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Analysis of interdomain taxonomic patterns in urban street mats

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Originality-Significance Statement

This study explores at the city-scale, the microbial assemblage of the communities forming mats in the street gutters of Paris, France. High-throughput sequencing of both bacterial and eukaryotic amplicons combined with statistical analyses revealed the importance of connectivity and co-occurrences in structuring these urban microbiomes while abiotic factors such as pH and conductivity appeared to be less important drivers. Various taxa were identified including primary producers, saprotrophic, symbiotic and parasitic organisms as well as bacteria involved in nutrient cycling. The results of this study highlight the importance and the diversity of street microbes, support the need to monitor this compartment but also to bring together urban biodiversity and waste management services, in order to elaborate action plans for cities.

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Summary

Streets are constantly crossed by billions of vehicles and pedestrians. Their gutters which convey stormwater and contribute to waste management, and are important for human health and well-being, probably play a number of ecological roles. Street surfaces may also represent an important part of city surface areas. To better characterize the ecology of this yet poorly explored compartment, we used filtration and DNA metabarcoding to address microbial community composition and assembly across the city of Paris, France. Diverse bacterial and eukaryotic taxonomic groups were identified, including members involved in key biogeochemical processes, along with a number of parasites and putative pathogens of human, animals and plants. We showed that the *beta* diversity patterns between bacterial and eukaryotic communities were correlated, suggesting interdomain associations. *Beta* diversity analyses revealed the significance of biotic factors (cohesion metrics) in shaping gutter microbial community assembly and, to a lesser extent, the contribution of abiotic factors (pH and conductivity). Co-occurrences analysis confirmed contrasting nonrandom patterns both within and between domains of life, specifically when comparing diatoms and fungi. Our results highlight microbial coexistence patterns in streets and reinforce the need to further explore biodiversity in urban ground transportation infrastructures.

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Introduction

Understanding urban microbiome structure might have implications on human health and risk management (e.g., in indoor environments including hospital and mass transit systems, urban recreational water, etc.) and promote our understanding on the impacts of long-term anthropogenic pressure on environmental resources (Khreis et al., 2016; Shamarina et al., 2017). Microbial diversity and species assemblages have been shown to respond to urbanization and to be largely influenced by site characteristics, land cover or architectural design (Kembel et al., 2012). Deciphering the microbiome compositions in transportation systems remains a key issue for urban development and city sustainability. In this respect, studies have reported microbial biodiversity in train stations (Dong and Yao, 2010; Patel et al., 2018) or subway systems (Dybwad et al., 2012; Dybwad et al., 2014; Leung et al., 2014; Afshinnekoo et al., 2015; Hsu et al., 2016; Fan et al., 2017). However, the street network system, which receives and conveys oil and other engine fluids that spill from transportation, dust, and atmospheric pollutants, along with human litter and waste, has surprisingly received much less attention (Janke et al., 2017).

Studies have reported the substantial roles of streets in conveying runoff and discharge of environmental, fecal and antibiotic-resistant bacteria to various surrounding water systems, such as streams (Baral et al., 2018), lakes (Zhang et al., 2016), waterways (Calderon et al., 2017) or groundwater (Voisin et al., 2018). Analyses of soils from a motorway or highly trafficked road in Copenhagen have revealed the presence of microbial polycyclic aromatic hydrocarbon degraders (Johnsen et al., 2006; Johnsen et al., 2014). An analysis of urban soils from the street

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green belts of the city of Chicago, USA, revealed that soils along a one-way street showed higher Shannon and phylogenetic diversities than other soils, including those along a two-way street, a feature that could be correlated with soil pH, moisture, and texture (Wang et al., 2018). We believe that the microorganisms living in the street, in street gutters and on pedestrian surfaces may present specific surface-associated diversity, such as the ones identified on concrete (Domingo et al., 2011; Li et al., 2012; Gomez-Alvarez et al., 2015; Jiang et al., 2016; Cayford et al., 2017; Cowle et al., 2017; Li et al., 2017) or buildings and monuments (Ragon et al., 2011; Chimienti et al., 2016; Gaylarde et al., 2017; Adamiak et al., 2018; Dyda et al., 2018).

Because surfaces are important to the interaction between bacteria and microeukaryotes, addressing the biodiversity and identifying the keystones species present in the street systems, including the ones made in concrete, stone and bitumen, is essential. In a previous study, we analyzed the eukaryotic diversity from street gutters biofilms/mats and found a gutter-specific diversity (Hervé et al., 2018). Our study aimed here at: *(i*) identifying microeukaryote-associated bacteria in street gutter biofilms, *(ii*) describing bacteria-eukaryote association patterns, *(iii*) identifying the factors structuring these complex microbial communities, and *(iv*) identifying potential keystone taxa in these biofilms. Because of the physical structure of these environmental biofilms, we hypothesized that biotic factors and microbial co-occurrences will play a significant role in the street gutter assembly.

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To focus on the bacterial communities that are specifically associated with microeukaryotes, we used a size fractionation approach. We expected that such procedure would give valuable information on resident bacteria along with the ecto- and endosymbiotic bacteria. Co-occurrence analysis was used to determine the intra- and interdomain (hereafter referring to domains of life) interactions and revealed different positive and negative patterns for bacteria alone, eukaryotes alone or eukaryotes and bacteria. To predict "keystone species" in gutter biofilms/mats, we used a cohesion algorithm (Herren and McMahon, 2017, 2018). Based on the biodiversity found in the street gutters, we discuss potential functional roles for these urban microbiomes.

Results

Complexity of the urban street gutter microbiome

Our aim was to understand the microbial community composition in street gutter biofilms, to infer putative interactions and interspecific cooperation between microeukaryotes (protists and Fungi) and bacteria as well as to identify taxa that might be involved in primary production, nutrient recycling or that might correspond to putative pathogens. Because we were interested in microeukaryote-bacterial interactions, the 60 gutter biofilms/mats sampled in Paris were filtered through a 5 µm membrane. We extracted the environmental DNA from the filter membrane and created two independent amplicon libraries for the 16S rRNA and 18S rRNA encoding genes. After amplicon sequencing, followed by sequence processing (see

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Experimental procedures), 3,981 OTUs and 5,899 OTUs were obtained for the 18S and 16S rRNA gene library, respectively.

The eukaryotes present in the street gutters were assigned to 9 taxonomic groups and to unclassified-eukaryotes. The highest richness was found among the Stramenopile (1,324 OTUs, including 764 OTUs belonging to the Bacillariophyta clade), followed by the Opistokonta (938 OTUs, among which we found 837 fungal OTUs), Rhizaria (461 OTUs), Alveolata (364 OTUs), and Archaeplastida (360 OTUs), and then by the Amoebozoa (79 OTUs), Hacrobia (63 OTUs) and Apusozoa (8 OTUs) (Fig. 1A). However, both the abundance and the diversity showed large variations depending on the sampling site, with some sites specifically rich in Fungi and others in Bacillariophyta (Supplementary Fig. S1). We also observed that Ciliophora, Chlamydomonadales, Fungi, Glissomonadida, Chrysophyceae and Oomycota were present in all samples. Within the fungal kingdom, Chytridiomycota was the most prevalent group, being present in 59 out of the 60 samples.

