Highlights

- Stone communities differ dramatically from surrounding soil and air communities
- Stone microbial communities are richer than soil and air communities
- Abundance of Cyanobacteria characterize stone microbial communities
- Stone communities share few rare taxa with soil and air
- Lithotype is the major determinant of the stone community structure

The tombstones at the Monumental Cemetery of Milano select for a specialized microbial community.

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Abstract
Subaerial biofilms play a central role in the ecology and biodeterioration of many outdoor monuments and pieces of art. It is well established that microorganisms can face a broad range of stress by living in these subaerial environments, but their origin, taxa determinants and physiological traits are debated.
Here, we hypothesized that the bacteria forming these biofilms originate from the surrounding air and soil and that the selective pressure of a life on rocks shapes the community. To verify this hypothesis, we studied the microbial communities of nine tombstones of the Monumental Cemetery of Milano, by collecting samples in three seasons. We analyzed the structure of these subaerial biofilms, compared them with the bacteria identified in the surrounding air and soil and found that only few rare taxa are shared among the three compartments and have been selected by the stone environment. In addition, we considered which parameters - among temperature, humidity, light, season and lithotype - concur to structure the microbial community.

Keywords: subaerial biofilm, stone, rock, Cyanobacteria, stress, microbial diversity

1. Introduction

The interface between air and rocks is an ancient and ubiquitous habitat supporting subaerial microbial growth as biofilms (Costerton and Woodely 2003; Gorbushina and Broughton 2009). Subaerial biofilms play a central role in the ecology of alpine and desert environments, soil and rhizosphere (Dang and Lovell, 2009; Wieler et al. 2019), but also in the biodeterioration of many outdoor monuments and pieces of art in stone, thus being a reason of concern for the conservation of our cultural heritage (Polo et al. 2012; Schreerer et al. 2009). The first microorganisms to colonize rocks are able to exploit the little moisture and compounds in the air, given the poor availability of water and organic material (Villa et al. 2016). In addition, rocks surface offers very little shelter - just pores and cracks in the stone surface – thus exposing these microorganisms to UV and dramatic temperature changes (Walker and Pace 2007).

In response to these stressful conditions, microorganisms on rocks typically grow as a well-organized multispecies community protected by a matrix of exopolymeric substances (Gorbushina 2007). Here, microorganisms distribute according to their nutritional and growth needs, the interactions are enhanced and the external stresses are mitigated by the presence of the matrix (Dang and Lovell, 2009). Compared to other biofilms, subaerial biofilms growing on rocks have very distinctive characteristics. Because of the broad range of stress they face, these subaerial biofilms are characterized by low taxonomical diversity, but high synergy and metabolic activity (Villa et al. 2015). Phylogenetically, subaerial communities growing on rocks are very similar throughout geographically separated regions, suggesting that the rock colonizers have a cosmopolitan distribution, and that, once they are transported to the rock, they adapt to the environment to form specialized biofilms (Walker and Pace 2007; Gorbushina and Broughton 2009). Microorganisms composing the subaerial biofilms on rock are able to tolerate drought, UV exposure and temperature fluctuations, thanks to the production of pigments, exopolymeric substances and efficient DNA repair systems (Pointing and Belnap 2012; Gómez-Silva 2018). It has been also shown that the permeability, the mineral composition and the texture of a rock, as well as its surface geometry and the degree of shading, shape the structure and the composition of these microbial communities (Guillitte 1995; Miller et al., 2012; Brewer and Fierer 2018). In addition, the airborne contribution to the development of subaerial biofilm cannot be neglected, especially in urban environments, where the presence of organic pollutants can be used as a carbon and energy source (Saiz-Jimenez, 1999; Mitchell and Gu, 2000).

Despite the importance of these communities for the ecology of rocks and the conservation of our cultural heritage, the origin of the microorganisms forming subaerial biofilms on rocks is still open for discussion. In arid and hyperarid regions, Wieler and colleagues reported that the taxonomic composition of biofilm communities is very different from the dust and the surrounding soil, suggesting that biofilms microbes are not simply deposited by dust, but they are actively responding to the rock selective pressure (Wieler et al. 2019). To the best of our knowledge, no other studies explore the origin of microorganisms in rock subaerial biofilms and verify the possibility that these communities are formed by air- and soil-borne bacteria, but strongly shaped by the selective pressure of a life on rocks.

To verify this hypothesis in an urban environment, we studied the microbial communities of nine outdoor tombstones of the Monumental Cemetery of Milano, by collecting samples in three seasons. We analyzed the structure of these subaerial biofilms and compared them with the bacteria identified in the surrounding air and soil, in order to clarify if the bacteria on stones originate from the surrounding environments and which phylotypes have been selected by the rocks' conditions. In addition, we considered which parameters (temperature, humidity, light, season and lithotype) could have concur to structure the microbial

community. By investigating the microbial presence of three interconnected compartments (stones, soil and air), we provide a complete picture of the environmental niche of rocks.

2. Materials and methods

2.1 Sampling

Nine tombstones at the Monumental Cemetery of Milano (Italy) have been sampled in three seasons (Table 1). For each tombstone and for each sampling campaign - July 2015, December 2015 and April 2016 - two samples of subaerial biofilms and one sample from the surrounding topsoil were collected. Subaerial biofilm samples were scraped from a surface of the tomb of about 25 cm^2. Sampling point for each point were chosen as representative of the visible patina on the statue and avoiding zone too degraded or that could alter the aesthetic of the monument.

In July 2015, December 2015 and April 2016, two samples of 300 liters of air were collected with an AGI-30 impinger (Ace Glass) with a flow rate of 4.55 l/min in 25 ml of phosphate saline buffer (PBS) as described in Polo et al. (2012). During the sampling days, for each tombstone, the temperature, the relative humidity and the exposure to light were recorded.

