains of *Cloacibacillus* (phylum Synergistetes) have been isolated from the intestinal tract of pigs and from anaerobic digester sludge [21,33]. Members of the Synergistetes are often found in insalubrious samples and should therefore be considered as potentially harmful to humans. The genus *Klebsiella* is ubiquitous in nature and can occur inhuman and animal intestinal content, water, sewage and soils. It has also been recovered from aquatic environments that receive wastewater. The genus can be associated with a variety of infections, from bacterial pneumonia to nosocomial urinary tract infections. *Treponema* (Spirochaetes) affiliated species were also detected in significant proportions in several sediment samples, with a peak at S3 and a relative abundance of 2.5% of all reads. *Treponema pallidum carateum* causes Pinta disease, an infectious skin condition that is commonly transmitted in tropical regions. A number of members of the Bacteroidetes are known as pathogens for animals (*Flavobacterium*) or as indicators of fecal contamination (*Bacteroides*). The latter were mainly found in the water samples W1, W4 and W5, which coincided with the highest plate counts for *E. coli*, the most widely used fecal indicator organism.

Two genera associated with pathogenicity and industrial activity were detected at relatively high abundance values:

Cloacibacterium (phylum Bacteroidetes) OTU counts peaked in samples W4 and W5, with relative abundance values of 9 and 16%, respectively, while *Aeromonas* (class Gammaproteobacteria) affiliated sequences accounted for 12% of the reads in sample W5. *Cloacibacterium* species have been isolated from untreated human wastewater [3]. *Aeromonas* is known as an opportunistic pathogen, although it is also ubiquitous in a wide variety of pristine aquatic ecosystems and is therefore unlikely to be a valid indicator for the human contamination of freshwater bodies [46]. However, both *Cloacibacterium* and *Aeromonas* have been associated with the Kraft pulping process used in paper production. Interestingly, a paper mill was located in the vicinity of the sampling point at both sites where these genera exhibited their peak read abundance values. *Cloacibacterium* has been isolated from paper mill pulp [47] and *Aeromonas* has been successfully used for the treatment of lignin rich paper mill liquor [25]. Besides their potential to act as a facultative pathogen in humans and as a pathogen in the fishery industry, *Aeromonas* species were found to increase in abundance and survive near the discharge point of a paper mill in the Albemarle Sound in North Carolina, USA [26]. This example shows how locally-defined sources of industrial pollution could influence the structure of the Jaboatão water communities and increase the pathogenic capacity of the water body. While the biological diversity sharply decreased at the sampling sites in the vicinity of the paper mills, a small subpopulation of resilient and adapted species prevailed and dominated the bacterial communities. At W5, the combined relative abundance values for *Cloacibacterium* and *Aeromonas* accounted for 28% of the sequence reads, while at the sites that were further away from the paper production facilities, their abundance proportions were well below one percent. Our data set contained a variety of potentially dangerous bacteria for public health, which would otherwise have gone unnoticed if relying on culture-dependent methods alone. The parsing of high-throughput 16S rDNA libraries of samples that are a risk to public health presents a possible strategy to assess the threat level, including microorganisms in the analysis that are unknown or not suspected to be present in the sample.

The present study shows how a massive level of anthropogenic contamination is capable of altering the microbial community structure and negatively affecting the biodiversity of a river ecosystem, particularly the water column. In this regard, the combination of several monometric descriptors such as the Shannon-Wiener diversity, the Berger-Parker index and OTU richness, as well as multidimensional approaches, such as the recording of log-abundance profiles for microbial communities, proved to be useful in terms of assessing the degree of disturbance in polluted rivers. Industrial on point contamination, which in this case involved the discharge of untreated domestic and paper mill wastewater and sludge, resulted in a decrease of bacterial diversity in water samples.

Pyrosequencing of the 16S rRNA gene is one feasible method of analyzing the taxonomic composition of river microbial communities. The rarefaction curves showed that increasing the sample size, within reasonable limits, would lead to the inclusion of the entire population of the ecosystem under study in the domain Bacteria.

The taxonomic assignment of NGS sequence tag libraries also confirmed fecal contamination, providing information on present pathogenic bacteria down to the genus level, most of which would not have been detected using culture-dependent methods.

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