The bacterial OTUs were assigned to 35 phyla and candidate phyla (Fig. 1B). The Proteobacteria phylum was the richest (2,097 OTUs, and 55.9 % of the total abundance) and was composed, in decreasing order, of Alphaproteobacteria (738 OTUs, 27.4 %), Deltaproteobacteria (509 OTUs, 1.9 %), Betaproteobacteria (411 OTUs, 13.7 %), Gammaproteobacteria (394 OTUs, 10.7 %) and Epsilonproteobacteria (8 OTUS, 0.6 %). The other dominant phyla corresponded to Bacteroidetes (1,284 OTUs, 24.7 % total abundance), Cyanobacteria (4.8 %), Actinobacteria (4.4 %), Firmicutes (1.8 %), Acidobacteria (1.3 %),

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Deinococcus-Thermus (1.3 %) and Verrucomicrobia (1.3 %). Members of the bacterial genera *Flavobacterium*, *Saccharibacteria* and *Hymenobacter* were present in all samples, suggesting a ubiquitous distribution of these bacteria in Parisian street gutters. In contrast with the 18S rRNA gene library, the abundance profiles at the bacterial phylum-level showed low variability among the 60 samples (Supplementary Fig. S1).

Diversity patterns of eukaryotic and bacterial communities

In the street gutters, bacterial richness corresponded on average to 657 OTUs, ranging from 210 to 1,246 OTUs. Eukaryotic richness was lower (mean value = 210 OTUs), ranging from 96 to 548 OTUs. Overall, bacterial richness was significantly higher than the eukaryotic richness across the samples (Wilcoxon test, p-value < $2.2e^{-16}$) (Fig. 2A).

Regarding the *beta* diversity, dissimilarity based on Canberra distances was significantly higher in eukaryotic than in bacterial communities (Wilcoxon test, p-value < $2.2e^{-16}$), suggesting more divergence in the eukaryotic community composition between the microbial mats compared to the bacterial community composition (Fig. 2B). A strong and positive correlation between eukaryotic and bacterial *beta* diversities was observed (partial Mantel test accounting for geographic distances, $r_M = 0.658$, p-value < 1.10^{-5}), indicating that among the microbial mats, changes in eukaryotic and bacterial community composition were correlated and followed a similar trend. At the scale of the city, no significant correlation was found between the microbial community composition and the geographic distances (partial Mantel test,

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158 accounting for water chemistry variables, r_M = 0.054, *p*-value = 0.1 for the eukaryotes and r_M =
159 0.033, *p*-value = 0.4 for the bacteria) (Supplementary Fig. S2).
160
161 **Factors shaping the street gutter microbiome**
162 Subsequently, factors shaping the street gutter microbiome were investigated. Because of the
163 observed correlation between *beta* diversity metrics, the contribution of biotic factors was
164 suspected. Thus, we used a recently developed statistical method named cohesion to quantify
165 the degree of connectivity (positive and negative cohesions) (Herren and McMahon, 2017).
166 Additionally, we evaluated the influence of two important physico-chemical parameters,
167 namely pH and conductivity, on the microbial community composition. Multivariate analysis of
168 deviance of the GLMs revealed similar trends for the effects of biotic and abiotic factors on
169 bacterial and eukaryotic community composition (Table I). The two-cohesion metrics (*p*-value <
170 0.005), conductivity (*p*-value < 0.005) and pH (*p*-value < 0.05) had a significant effect on
171 community composition. The contribution of pH (Dev = 1960 and Dev = 726 for bacterial and
172 eukaryotic community, respectively) to community composition was always lower than the
173 contribution of conductivity (Dev = 2635 and Dev = 1061 for bacterial and eukaryotic
174 community, respectively). However, in the present dataset, biotic factors (positive and negative
175 cohesions) always show a much higher explained deviance compared to the tested abiotic ones,
176 indicating that cohesion explains more of the variation than pH and conductivity.
177
178 Regarding the cohesion metrics, we observed different patterns between bacterial and
179 eukaryotic communities. For bacteria, positive cohesion (Dev = 7,639) appears to be more

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important than negative cohesion (Dev = 5,410) in explaining community composition. Opposite results were observed for the eukaryote where negative cohesion (Dev = 2,027) contributed more than positive cohesion (Dev = 1,781), suggesting that connectivity patterns differ between bacteria and eukaryotes. The same analysis was performed after merging both the bacterial and eukaryotic OTU matrices. The four variables remained significant, with biotic factors (Dev = 8,040 and Dev = 5,349 for positive and negative cohesion, respectively) still showing higher explained deviance compared to abiotic factors (Dev = 2,567 and Dev = 3,420 for pH and conductivity, respectively).

Bacterial and eukaryotic OTU co-occurrences

Because biotic factors appeared to be the highest driving forces in the street gutter microbiome, we computed co-occurrence probabilities to identify co-occurring taxa (see Experimental procedures). We found 228,695 positive and 83,749 negative significant co-occurrences, representing 16.2 % of the nonrandom co-occurrences. To gain insight into the intra- and interdomain co-occurrence patterns (eukaryote-eukaryote, eukaryote-bacteria and bacteria-bacteria), we then inspected these co-occurrences at bacterial phylum and eukaryotic clade level (Supplementary Fig. S3, S4 and S5). *Circos* visualizations revealed that in several clades, including Apusozoa, Bacillariophyta, Alveolata or Rhizaria, the patterns of positive and negative co-occurrences can be significantly different for their eukaryotic-eukaryotic interactions (Supplementary Fig. S3). Among the bacteria-bacteria interactions, we see a general pattern in the much higher number of intra-phylum positive co-occurrences compared to the negative ones, which might indicate synergistic relationships (Supplementary Fig. S5).