2.2 DNA extraction

Air samples were filtered through sterile polycarbonate membranes (pore size 0.2 mm), and then put into tubes with 1.8 ml of lysis buffer and vortexed for 5 min. Total DNA was extracted directly from stone surface samples and air samples as described by Ausubel et al. (1994), with the addition of two cycles of mechanical lysis with bead beater (max speed, 30 s each; Precellys 24, Bertin) and three thermal cycles -80°C/ +70°C before the addition of lysozyme in order to break the cellular walls. DNA from soil samples was extracted with the DNeasy PowerSoil (MO BIO, Qiagen).

The bacterial 16S rRNA regions V3 and V4 have been amplified with primers CS1_V3F (341F) e CS2_V4R(806R). For each sample, three reactions of amplification have been run and pooled.

2.3 Sequencing Analysis

Amplicons of the bacterial 16S rRNA regions V3 and V4 were sequenced at the Core Facility of the University of Illinois (http://www.rrc.uic.edu/). The generated reads were demultiplexed by sample, the adapters have been removed and quality filtered. The remaining sequences were dereplicated, screened for chimeras and clustered into amplicon sequence variants (ASV) using the DADA2 pipeline as described (Callahan et al., 2017; https://benjjneb.github.io/dada2/tutorial.html). Taxonomy was assigned to each ASV with the database Silva SSU 138 Ref.NR 99 (Quast et al., 2013). All ASVs not assigned to the Bacteria kingdom have been removed prior to any other analysis. A summary of the reads obtained, filtered and analyzed is presented in the Table A.1. Normalization was performed by randomly sub-sampling ASVs to the number of reads present in the sample with the fewest reads.

2.4 Diversity and statistical analyses

All diversity analyses were performed in the R environment using the packages vegan (v2.5-1; Oksanen et al. 2018), phyloseq (Mcmurdie et al. 2013) and ggplot2 (Wickham et al. 2009). Rarefaction curves of the observed ASVs and alpha diversity indices (Observed, Chao1; ACE, Shannon, Simpson, Inversed Simpson, Fisher) were calculated.

Ordinations were created using non-metric multidimensional scaling NMDS (Ramette et al. 2007) applying the Bray– Curtis distance measure. Considering the small scatter of the Shepard plot and the low NMDS stress value (stress = 0.147 for all samples and stress = 0.161

for tombs samples only), two dimensions were used to ordinate samples (Oksanen et al., 2018). The effect of the environmental parameters on the community composition was examined. The communities were evaluated using the envfit function (vegan) for correlation with physical parameters and lithotypes of the stone substrate samples. The R code is available as file A.2.
To determine the shared taxa among the three compartments (stone, soil and air), the ASVs and taxa were imported into a relational database on MySQL server (version 8.0.22). Then with the use of function and query, ASVs present at least in one sample for each compartment have been subset and used to build Venn diagrams.

3. Results

3.1 The tombstones at the Monumental Cemetery of Milano

The microbial communities growing on the stone surface of nine tombs at the Monumental Cemetery of Milano were analyzed by collecting two samples each, in summer (July 2015), winter (December 2015) and spring (April 2016). The tombstones are made of different lithotypes: calcitic-marble (tombs 1, 3, 6, 7, 9), limestone (tombs 2 and 5), feldspathic-arenite sandstone (tomb 8) and granite (tomb 4) (Fig. 1). By the start of the sampling campaign, tomb 6 was completely restored and tomb 8 was in the process. For each sampling point, the temperature, the relative humidity (RH) and the exposure to light were recorded (Table 1). During spring, temperature ranged between 23°C and 30°C with 17% to 36 % RH; during summer, temperature ranged between 31°C and 35 C with 43% to 51 % RH; during winter, temperature ranged between 14°C and 21°C with 22% to 36 % RH. As expected, minimum light exposure was recorded during winter and the maximum during summer, with important variations among the tombs, depending on the position and orientation of the point sampled.

3.2 The composition of the microbial communities on stones, in soil and air.

To determine the composition of the microbial communities growing on the stone surface of tombs and the microorganisms in the surrounding topsoil and air, reads from all samples were pooled and processed together. A summary of the sequencing results is presented in the Table A.1.
16019 ASV have been identified by analyzing the microbial community on stones. Despite the use of primers selective for Bacteria, we could not classify 1.1% of the ASV and 0.07% have been assigned to the Archaea or Eukaryota domains after the taxonomic classification and subsequently removed. The composition of the community varies according to the sampled tombstone and the season, indicating a shift of the community structure with the change of physical stimuli. At the phylum level, the microbial communities on the tombs surface is generally dominated by Cyanobacteria (from 11% of the spring sample from tomb 4 to 77% of the summer samples from tomb 8, belonging to the class Oxyphotobacteria) and Proteobacteria (from 8% of the winter sample from tombstone 6 to 51% of the summer sample from tombstone 3; mainly from the class Alphaproteobacteria) (Table 2; Table A.3). Except for the tomb 8, during spring the percentage of Cyanobacteria decreases compared to winter's and often summer's samples (samples 3, 5, 6 and 9), corresponding to a general increase in Bacteroidetes and Acidobacteria. For example, the microbial community of tombstone 4 is dominated by Cyanobacteria and Proteobacteria in summer (67% and 21% respectively) and winter (60% and 24% respectively), with a smaller presence of Bacteroidetes (7 in summer and 4% in winter) and Acidobacteria (2% in summer and 4% winter), while in spring, they represent the 11% of the community, mostly replaced by Proteobacteria (47%), Bacteroidetes(20%) and a small fraction of Acidobacteria (7%).