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We then focused on the co-occurrences associated with the two eukaryotic clades that present the highest diversity and abundances. For the Bacillariophyta, the highest number of positive co-occurrences was with themselves (71.2 %), followed by the ones with Archaeplastida (7.5 %), other Stramenopiles (5.4 %) and Fungi (4.6 %), while their negative co-occurrences were with Fungi (44.0 %), other Stramenopiles (17.3 %), Rhizaria (13.8 %) and Alveolata (12.7 %) (Fig. 3A, left panel). For the Fungi, we found 2.9 % of their positive co-occurrences and 55.3 % of their negative ones with Bacillariophyta (Fig. 3A, right panel), suggesting interferences or competition for space and/or resources in street gutter biofilms/mats. Looking at the co-occurrences with bacteria, the Bacillariophyta presented 17,765 positive and 4,945 negative co-occurrences, whereas the Fungi presented 11,455 positive and 13,648 negative co-occurrences. For these two clades, the highest number of positive and negative co-occurrences was with the Proteobacteria (Bacillariophyta: 38.2 % positive and 39.3 % negative; Fungi: 31.0 % positive and 40.2 % negative), followed by the Bacteroidetes (Bacillariophyta: 28.0 % positive and 30.0 % negative; Fungi: 29.1 % positive and 34.2 % negative) (Fig. 3B). For the other bacterial phyla, we found that most of them (except Firmicutes, Fusobacteria and Saccharibacteria) showed more positive than negative co-occurrences with Bacillariophyta. The Fungi presented more negative than positive co-occurrences with most of the phyla, except for the Actinobacteria, Chroroflexi and FBP (Fig. 3B). At the order level, the main differences in co-occurrences between the bacteria and diatoms were for the OPB35_soil_group (Verrucomicrobia), SC-I-84 (Betaproteobacteria) and OM190 (Planctomycetes), with ratios of positives/negatives co-occurrences of 293, 164 and 134,

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respectively. The other orders, such as Aeromonadales (Gammaproteobacteria) and Corynebacteriales (Actinobacteria), showed more negative than positives co-occurrences with ratios of 0.019 (2/103) and 0.044, respectively. Between the bacteria and Fungi, the main differences were for Frankiales (Actinobacteria) and Longimicrobiales (Gemmatimonadetes) with ratios of 235 or 63, respectively, whereas Bradymonadales (Deltaproteobacteria), OM190 (Planctomycetes), and Chromatiales (Gammaproteobacteria) showed more negative co-occurrences, with ratios of 0.037, 0.041, and 0.047, respectively. Altogether, these analyses demonstrated differences in intra- and interdomain co-occurrence patterns.

Identification of highly connected taxa

The cohesion algorithm mentioned above was also used to identify the highly connected OTUs within the gutter microbial mats, which can be assimilated as keystone taxa (Herren and McMahon, 2018). Because both positive and negative cohesions had a significant impact on the microbial community composition (Table I), the 15 bacterial and eukaryote OTUs presenting the highest positive or negative connectedness were further investigated. In fact, since an OTU could be ranked among the 15 most positive but also among the 15 most negative connectedness values, we identified 25 OTUs for the eukaryotes (10 positives, 10 negatives and 5 OTUs that were among the most positive and negative) and 28 OTUs for the bacteria (13 positives, 13 negatives and 2 OTUs that were among the positive and negative) as potential keystone taxa. When necessary, the taxonomy assignment of some of the most connected OTUs was improved by BLASTn analyses and phylogenetic trees reconstruction.

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246 The data presented in Fig. 4A revealed that the eukaryotes presenting the highest positive
247 and lowest negative connectedness values corresponded to Fungi (these included eight OTUs
248 from the Ascomycota lineage and four Chytridiomycota, two Basidiomycota and one
249 Blastocladiomycota), Bacillariophyta (three OTUs assigned to the genus *Melosira*), Cercozoa (six
250 members of the class Sarcomonadea), and Pythiales (the single OTU corresponded to the genus
251 *Pythium*). For the bacteria, we identified thirteen members of the Proteobacteria lineage (eight
252 Alphaproteobacteria, two Betaproteobacteria, one Deltaproteobacteria, one
253 Gammaproteobacteria and one unclassified with a best BLASTn corresponding to the genus
254 *Paracoccus*), eight Bacteroidetes (four Sphingobacteriales, two Cytophagales, one
255 Flavobacteriales and one Bacteroidetes Order II. *incertae sedis* likely to correspond to the order
256 Rhodothermales), three Planctomycetes (two from the family Planctomycetaceae and one with
257 best blast to uncultured bacterium), two Actinobacteria, one Acidobacteria and one
258 Verrucomicrobia (Fig. 4B).
259
260
261 **Discussion**
262 Even if more data are needed on the total surface area covered by streets in cities, existing
263 evaluations vary from 10 to 50 %, with about 25 % for Paris. Depending on the city size, the
264 street transportation systems correspond to hundreds to thousands of kilometers. Therefore,
265 the number of microorganisms living worldwide in streets and street gutters is likely to be
266 enormous and undoubtedly these organisms might play fascinating roles in urban
267 environments including in food webs, biogeochemical cycles, pollutants detoxification, or waste

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and stormwater treatments. Additionally, these microorganisms should require further attention for human health and well-being concerns.

Biofilm assembly in gutter mats

Using a 5 µm filtration procedure to enrich the bacterial fraction that was closely associated with the eukaryotes from sixty gutter biofilms sampled across Paris, we describe here the molecular diversity of both bacteria and eukaryotes via a metabarcoding approach (Fig. 1). A comparison of the *beta* diversity of these two domains of life revealed a positive and significant correlation. We also found that both the street water pH and conductivity had a significant effect on bacterial and eukaryotic community composition (Table I). Water chemistry is known to have an impact on aquatic biofilm community structure (Besemer, 2015). At the city scale, we did not observe any significant correlation between microbial community dissimilarity and geographical distance (Supplementary Fig. S2), indicating no spatial autocorrelation (see: (Hanson et al., 2012)). This suggests no dispersal limitation among the street gutter microbial communities of Paris. Microbial dispersal could be facilitated by rains, water transport, wind and cleaning processes (i.e., street sweeping and washing and vacuum vehicles).

Regarding the factors shaping the microbial *beta* diversity, the multivariate analysis of deviance of GLMs indicated that the biotic factors (cohesion metrics) explained more variation than the abiotic factors (pH and conductivity). Nonetheless, further studies should include measurements of more abiotic variables to confirm our results. The importance of cohesion, *i.e.,* the degree of connectivity of a microbial community, might be explained by the physical

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structure of the microbial community being sampled. Within a biofilm, microorganisms are enclosed in a matrix containing extracellular polymeric substances (Battin et al., 2016). These microbial aggregates present enhanced intercellular communications and a higher level or organization compared to single cells (Flemming and Wuertz, 2019). Overall, supported by three different statistical approaches (Mantel test, co-occurrences and cohesion analyses), our study highlights the importance of microbial associations in street gutters.

Bacteria and eukaryotes diversity in biofilms/mats from street gutters

The 5,899 bacterial OTUs were assigned to 35 phyla and candidate phyla, in decreasing order beginning with the Proteobacteria (55.9 % of the total abundance), followed by the Bacteroidetes (24.7 %), Cyanobacteria (4.8 %), Actinobacteria (4.4 %), Firmicutes (1.8 %), Acidobacteria (1.3 %), Deinococcus-Thermus (1.3 %) and Verrucomicrobia (1.3 %). The Alphaproteobacteria, which corresponded to 27.4 % of the total abundance, was dominated by the order Sphingomonadales (58.8 % of the relative abundance within this class). Interestingly, among the 126 Sphingomonas-related OTUs, three of them could be defined as keystones species (see below). This bacterial order that presents interesting metabolic pathways, including carotenoid pigment synthesis (Siddaramappa et al., 2018), was found in various environments from the caves (Marques et al., 2019) to thermal springs (Pedron et al., 2019) and clouds (Amato et al., 2017), and recently, in the drinking water distribution system in Paris (Perrin et al., 2019). Microbial biofilm diversity and community composition have been addressed in several urban drinking and draining water distribution systems, and enrichments of specific communities within biofilms have been reported (Bertelli et al., 2018; Bruno et al.,

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2018; Douterelo et al., 2018; Douterelo et al., 2019; McLellan and Roguet, 2019). Interestingly, in the street gutter biofilms/mats, we identified 21 OTUs (three *Arcobacter*, twelve *Acinetobacter*, five Aeromonas and one *Trichococcus*) from genera that were previously shown to be dominant in sewer pipe (McLellan and Roguet, 2019). Among these OTUs, five of them (one *Arcobacter*, two *Acinetobacter*, one *Aeromonas* and one *Trichococcus*) were among the 50 most abundant OTUs in our dataset.

We also identified several bacteria that could correspond to potential opportunistic pathogens for human, animals or plants. In particular, we found OTUs assigned to the Enterobacteriaceae family (i.e., *Escherichia-Shigella*, *Serratia*, *Pectobacterium*, *Enterobacter*, *Dickeya,* etc.), Legionellaceae (i.e., 16 OTUs of the genus *Legionella*), Pseudomonadaceae (i.e., 17 OTUs of the genus *Pseudomonas*), Aeromonadaceae (i.e., three rare and five abundant OTUs of the genus *Aeromonas*), Streptococcaceae (i.e., *Streptococcus*), Enterococcaceae (i.e., *Enterococcus*), Mycobacteriaceae (i.e., *Mycobacterium*), Dietziaceae (i.e., *Dietzia*) and Corynebacteriaceae (i.e., *Corynebacterium*). Furthermore, we found that the Rickettsiales order, harboring parasitic and mutualistic intracellular bacteria, was prevalent in the gutter biofilms, with 97 OTUs present in 90 % of the samples. Within this order, three OTUs belonging to the genus of *Rickettsia* were identified. Many members of this latest genus are arthropod endosymbionts and include some species that are causative agents of human diseases such as typhus and spotter fever (Perlman et al., 2006). Four OTUs were assigned to *Candidatus Odyssela*, a bacterial genus with members that live intracellularly in Amoebozoa (Birtles et al., 2000). We are aware that to further characterize the presence of putative pathogens in the

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street surfaces, the complete bacterial community should be analyzed (using 0.2 µm filters), along with the virome (Fresia et al., 2019).

Among the 3,981 eukaryotic OTUs we identified putative symbiotic eukaryotes. Among them, six OTUs were assigned to Glomeromycota, which forms arbuscular mycorrhizas with the roots of many land plants. Most likely, these sequences were amplified from fungal spores which would indicate that gutters could act as vector. A number of known plant and algae (including diatoms) parasites belong to the Oomycetes clade (Kuhn and Hofmann, 1999; Scholz et al., 2016; Garvette et al., 2018; Leonard et al., 2018). Among the Oomycetes, 14 OTUs assigned to the *Phytophthora* genus were identified in eighteen samples. This genus is composed exclusively of plant pathogens targeting a wide range of hosts. Thus, street gutters might act as a reservoir of plant pathogens. We also found a keystone species belonging to the *Pythium* genus, along with a number of other parasite-related sequences, including from *Pirsonia* and "pseudofungus," such as *Hyphochytrium*. Finally, regarding the fungal diversity, 239 out of 837 fungal OTUs were assigned to unclassified fungi, representing a potential fraction of fungal dark matter (Grossart et al., 2016) in urban environments and thus, making street gutters potential hotspots of hidden diversity.

Keystone taxa in street gutters and co-occurrences between bacteria and eukaryotes

As mentioned above, cohesion metrics were used to identify the most highly connected OTUs, which might correspond to potential keystone taxa that influence community structure (Herren and McMahon, 2018). Among the 53 keystone OTUs that we identified, three of them

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correspond to a primary producer, and in particular to the diatom genus *Melosira*, further confirming the importance of diatoms in street gutters (Hervé et al., 2018). Indeed, among the 100 most abundant OTUs, 26 corresponded to this clade with 24 related to raphid-pennate species and two to *Melosira* (abundance ranked 1st and 20th). Even if the dominance of pennate diatoms in fouling biofilm is a ubiquitous observation (see: (Richard et al., 2017; Zhang et al., 2017)), the presence of *Melosira*, a true cosmopolitan centric form capable of forming filaments, suggests that it might live and proliferate entangled within the urban biofilm matrices. Moreover, we found that diatoms present, mostly intraclade positive co-occurrences, and their negative co-occurrences were dominated by interactions with Fungi (Fig. 3A) and, in particular, with the most abundant and diverse subphylum, the Pezizomycotina. This observed pattern of negative fungi-diatoms associations could be explained by antagonistic interactions, such as allelopathic interactions between aquatic fungi and diatoms that have previously been reported and showed a negative impact of fungi on primary production (Allen et al., 2017).

Among the fifteen keystone OTUs related to Fungi, seven of them were related to Pezizomycotina (four Eurotiomycetes, two Dothideomycetes and one unclassified Pezizomycotina). BLASTn analyses revealed the proximity of these OTUs to *Bradymyces* and to *Knufia,* two fungal genera that have been found in various environments such as outdoor rocks or indoor granites surface (Reblova et al., 2016; Tesei et al., 2017). A putative member of the Cucurbitariaceae also corresponded to a keystone taxa, which suggests that members of this family usually regarded as saprotrophs or necrotrophs could play roles in street microbial community structure (Doilom et al., 2013; Jaklitsch et al., 2018).

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Keystone taxa found here also belong to the Glissomonadida, an important cercozoan order of predominantly biflagellate gliding bacterivores. By recognizing the importance of micropredators in the structure of microbial communities, we can hypothesize that bacteria are used as food source in this environment and that Cercozoans play important roles in biofilms/mats formation (Cavalier-Smith and Chao, 2003). A putative phytopathogen belonging to the genus *Pythium* (best BLASTn hit) was also identified as a eukaryotic keystone taxon. The Pythiaceae that are well-known plant pathogens (Meng et al., 2009) with saprotrophic behavior, have been isolated from sediments and plant materials in decomposition in freshwater inland ecosystems (Kachour et al., 2016). Whatever their urban origin (i.e., plants covering green roofs and green walls, parking lot and sidewalk, etc.), Pythiaceae might contribute to plant organic matter decomposition in the street network system (also see below).