Actinobacteria presence strongly increases during winter for all the tombstones, reaching 14% in tombstone 9.
For each tombstone, a sample of the soil surrounding the tomb has been collected in concomitance with the tombstone samples and the microbial community analysed. Unfortunately, DNA extraction and sequencing were not successful for some of the samples (spring samples for soil around tomb 1, 3, 6, 8, 9; summer samples for soil around tombs 4 to 8) and since the tomb 7 was surrounded by concrete, it was not possible to collect any soil sample. The composition of the soil communities is much more variable than the one retrieve from the stone, both depending on the sampling point and time. Despite the variability, in most of the samples we can observe a strong presence of Proteobacteria (from 13% to 93%, both Alphaproteobacteria and Gammaproteobacteria) and Actinobacteria (from 0% to 50%), followed by Chloroflexi, Acidobacteria, Gemmatinomonadetes, Bacteroidetes and Firmicutes. Compared to the samples collected from the tombstones, it is evident the very low presence of Cyanobacteria. The only exception is the soil sample collected close to the tomb 3 and 8 during winter, where Cyanobacteria reach the 73% and 8% respectively (Table 2; Table A.3).
For each season, two samples from air surrounding the sampling points have been collected and analysed. The air samples are dominated by the presence of Proteobacteria, with lower percentage of Acidobacteria, Actinobateria and few others (Table 2). In summer Alpha and Gammaproteobacteria constitute 67% of the air bacteria, while in winter the Alphaproteobacteria reach 93% of the community and by spring the Gammaproteobacteria take over with 98%. As for the soil samples, in the air Cyanobacteria are almost absent (Table 2; Table A.3).
In summary, the microbial communities in the three compartments -stone, soil and air- are quite different in terms of dominating taxa. On tombstones, the communities are dominated by Cyanobacteria and Proteobacteria. While the presence of Proteobacteria is shared among the three compartments, it is striking how Cyanobacteria, abundant on stones, are almost absent in air and soil samples.

3.3 The diversity of the microbial communities on stones, in soil and air.
In a microbial community, the presence of each microorganism is the results of its interactions with other species and with the environment surrounding them (Hibbing et al. 2010). To describe these interactions, we established the richness of each sample from the sequencing data. The alpha diversity of the microbial communities on stones, soil and air has been explored with a number of different indexes (Table A.4), well represented and generally in agreement with the observed phylotypes and Chao1 index that we report in Figure 2. As evident in Figure 2A and 2B, stone communities are statistically richer than soil and air communities. Despite the observed trend, the variability from sample to sample is high, both in air, soil and stone communities. The variability for air and soil samples can be attributed to the seasonality, since the samples collected during winter are the least diverse, having the lowest observed number of phylotypes and Chao1 index. The decrease of richness during winter is a trend shared only by a part of the stone samples, namely by samples collected on tombs 1, 3, 5 and 6 (Fig. 2C and 2D). Since these four sampling tombs have different lithotypes and physical parameter (temperature, light or relative humidity in Table 1), the reduced diversity in winter cannot be ascribed to a single variable. Nevertheless, the tombs in marble (with an asterisk in Fig. 2C and 2D) seem to support a community with higher diversity (observed phylotypes from 78 ± 8 to 80 ± 4; Chao1 index from 213 ± 34 to 200 ± 22), except for the tomb 6 that has been recently restored and has one of the lowest diversity among the tombs (observed phylotypes 56 ± 24; Chao1 index 108 ± 62). The less diverse stone samples correspond to the tomb 4, a tomb in granite (observed phylotypes 62 ± 8; Chao1 index 99 ± 24).

Non-metric Multi Dimensional Scaling (NMDS) based on Bray–Curtis distance was used to examine the strength of clustering of the microbial communities of stones, soil and air (Fig. 3A). The samples from the stones cluster tightly and separated from the soil and air samples, confirming the differences observed with the alpha diversity indexes. If we focus our attention on the samples from the tombs only (Fig. 3B), they better cluster according to the lithotype (by colors) than to the season (by shape). The samples collected on marble tend to cluster together, with the exception of the winter sample from tomb 6. As anticipated, tomb 6 was completely restored just before the sampling campaign and this could be the reason for such a diverse microbial community. While the samples collected on limestone partially overlap with the samples from marble, the granite samples are very different.

3.4 Shared taxa among stones, soil and air

The microbial communities on tombstones, in soil and air shared a very limited subset of taxa. The 52 shared ASVs represented the 3% of the stone, the 6% of the soil and the 39% of the air ASVs (Fig. 4), and they are mainly Gammaproteobacteria, within the families of the Xanthomonadaceae and Burkholderiaceae (Table A.5). Interestingly, the shared ASVs correspond to taxa with low abundance in the stone communities. For example, ASV85 is the most represented among the shared ASVs with 1562 reads, while ASVs exclusively present in the stone communities are ten times more represented. On top of these 52 ASVs, stone communities share only other 9 ASVs with air, corresponding to members of the phyla Bacteriodetes, Cyanobacteria, Firmicutes, Proteobacteria. Stone and soil communities share instead other 63 additional ASVs, corresponding to members of the phyla Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Deinococcus, Firmicutes and Proteobacteria (Table A.5). Despite these phyla are represented in both soil and stone communities, most ASVs differ between the two compartments.

3.5 Parameters affecting the microbial communities on tombstones

Light, temperature and humidity are known to influence the development of microbial communities. We evaluated the correlation between these environmental parameters during the sampling campaign and the analyzed microbial communities on stones, soil and air, but we did not find any statistical significance ($p>0.1$ for minimum and maximum light, temperature and relative humidity). Nevertheless, the correlation with these parameters might be complex and require more data to be revealed.