The 25 bacterial keystone taxa identified here belong to Proteobacteria (thirteen OTUs), Bacteroidetes (eight OTUs), Planctomycetes (three OTUs), Actinobacteria (two OTUs), one Acidobacteria, and one Verrucomicrobia (Fig. 4B). The presence of three keystone taxa belonging to Sphingomonadaceae (Proteobacteria, Alphaproteobacteria), a family that harbors aromatic hydrocarbon-degrading species (Shi et al., 2001; Marques et al., 2019; Sanchez-Gonzalez et al., 2019) suggests the need to develop studies on the mechanisms of aromatic compound biodegradation in the street gutters. Interestingly, the Sphingobacteriales (4 keystone OTUs) correspond to another clade to investigate for putative roles in street gutter

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400 bioremediation. For example, this order was found to be enriched in urban rivers influenced by
401 wastewater treatment plant effluents (Drury et al., 2013).
402
403 As mentioned above, bacteria-eukaryote interactions are known to be important for the
404 functioning of various ecosystems, and street gutters appear to be no exception. Indeed, co-
405 occurrence analysis demonstrated preferential patterns that are phylum-specific, and in
406 particular, the many nonrandom bacterial-fungal and bacterial-diatom co-occurrences (Fig. 3).
407 As in freshwater or marine water environments, our data demonstrated that Proteobacteria
408 and Bacteroidetes are the main bacterial phyla interacting with the diatoms; hence, these
409 bacteria are expected to influence their growth, behavior and physiology in street biofilms
410 (Amin et al., 2012; Buchan et al., 2014; Sison-Mangus et al., 2014; van Tol et al., 2017). The
411 Cyanobacteria phylum (4.8 % of the total abundance) presented very few negative co-
412 occurrences with the diatoms (493 positives and 29 negatives), a result which suggests a
413 foreseeable association between diatoms and autotrophic nitrogen-fixing Cyanobacteria (Amin
414 et al., 2012). We also found contrasting patterns in intra- and interphylum interactions when
415 comparing diatoms and Fungi, especially for some order like the Planctomycete lineage OM190,
416 Verrucomicrobia Subdivision 3, Aeromonadales, Corynebacteriales, Frankiales, Bradymonadales
417 or Chromatidales. We believe that a number of these nonrandom species co-occurrence and
418 enrichment patterns might correspond to differences in trophic level interactions between
419 diatoms and Fungi with their associated microbial communities, for nutrient fluxes or energy
420 production.
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Putative functional roles of microbes in street gutters

While the present study is only based on small subunit rRNA gene amplicons functional inference can be proposed based on the presence of certain taxa. Regarding the carbon cycle, various phototrophic bacteria were observed, including purple sulfur bacteria (11 OTUs assigned to the genus *Rheinheimera*), purple non-sulfur bacteria (*Rhodobacter* and *Roseomonas*) and oxygenic photosynthetic Cyanobacteria (92 OTUs). Methylotroph (*Methyloversatilis* and *Methylobacterium*) and methane-oxidizing bacteria (*Candidatus* Methylomirabilis) were also detected. The presence of ammonia-oxidizing bacteria (*Nitrosospira* and *Nitrosomonas*) and nitrite-oxidizing bacteria (*Candidatus* Methylomirabilis, and *Nitrospira*) suggest a role of gutter mats in nitrogen cycling. Similarly, the presence of iron-reducing (*Ferribacterium*) and iron-oxidizing bacteria (*Sideroxydans*) as well as sulfate-reducing bacteria (*Desulfomicrobium, Desulfobulbus, Desulfovibrio, and Desulfobacca*) are early evidence of the contribution of the gutter microbiome to urban nutrient cycling. Meanwhile, we also found OTUs related to bacteria that have been shown to be predominant in activated sludge and contribute to pollutant degradation such as members of the Nocardiaceae (i.e., *Gordonia*, *Nocardia*, *Rhodococcus*, *Nocardioides, etc.*) or the Propionibacteriaceae (i.e., *Propioniciclava*).

Among the bacterial taxa present in all gutter samples, we found *Flavobacterium, Saccharibacteria* and *Hymenobacter*. Saccharibacteria, formerly known as Candidate phylum TM7, still lacks isolates from axenic culture. Thus, gutter mats could represent samples of choice for targeted isolation of this phylum. Regarding the richness of this clade in the gutter mats, 116 OTUs were assigned to this phylum, with an average of 11 OTUs per sample. This

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phylum was also reported to be diverse in activated sludge, with some phylotypes able to utilize glucose, amino acids and N-acetylglucosamine (Kindaichi et al., 2016). The latter compound is likely to be abundant in the street gutters due to the abundant presence of fungi that possess chitin cell walls. Both *Flavobacterium* (165 OTUs identified) and *Hymenobacter* (82 OTUs identified) belong to the Bacteroidetes phylum. Further research effort should focus on isolating and characterizing members of these two genera to better understand their metabolic potential and functional role in the streets. More generally, the taxonomic inventory generated in the present study could provide a foundation for investigating gutter functional ecology via both *in situ* and *in vitro* experiments to better understand the role of their microorganisms in the urban landscapes.

Experimental procedures

Field sampling strategy, DNA preparation and sequencing

The sampling procedure, used herein to address the street gutter microbial communities in a metropolitan area of Paris, has been previously described (Hervé et al., 2018), except that here we focused on 60 gutter mats for which both the 16S rRNA and 18S rRNA genes could be successfully amplified. Briefly, samples were homogenized on a rotating wheel, prefiltered using a 300 µm mesh to remove putative debris, and then filtered through a 5 µm TMTP filter (Millipore). The filters were stored at -80°C until they were used. The pH and the conductivity of the samples were measured on arrival in the laboratory (within a few hours after the sampling) using a Benchtop Multiparameter Meter (Thermo Fisher Scientific).

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Total environmental DNA was extracted from the filters using a PowerBiofilm DNA Isolation kit according to the manufacturer's recommended procedure (MO BIO). To prepare the 16S and 18S rRNA gene libraries, PCR amplifications were performed with primers that contained a tag preceded by 2-4 random bases at the 5' end. The V4 region of the 18S rRNA gene region was amplified using the D512for (5'-NtagATTCCAGCTCCAATAGCG-3') and D978rev (5'-NtagGACTACGATGGTATCTAATC-3') primers (Zimmermann et al., 2011). The V3-V4 region of the 16S was amplified with the 341F (5'-NtagCCTACGGGNGGCWGCAG-3') and 805R (5'-NtagGACTACHVGGGTATCTAATCC-3') primers (Herlemann et al., 2011). Preparation of the DNA libraries and sequencing were performed by Fasteris SA, using the following procedure. PCR amplifications were performed using 1 µL of DNA (5-10 ng) in the following mix: 1.00 µL DNA, 1.00 µL Forward Primer (10 µM), 1.00 µL Reverse Primer (10 µM), 0.75 µL DMSO, 0.25 µL BSA (10x), 8.50 µL H2O, and 12.50 µL PCR Master Mix 2x (KAPA2G Robust HotStart DNA polymerase ReadyMix, KAPA Biosystems). The following amplification program was used: 95°C 5min / 30 x (95°C 15 sec / 52°C 15 sec /72°C 30 sec) / 72°C 3 min. The PCR products were checked on agarose gels, purified using Agencourt AMPure XP beads (Beckman Coulter), and quantified using a Qubit dsDNA HS assay kit. The PCR products were then normalized and pooled (2 pools). The libraries were prepared using 1 µg DNA from the pools and the Illumina TruSeq Genomic Nano Library Preparation Kit. The supplier's protocols were followed, with the exception of the use of a modified End-Repair mix to avoid production of chimeric constructs, and no PCR cycle was done to finalize the libraries. The resulting libraries were quantified by qPCR and sequenced using a MiSeq 2 x 300 paired-end run.

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489 ***Sequence processing***

490 Amplicons of the 16S and 18S rRNA gene sequences were analyzed independently with *mothur*
491 software version 1.39.5 (Schloss et al., 2009). Reads were processed largely following the
492 Schloss standard operating procedure for Illumina MiSeq data (Kozich et al., 2013). First, contigs
493 between the read pairs were assembled. Then, barcode, primer sequences and low-quality
494 sequences were removed (minimum length of 400 bp and maximum length of 470 bp for 16S
495 rRNA genes, minimum length of 370 bp and maximum length of 460 bp for 18S rRNA genes,
496 removing any sequences with ambiguous bases and removing any sequences with
497 homopolymers longer than 8 bp). Subsequently, sequences were aligned to the SILVA reference
498 database release 128 (Quast et al., 2013) and preclustered (pre.cluster, diffs = 1). Singletons
499 were excluded, and chimeras were removed with VSEARCH (Rognes et al., 2016) implemented
500 in *mothur*. Then, sequences were classified using the k-nearest neighbor (*knn*) algorithm
501 implemented in *mothur* and the BLASTN search method with the SILVA reference database
502 release 128. After classification, unknown sequences along with nonbacterial, chloroplast and
503 mitochondria and noneukaryotic were excluded from the rRNA gene datasets. To account for
504 differences in sampling efforts, 8,631 and 4,244 sequences from the 16S and 18S rRNA gene
505 datasets respectively, were then randomly subsampled from each sample (Weiss et al., 2017).
506 Operational taxonomic units (OTUs) were generated using the OptiClust algorithm (Westcott
507 and Schloss, 2017), with an OTU being defined at the 97 % and 99 % sequence similarity level
508 for the 16S and 18S rRNA gene reads, respectively. Finally, sequences were classified using the
509 *knn* algorithm implemented in *mothur* and the BLASTN search method (cut-off of 80 %) with the

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SILVA reference database release 128 and the PR2 database (Guillou et al., 2013) for the 16S and 18S rRNA gene reads, respectively. The raw sequence data have been deposited in the NCBI Sequence Read Archive under the BioProjects PRJNA546091 (16S rRNA genes) and PRJNA316490 (18S rRNA genes).

Diversity and statistical analysis

All statistical analyses were computed using R software version 3.4.4. *Alpha* and *beta* diversity metrics were computed using the *vegan* package (Oksanen et al., 2015). To test the relationship between the bacterial and eukaryotic community matrices and between microbial community matrices and geographical location, partial Mantel tests were performed using the *ecodist* package (Goslee and Urban, 2007)□, with Pearson correlation coefficient and 10^6 random permutations. Microbial community matrices were computed with Canberra distance. Geographical (latitude and longitude) and water chemistry (pH and conductivity) matrices were computed with Euclidean distances.

Cohesion (positive and negative) metrics were computed for bacterial, eukaryotic and microbial (bacterial and eukaryotic matrices merged) communities using an OTU persistence cutoff of 10 %, the "focal taxon" null model and 1,000 iterations (Herren and McMahon, 2017). After this persistence cutoff had been applied, the following were identified: 1,570 bacterial OTUs, 425 eukaryotic OTUs, and 1,995 microbial OTUs. Cohesion analysis was also used to identify the most connected OTUs, which have been shown to be potential keystone taxa of a community (Herren and McMahon, 2018). Here, we used the 15 bacterial and eukaryotic OTUs

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with the highest positive connectedness and the lowest negative connectedness, which corresponded to 1 % and 3.5 % of the bacterial and eukaryotic richness, respectively, as recommended by Herren *et al*. (Herren and McMahon, 2018).

The impacts of the abiotic (pH, conductivity) and biotic (cohesion metrics) variables of community structure were investigated with generalized linear models (GLMs) for multivariate abundance data using the *mvabund* package (Wang et al., 2012), a method that has been shown to have more statistical power than distance-based multivariate analysis such as PERMANOVA (Warton et al., 2012). After the data had been examined, a negative binomial distribution was assumed. Subsequently, the statistical significance of the GLMs was tested with an analysis of deviance of the GLMs, performed with Monte Carlo resampling (1,000 iterations) and likelihood ratio tests.

For the evaluation of the co-occurrence probability between and among bacterial and eukaryotic OTUs, the Veech probabilistic model of species co-occurrence with a hypergeometric distribution (Veech, 2013) was applied using the *cooccur* package (Griffith et al., 2016). The same OTU persistence cutoff of 10 % used for the cohesion and GLM analyses was also applied to avoid detection of spurious co-occurrences. Negative and positive co-occurrences (p-value < 0.05) were then analyzed according to the taxonomic classifications of the OTUs and subsequently visualized with *Circos* v0.63-9 (Krzywinski et al., 2009).

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559 **Figure Legends**

560

561 **Fig. 1. Global abundance and richness of eukaryotic and bacterial OTUs from 60 street gutter**
562 **mats.** (**A**) Richness (*Left*) and Abundance (*Right*) obtained from high-throughput sequencing of
563 18S rRNA gene amplicons. (**B**) Richness (*Left*) and Abundance (*Right*) obtained from 16S rRNA
564 gene amplicons assigned to the phylum level.

565

566 **Fig. 2. Alpha and beta microbial diversity in street gutter mats.** (**A**) OTU richness. (**B**)
567 Dissimilarity distances were calculated using Canberra distance. Statistical significance between
568 bacterial and eukaryotic communities were tested using Wilcoxon signed-rank test.