Two additional variables, the season and the lithotype, have been also evaluated as drivers of the microbial communities on tombstones. While no correlation has been found for the season ($p>0.1$), the microbial community composition correlates well with the lithotype of the tombstones ($p=0.002$; Fig. A.6). Our analysis highlights the importance of the lithotype for the development of subaerial microbial communities on stones.

4. Discussion

Stone surfaces exposed to air are challenging environment for microorganisms because of the exposure to UV, the extreme temperature fluctuations and the low water and resources availability (Viles and Cutler 2012). Bacteria in these communities are organized in biofilms, structures that provide protections for physical stress and facilitate mutualistic interactions (Gorbushina and Broughton, 2009).

In this study, we analyzed the subaerial biofilms growing on nine tombstones at the Monumental Cemetery of Milano, in Italy, and compared them with the microbial communities in the topsoil and the air surrounding the tombstones. The biofilms on the stones are very different from the soil and air communities, both in their taxonomical composition

and diversity, being richer than soil and air communities. The diversity indexes of soil and air microbial communities are comparable with others reported for similar communities in urban environments. Urban topoil is usually compacted by the use as walking paths and/or the presence of a pavement, with a consequent reduction of the carbon, nitrogen and gas available to support microbial growth, compared to deeper soil (Hu et al. 2018; Yu et al. 2019). In the air, on the other hand, microorganisms are thought to survive shortly because of the exposure to UV light and reduced water activity (Fahlgren et al. 2010), but recent studies in urban regions revealed that are not necessarily less diverse (Duecker er al. 2018). It is thus no surprising that stone communities yielded more diverse communities than soil and air and, indeed, its diversity indexes are also comparable with other subaerial biofilms growing on historical stones in temperate regions (Chimienti et al. 2016; Li et al. 2016; Schröer et al. 2020).

The bacterial communities in the three compartments are also different in terms of taxonomic composition. As recurrently observed (Villa and Cappitelli 2019), biofilms on rocks were composed of a core microbiome dominated by Cyanobacteria and Proteobacteria taxa. Cyanobacteria are fundamental first colonizers and contributors to the ecosystem functions, because of their photoautotroph metabolism, based on the ability to exploit light exposure for energy and organic matter production and collecting micronutrients, oxygen, carbon dioxide and water from the surrounding air (Crispim and Gaylarde 2005; Gorbushina and Broughton, 2009; Cappitelli et al. 2012). Within the stone communities, the most represented Cyanobacteria family was the Chroococcidiopsaceae. Strains of this family are well-known stone-dwelling microorganisms (Ennis et al. 2020), but they also inhabit even hot and cold deserts, being able to survive radiations and desiccation, possibly because of thick multi-layered polysaccharidic envelopes, efficient DNA repair systems and avoidance of protein oxidative damage (Billi et al. 2000; Fagliarone et al. 2017). We observed that Chroococcidiopsaceae, and Cyanobacteria in general, are very scarce is in soil and air, suggesting that the interaction between stone biofilms and the soil and air communities is limited for phototrophs, and that it is within the biofilm communities on stones where these bacteria are actively growing and flourishing. In addition to Cyanobacteria, stone subaerial biofilms were also composed by abundant Proteobacteria, that, together with other phyla such as Actinobacteria and Chloroflexi, are fundamental to maintain the nutrients balance in the self-sustaining ecosystem of subaerial biofilms (Villa and Cappitelli 2019; Zanardini et al. 2019).
Air and soil samples instead were dominated by heterotrophic communities, as previously observed in urban environments with taxa from the Proteobacteria, Actinobacteria, Acidobacteria (for example, Xu et al. 2014; Yan et al. 2014; Gill et al. 2017; Brewer and Fierer 2018; Yu et al. 2019). Despite the strong differences among stone, soil and air communities, we could identify a small group of shared ASVs that belong to the Proteobacteria families of Sphingomonadaceae and Xanthomonadaceae. Bacteria from these taxa have metabolic capacities that could be advantageous to survive and grow in niches with low nutrient availability and exposure to stressors. Several Sphingomonadaceae species can degrade recalcitrant aromatic compounds and can produce exopolysaccharides that might be used as a defense from desiccation and UV and temperature exposure, while some other species can tolerate multiple stressors such as differences in pH, temperature and salt concentrations (Glaeser and Kämpfer 2014; de Vries et al. 2019). In the Xanthomonadaceae family, several ASVs belong to the *Lysobacter* genus, known for its gliding motility, slime and exoenzymes production, such as chitinase (Reichenbach 2006), very useful abilities in a subaerial biofilm growing on rocks. The identification of these bacteria as main constituent of the shared community among the three compartments supports the hypothesis that only

metabolically versatile and flexible microorganisms are able to face the extreme conditions of life on rocks, as suggested for fungi (Zakharova et al. 2013), and that probably fewer of them can migrate from one compartment to the other. Similar observations have been reported also for deserts, with very different soil and rock communities dominated by different phyla, despite their proximity (Lee et al. 2016).

Our results reflect the strong niche filtering of rocks and possibly the presence of a microbial seed bank. As previously demonstrated for biological rock crusts, soil and dust microbial communities can be very different from biofilm communities, and still sharing a subset of taxa that persist and form a subaerial biofilm (Wieler et al. 2019). The ubiquity and the difference in abundance of the shared rare taxa identified (from very abundant in air and soil to low abundant in stone communities) suggest that they might constitute a microbial seed bank, a reservoir of rare taxa that activate and grow only under specific environmental conditions (Lennon and Jones 2011). The identification of a seed bank has been previously used to indicate directional recruitments driven by the water flow in a complex network of soil, rivers and lakes (Ruiz-Gonzalez et al. 2017). Similarly, we could hypothesize that the abundance of these rare taxa indicates the directional recruitment among the three compartments, with the air and soil communities recruiting more from the seed bank than the stones communities. This hypothesis seems to be supported also by the striking difference in the Cyanobacteria presence, certainly recruited elsewhere than from air and soil communities. Further studies will be necessary to confirm it and reveal its importance.