569

570 **Fig. 3. Circos representations of co-occurrences of the Fungi and Bacillariophyta OTUs**. (**A**) *Left*
571 *panel*, Bacillariophyta-eukaryote co-occurrences, which correspond to 84 diatom OTUs, co-
572 occurring positively with 259 eukaryotic OTUs and negatively with 238 eukaryotic OTUs. *Right*
573 *panel*, Fungi–eukaryote co-occurrences, which correspond to 95 Fungi OTUs, co-occurring
574 positively with 373 eukaryotic OTUs and negatively with 304 eukaryotic OTUs. (**B**) *Left panel* –
575 Bacillariophyta-bacterial co-occurrences, which correspond to 84 diatom OTUs presenting
576 positive co-occurrences with 1,194 bacterial OTUs and negative co-occurrences with 805
577 bacterial OTUs. *Right panel* - Fungi-bacterial co-occurrences. These data involved 94 fungal
578 OTUs, co-occurring positively with 1,341 bacterial OTUs and negatively with 1,099 bacterial
579 OTUs. The color code used for the different clades is presented below the figures, and black and
580 the white correspond to the positive and negative co-occurrences, respectively.

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582 **Fig. 4. Taxonomic distribution of the most highly connected OTUs.** The data correspond to the
583 15 bacterial and eukaryotic OTUs that show the highest positive connectedness and the lowest
584 negative connectedness. Since some OTUs could be among both the most positively and
585 negatively connected, 25 and 28 eukaryotic and bacterial OTUs are presented here,
586 respectively. (**A**) Eukaryotic OTUs. (**B**) Bacterial OTUs. The presented taxonomic assignment
587 varies among the OTUs from the kingdom, phylum, subphylum to class. The green, blue and red
588 bars correspond to the positively, positively and negatively, or negatively connected OTUs.

589

590 **Table I**. Multivariate analysis of the deviance of generalized linear models fitting microbial
591 communities to biotic and abiotic factors.

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Supplementary Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Relative abundance and richness of bacterial and eukaryotic OTUs in street gutter mats. (**A**) Abundance (top panel) and diversity (bottom panel) obtained from 18S rRNA gene amplicons. (**B**) Abundance (top panel) and richness (bottom panel) obtained from high-throughput sequencing of 16S rRNA gene amplicons assigned to the phylum level. The 60 samples were sorted according to the abundance of the Bacillariophyta clade.

Fig. S2. Relationship between geographical distance and (**A**) **eukaryote and** (**B**) **bacterial community dissimilarity.** Community dissimilarity was computed based on Canberra distances. The significance of the relationship was tested using partial Mantel tests, to control for the effect of water chemistry (pH and conductivity).

Fig. S3. Eukaryote-Eukaryote co-occurrence patterns. Circos visualization of the eukaryote-eukaryote positive (black) and negative (white) co-occurrences. The total set corresponds to 12,128 positive co-occurrences involving 425 eukaryotic OTUs and 3,908 negative co-occurrences involving 408 eukaryotic OTUs.

Fig. S4. Eukaryote-Bacteria co-occurrence patterns. Circos visualization of the eukaryotic-bacterial positive (black) and negative (white) co-occurrences. The set corresponds to 60,776

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847 positive co-occurrences involving 425 eukaryotic OTUs and 1,546 bacterial OTUs and 38,122
848 negative co-occurrences involving 425 eukaryotic OTUs and 1,515 bacterial OTUs.
849
850 **Fig. S5. Bacteria-Bacteria co-occurrence patterns**. Circos visualization of the bacteria-bacteria
851 positive (black) and negative (white) co-occurrences. The total set corresponds to 155,790
852 positive co-occurrences involving 1,557 bacterial OTUs, and 41,719 negative co-occurrences
853 involving 1,553 bacterial OTUs.
854

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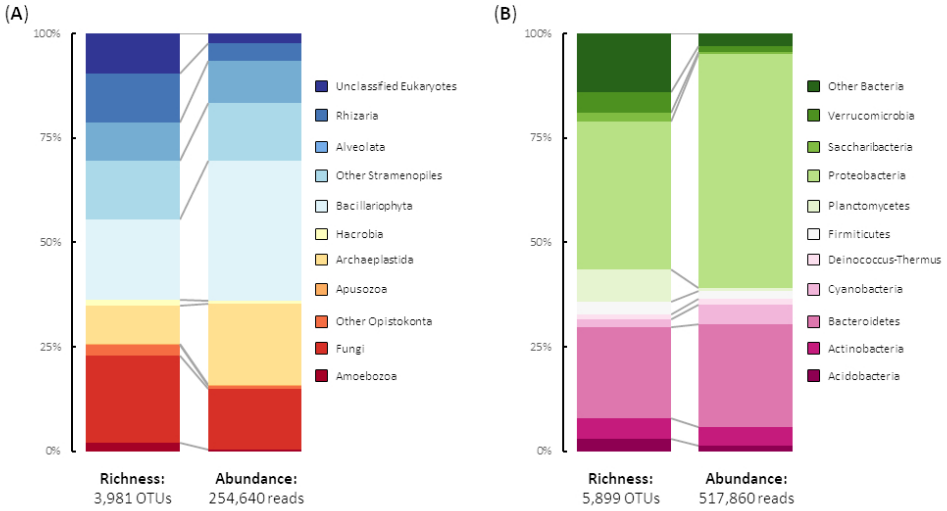


Fig. 1. Global abundance and richness of eukaryotic and bacterial OTUs from 60 street gutter mats. (A) Richness (left) and Abundance (right) obtained from high-throughput sequencing of 18S rRNA gene amplicons. (B) Richness (left) and Abundance (right) obtained from 16S rRNA gene amplicons assigned to the phylum level.

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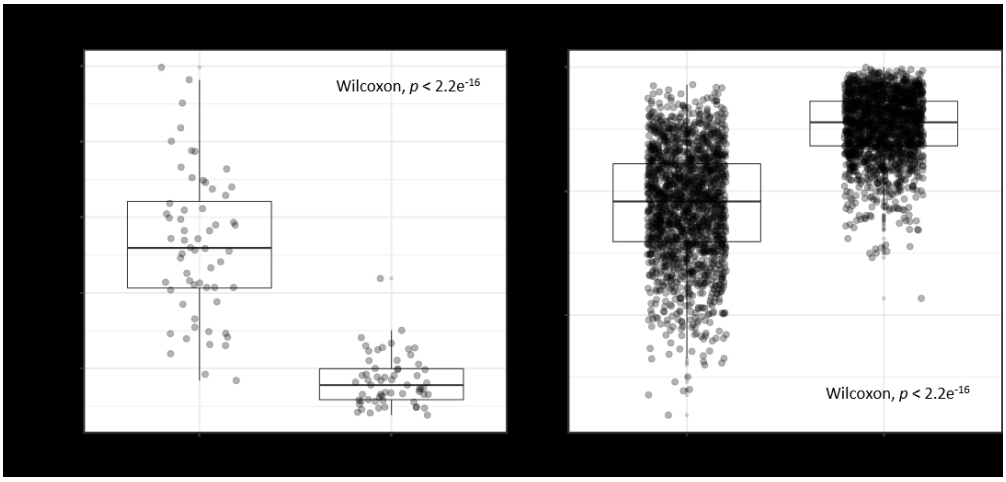


Fig. 2. Alpha and beta microbial diversity in street gutter mats. (A) OTU richness. (B) Dissimilarity distances were calculated using Canberra distance. Statistical significance between bacterial and eukaryotic communities were tested using Wilcoxon signed-rank test.