In this study, we also verified that nor seasons or any physical parameter among the considered could shape the microbial communities in the three compartments so much to justify their diversity. During winter, for example, we observed a reduction of diversity for all soil and air samples, but only for some tombstone samples. The presence of the exopolymeric matrix protecting microorganisms if the subaerial biofilms from temperature extremes and enhancing water retention (Flemming and Wingender 2010; Nwodo et al., 2012) might explain the higher stability and resistance of stone communities. On the other hand, we found that lithotype was a driver for diversity within the subaerial biofilm communities growing on tombstones. Samples collected from the granite tombstone (tomb 4), irrespectively of season, enrich for a less diverse community than the other lithotypes, being composed by a minor number of ASVs. It has been previously observed that bioreceptivity (the ability of a material to be colonized by microorganisms, Guillite 1995) for granite is low and that microbial communities are enriched with acid tolerant taxa (Brewer and Fierer 2018). While this might be due to granite physical and chemical composition, the similar mineralogy of granite and feldspathic-arenite sandstone suggests that other inherent properties of the stone must be considered too, such as surface roughness, open porosity, capillary water and bulk density (Miller et al. 2012; Vazquez-Nion et al. 2018). Since these properties are extremely variable in rocks and artworks, it is very complex to establish with certainty the reason for a less diverse community on the granite tombstone than on other lithotypes. In our study the microbial communities of limestone and marble tombstones are mainly composed by Cyanobacteria and Alphaproteobacteria. Compared to previous studies (Chimienti et al. 2016; Mihajlovski et al. 2017; Brewer and Fierer 2018; Pinheiro et al. 2019), the presence of Actinobacteria is lower, despite their abundance in the soil samples. In addition, since several examples of limestones and marbles biodeterioration by phototrophs excretion of corrosive organic acids have been reported in urban and temperate environment (reviewed in Warscheid and Braams 2000), the constant and significant presence of Cyanobacteria is worrisome for the conservation of these artworks. Two interesting cases are the marble tombstones 6 and 9 that, at the moment of the first sampling campaign (summer), have been recently restored. We expected to observe a very low diversity community, composed by early colonizers, but the diversity indexes and the taxonomic composition were instead in line with the other

tombstones. The presence of a microbial community very similar to older and more mature communities present on not restored tombstones might be due to the high bioreceptivity of marble or to the difficulty of completely removing the pre-existing community with the available restoration methods.

5. Conclusions

Our analysis clearly shows that the tombstones microbial communities are very different from the microorganisms in the surrounding soil and air, irrespectively of the stone lithotype or the season of the sampling campaign. The selective pressure of living on a stone surface exposed to air is therefore the main driver of the microbial diversity in the system analyzed. Nevertheless, we identified a core community of ASVs shared among the three compartments – stone, soil and air – made of bacterial species particularly resistant to stress and flexible enough to migrate from one compartment to the other. This observation supports the theory that stone-dwelling microorganisms originate from the surrounding soil and air, and that rock conditions pose as a strong selective pressure and select for specific taxa to build subaerial biofilms.
Finally, as previously reported, we confirm that the lithotype is a major determinant of stone microbial communities' structure, affecting their diversity more than season or other physical parameters.

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Declaration of competing interest

The authors declare that they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online.

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Figure captions

Table 1 Summary of the physical parameter recorded for each tombstone in summer (July 2015), winter (December 2015) and spring (April 2016).

Fig. 1 The nine tombstones from the Monumental Cemetery of Milano investigated in this study. The number of each tombstone is indicated on the bottom left corner of each picture.

Table 2 Relative abundance of the major phyla identified in bacterial communities from the stones, in the soil surrounding the stones and the air in three seasons (summer, winter, spring), expressed as percentage.

Fig. 2 Alpha diversity of stone, soil and air samples. Boxplots of the observed phylotypes (A) and the Chao1 index (B) for the stone, soil and air samples. Given a significant *p*-value obtained by ANOVA analysis (*p*-value observed phylotypes = 0.0319; *p*-value Observed phylotypes = 0.0962) obtained by ANOVA analysis, the Tukey's *post hoc* analysis was run (Tukey's HSD, $p < 0.05$) and results are summarized with letters on top of boxplots: boxplots sharing the same letter are not significantly different from each other. Observed phylotypes (C) and Chao1 index (D) according to sample type (circle for air, triangle for soil and square for stone) and season (spring in yellow, winter in blue and summer in orange). Asterisks in C) and D) indicate the tombstones in marble.

Fig. 3 Non-metric dimensional scaling (NMDS) ordination based on Bray–Curtis distance of the microbial communities. (A) Microbial communities of stones, soil and air (stress = 15.7%), the distribution is colored according to the type of sample: red for stone, grey for soil and yellow for air. (B) Microbial communities of stones only (stress = 16.1%), the distribution is colored according to the lithotype and shaped according to the season. The sample name is indicated with the type of sample, the sampling point and the season (Su, for summer; W, winter; Sp, for spring).

Fig. 4 Venn diagram showing the number of unique and shared ASVs in the three compartments: stone, soil and air.

Table A.1 Summary of the sequencing results as reads number (#) and percentage of the initially obtained (%) after each bioinformatic step.

File A.2 R code used in this study.

Table A.3 Relative abundance of the major class identified in bacterial communities from the stones, in the soil surrounding the stones and the air in three seasons (Su for summer, W for winter, Sp for spring), expressed as percentage of each sample.

Table A.4 Alpha diversity of stone, soil and air samples calculated indexes (Observed unique phylotypes, Chao1, ACE, Shannon, Simpson, Inverse Simpson, Fisher), the variable describing each sample (season, type, sampling point) and the average of the Observed unique phylotypes and the Chao1 indexes per stone.

Table A.5 List of unique and shared ASVs in the three compartments: stone, soil and air.

Fig. A.6 Correlation between environmental parameters (minimum and maximum light, temperature and relative humidity), season, lithotype, and microbial communities. The lithotype is the only variable that significantly affects the microbial communities on tombstones (p=0.002).

Figure 1

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Figure 2

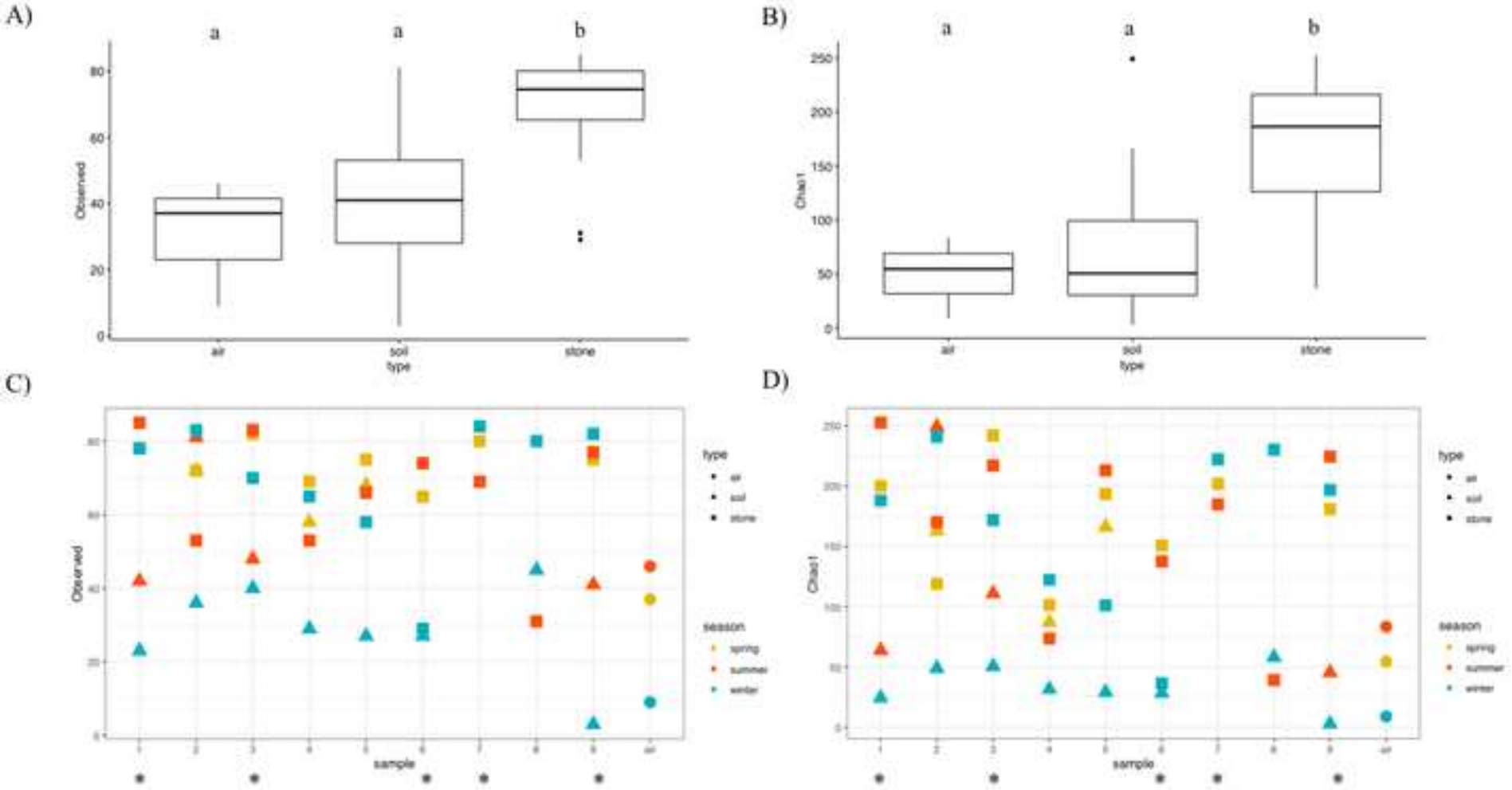

Figure 3

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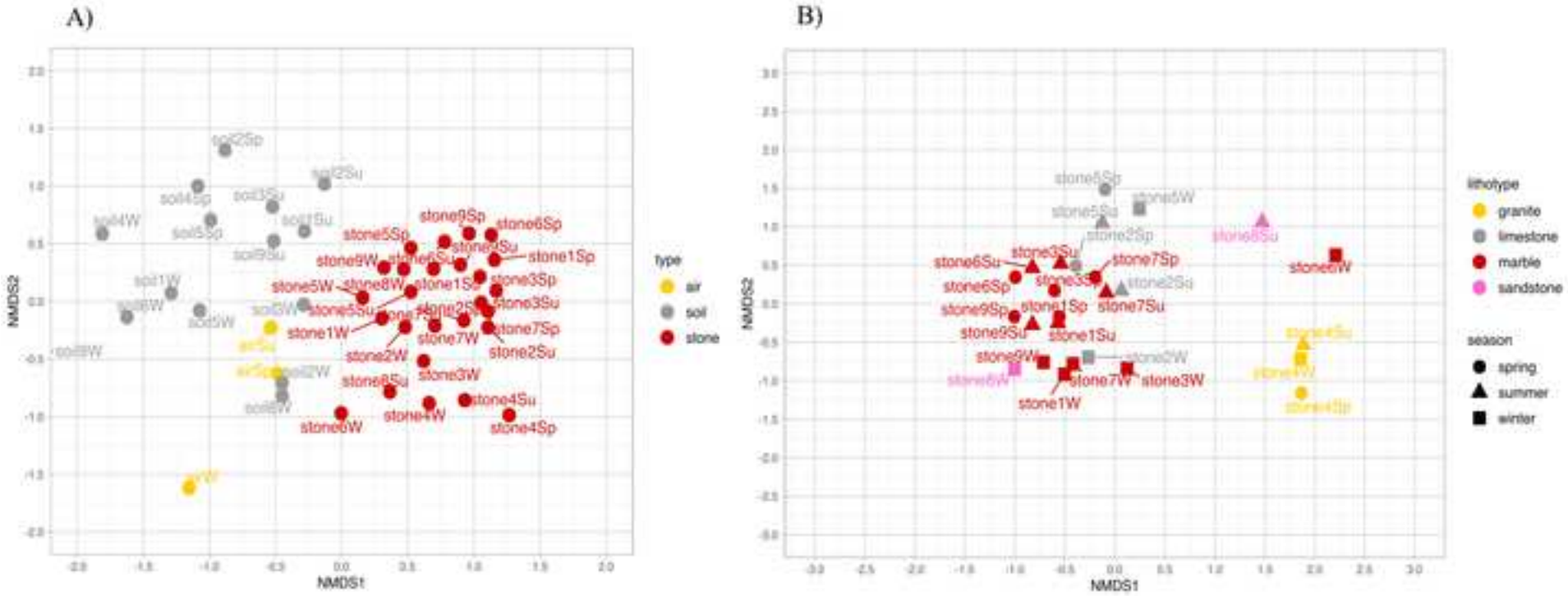
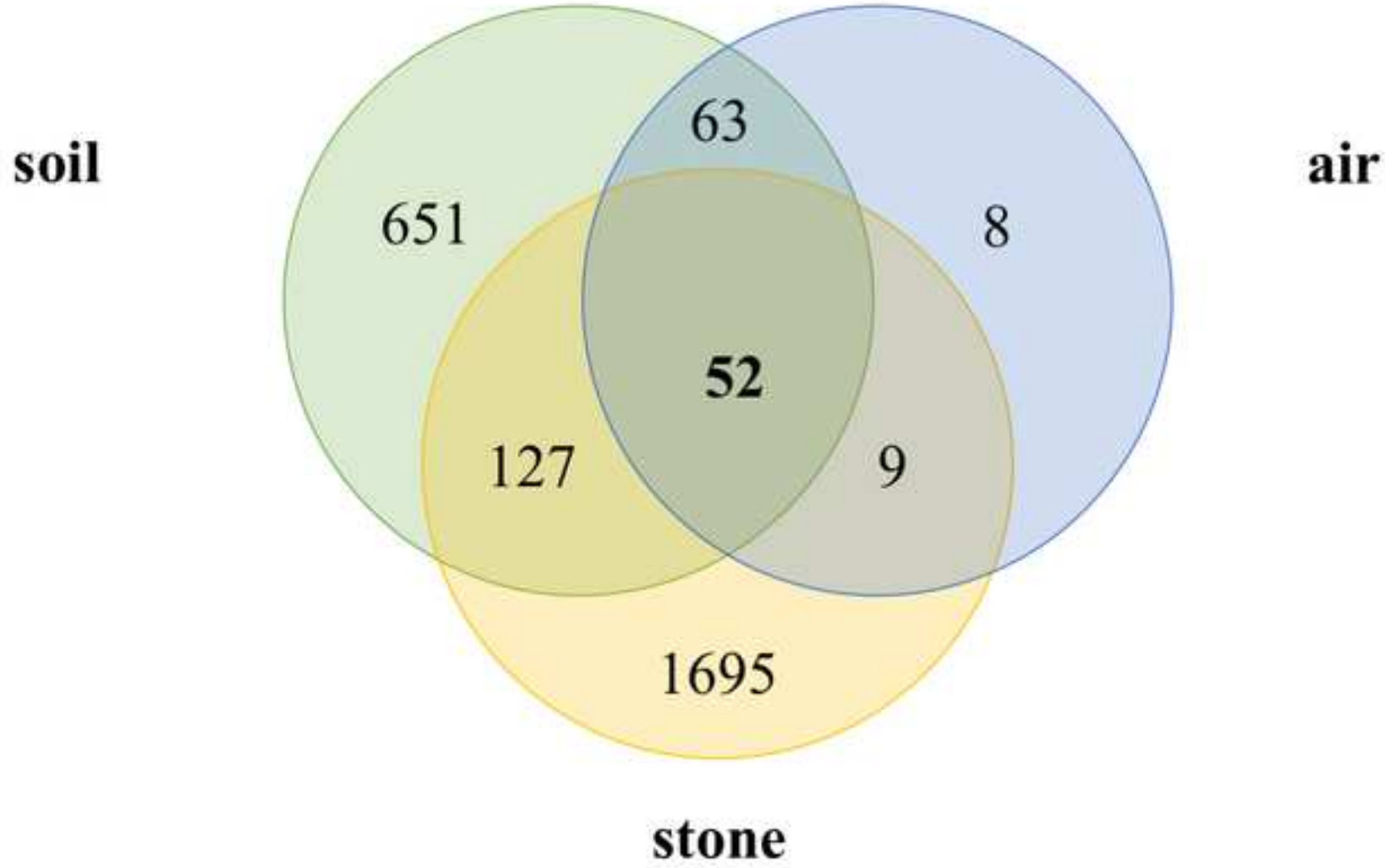