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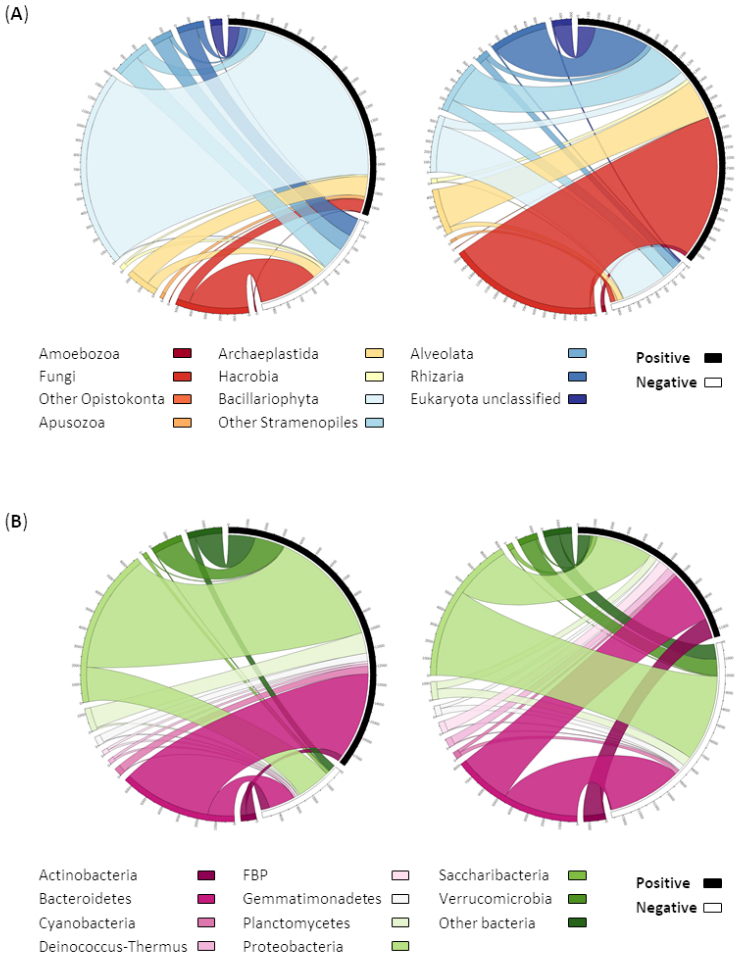


Fig. 3. Circos representations of co-occurrences of the Fungi and Bacillariophyta (diatoms) OTUs. (A) Left panel, Bacillariophyta-eukaryote co-occurrences, which correspond to 84 diatom OTUs, co-occurring positively with 259 eukaryotic OTUs and negatively with 238 eukaryotic OTUs. Right panel, Fungi-eukaryote co-occurrences, which correspond to 95 Fungi OTUs, co-occurring positively with 373 eukaryotic OTUs and negatively with 304 eukaryotic OTUs. (B) Left panel – Bacillariophyta-bacterial co-occurrences, which correspond to 84 diatom OTUs presenting positive co-occurrences with 1,194 bacterial OTUs and negative co-occurrences with 805 bacterial OTUs. Right panel - Fungi-bacterial co-occurrences. These data involved 94 fungal OTUs, co-occurring positively with 1,341 bacterial OTUs and negatively with 1,099 bacterial OTUs. The color code used for the different clades is presented below the figures, and black and the white correspond to the positive and negative co-occurrences, respectively.

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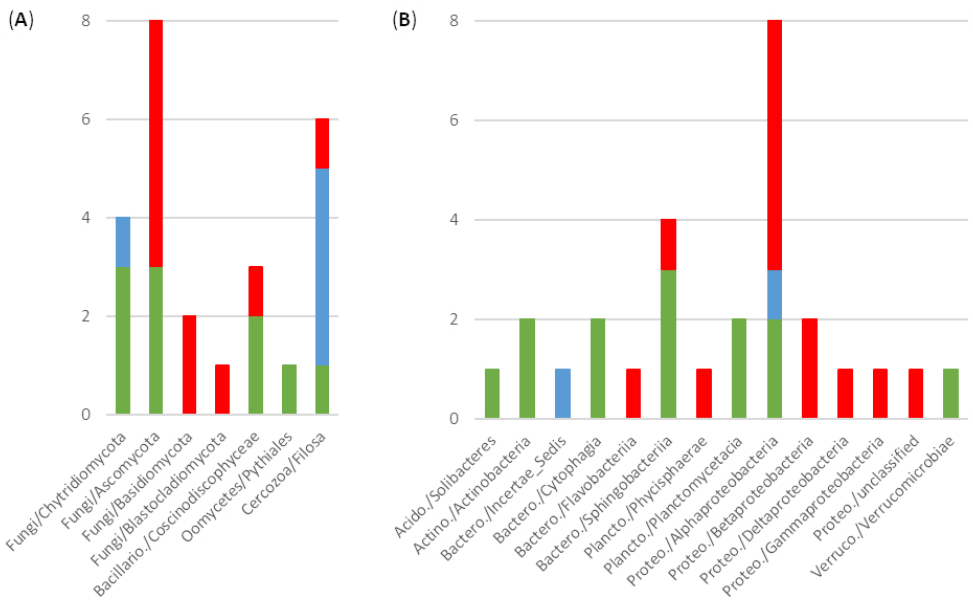


Fig. 4. Taxonomic distribution of the most highly connected OTUs. The data correspond to the 15 bacterial and eukaryotic OTUs that show the highest positive connectedness and the lowest negative connectedness. Since some OTUs could be among both the most positively and negatively connected, 25 and 28 eukaryotic and bacterial OTUs are presented here, respectively. (A) Eukaryotic OTUs. (B) Bacterial OTUs. The presented taxonomic assignment varies among the OTUs from the kingdom, phylum, subphylum to class. The green, blue and red bars correspond to the positively, positively and negatively, or negatively connected OTUs.

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Table I. Multivariate analysis of the deviance of generalized linear models fitting microbial communities to biotic and abiotic factors.

Factor	Bacteria		Eukaryotes		Bacteria and Eukaryotes	
	Deviance	*p* value	Deviance	*p* value	Deviance	*p* value
Positive Cohesion	7,639	$< 1x10^{-3}$	1,781	$< 1x10^{-3}$	8,040	$< 1x10^{-3}$
Negative Cohesion	5,410	$< 1x10^{-3}$	2,027	$< 2x10^{-3}$	5,349	$< 1x10^{-3}$
pH	1,960	0.018	726	0.018	2,567	0.041
Conductivity	2,635	$< 1x10^{-3}$	1,061	$< 2x10^{-3}$	3,420	$< 2x10^{-3}$

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