Figure 4



				max light (lux)			min light (lux)			temperature (℃)			relative humidity		
ID		**Lithotype**		**summer**	**winter**	**spring**	**summer**	**winter**	**spring**	**summer**	**winter**	**spring**	**summer**	**winter**	**spring**
1	putto's	calcitic-marble		44610	94090	57760	16075	83460	48990	31.3	20.8	25.97	50.61	21.9	21.09
2	Arton's	limestone		17118	1404	7664	7976	1374	6398	32.61	17.13	27.08	48.84	27.76	20.87
3	de Daninos'	calcitic-marble		48870	44670	46550	9590	40080	28920	32.64	20.62	27.64	48.17	23.15	19.36
4	31-32-33	granite		49120	2450	92300	16140	1419	24170	32.88	19.22	27.18	50.81	29.88	20.59
5	71-72	limestone		1883	706	3151	1446	646	2262	31.74	14.6	27.06	50.13	35.9	17.12
6	Bernocchi's family	calcitic-marble	R	21760	3260	41870	20390	2885	22440	33.35	13.7	29.38	46.87	28.1	21.41
7	729	marble		9807	3316	7649	9371	3427	4093	35.37	18	27.5	42.97	27.1	20.19
8	Besenzanica's family	feldspathic-arenite sandstone		963	145	626	607	114	531	32.33	16	22.97	47.81	25.4	35.81
9	Toscanini's	marble	R	19560	2292	12668	17989	2217	12305	34.41	17.1	27.88	46.38	30.2	28.49

Table 1 Summary of the physical parameter recorded for each tomb in summer (July 2015), winter (December 2015) and spring (April 2016).

Table 2

Click here to access/download;Table;Table 2.xlsx

	summer								winter								spring								
Sample / **Phylum**	stone 1 Su	stone 2 Su	stone 3 Su	stone 4 Su	stone 5Su	stone 6 Su	stone 7 Su	stone 9 Su	stone 1 W	stone 2 W	stone 3 W	stone 4 W	stone 5 W	stone 6 W	stone 7 W	stone 8 W	stone 9 W	stone 1 Sp	stone 2 Sp	stone 3 Sp	stone 4 Sp	stone 5 Sp	stone 6 Sp	stone 7 Sp	stone 8 Sp	stone 9 Sp
Cyanobacteria	51	59	20	67	41	49	49	42	48	45	43	60	59	91	39	49	53	29	28	23	11	22	55	28	77	46
Proteobacteria	31	26	51	21	37	37	36	29	28	28	40	24	19	8	25	27	21	29	44	40	47	45	28	39	13	25
Acidobacteria	7	10	17	2	4	4	6	3	3	4	3	4	6	0	5	1	3	3	11	15	7	12	4	14	0	19
Bacteroidetes	9	3	9	7	12	8	5	18	9	7	4	4	7	0	8	9	12	34	9	16	20	16	11	14	6	8
Deinococcus-Thermus	0	1	1	1	0	0	1	3	3	8	2	1	1	0	8	2	6	0	2	1	6	1	0	1	0	1
Actinobacteria	0	1	1	1	6	1	2	5	6	7	8	6	5	1	14	11	4	2	3	3	3	3	1	3	3	1
FBP	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	1	2	0	0	1	0	0	0	0
Chloroflexi	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0
Armatimonadetes	1	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	4	0	0	0	0	0
Gemmatimonadetes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Nitrospirae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Verrucomicrobia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Firmicutes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Planctomycetes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chlamydiae	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Patescibacteria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
WPS-2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elusimicrobia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Latescibacteria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fibrobacteres	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dependentiae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	summer	winter	spring		summer				winter								spring		
Sample / **Phylum**	air Su	air W	air Sp		soil 1 Su	soil 2 Su	soil 3 Su	soil 9 Su	soil 1 W	soil 2 W	soil 3 W	soil 4 W	soil 5 W	soil 6 W	soil 8 W	soil 9 W	soil 2 Sp	soil 4 Sp	soil 5 Sp
Cyanobacteria	0	0	0		1	0	0	0	0	1	73	0	0	0	8	0	0	1	1
Proteobacteria	67	97	98		52	45	63	56	32	93	13	39	42	27	64	96	43	34	45
Acidobacteria	13	0	0		5	14	7	0	0	2	6	11	1	7	0	4	22	9	8
Bacteroidetes	1	1	0		1	1	0	0	1	0	4	3	0	0	0	0	12	42	17
Deinococcus-Thermus	0	0	0		0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Actinobacteria	6	0	0		34	13	26	42	47	2	3	32	42	50	0	0	4	6	22
FBP	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chloroflexi	4	0	0		5	4	0	1	14	1	0	5	7	8	0	0	2	2	2
Armatimonadetes	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gemmatimonadetes	2	0	0		2	14	3	1	0	0	0	3	3	1	0	0	10	2	1
Nitrospirae	2	0	0		0	3	0	0	0	0	0	3	0	2	0	0	4	2	1
Verrucomicrobia	2	0	0		0	2	1	0	2	0	0	0	0	0	0	0	3	0	3
Firmicutes	1	2	1		0	0	0	0	3	1	0	0	5	6	27	0	0	0	0
Planctomycetes	1	0	0		0	3	0	0	0	0	0	3	0	0	0	0	1	0	0
Chlamydiae	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Patescibacteria	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
WPS-2	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elusimicrobia	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Latescibacteria	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fibrobacteres	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dependentiae	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0