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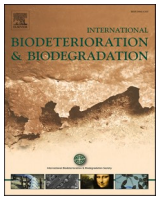

Close but different: Metabarcoding analyses reveal different microbial communities in ancient Roman nymphaea

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ABSTRACT

In this study, we used a metabarcoding approach to characterize the prokaryotic and eukaryotic communities colonizing two adjacent but structurally different Roman nymphaea in the archaeological park of Baia (Phlegraean Fields, Campania region, Italy). These two environments likely belonged to a thermal complex and are still mostly unearthed because of the effect of bradyseism. This condition, together with infiltrations by underground brackish water, exposed the sites to different degrees of biodeterioration. We sampled 17 biofilms across the two sites and found differences in microbial communities both between sites and within one site. No particular patterns of diversity were detected when considering the composition of the substratum. At finer taxonomic level, the influence of marine aerosols and submerged water was highlighted by the presence of several marine taxa. Moreover, we found some bacteria previously reported elsewhere from thermal vents or deep waters, confirming that cave environments often harbour extremophiles. Overall, this study provided a detailed overview of whole community colonizing the nymphaea and confirmed the metabarcoding approach as a powerful tool to gather taxonomic information useful for restoration purposes.

1. Introduction

Biodeterioration represents one of the major threats to the conservation of cultural heritage because it can cause aesthetic and structural changes (Di Carlo et al., 2022). Aesthetic changes, collectively indicated with the term "patina", have usually been attributed to chemical processes as redox reactions or mineral deposits. However, now it is widely recognized that many patinas are due to the presence and metabolic activity of many and diverse phototrophic, chemoorganotrophic, and chemolithotrophic microorganisms (Krumbein, 2003). These microorganisms are mostly bacteria, cyanobacteria, fungi, microalgae, lichens and bryophytes, capable of utilising the substrates of historic materials (stone and building materials) as source of nutrients or anchorage (reviewed in Beata, 2020). Stone monuments contain many and different minerals like calcium, iron, magnesium, sodium and their salts, to which other elements deriving from coatings like plaster or paints can be added (Negi and Sarethy, 2019). This heterogeneity in the mineralogical composition of the substratum, combined with different action of environmental variables (e.g., incidence of UV radiation, water availability, presence of pollutants) creates a potential plethora of micro-environments in which different microbial communities can settle and thrive. The characterization of these microbial communities is important to assess potential damage to cultural heritage and to determine the best cleaning procedures (Pyzik et al., 2021). Indeed, some commonly used, non-invasive cleaning treatments as electromagnetic radiation (EMR) and UV-C rays have proven to be particularly effective against some groups of microorganisms but not over others (Pfendler et al., 2017; Cennamo et al., 2020a,b). Despite some studies (reviewed in Pyzik et al., 2021) have tried to summarise the most common taxa colonizing cultural heritage of different materials, predicting the taxonomic composition of a biofilm based on the substratum, the environmental conditions and the colour of patinas is a daunting task.

In recent years, the application of next-generation sequencing (NGS) approaches as metabarcoding has accelerated and expanded our comprehension of the taxonomic diversity of microorganisms colonising cultural heritage compared to classical Sanger sequencing method (Marvasi et al., 2019; Branysova et al., 2022). Such studies focused on the characterization of solely bacteria (Lepinay et al., 2018), fungi

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(Trovão et al., 2019), phototrophic organisms, or a mix of them (Pfendler et al., 2018; Cennamo and De Luca, 2022; Alaoui-Sosse et al., 2023) from diverse substrates and targeted specific regions of the genome. These studies allowed unprecedented insights in the relative abundance and overall diversity of microorganisms associated to cultural heritage, regardless their effective role in biodeterioration processes. Indeed, the dominance of some microorganisms over others is determined by the interaction of environmental conditions and chemical properties of the substratum, which can trigger a different ecological succession every time and a different degradation process (Kumbaric et al., 2012; Favero-Longo and Viles, 2020). Consequently, the knowledge of all microbial taxa present on a particular substratum is important to predict possible outcomes of biodeterioration.

In this study, we used a metabarcoding approach to characterize prokaryotic and eukaryotic communities in two adjacent but structurally different nymphaea in the Archaeological Park of Baia along the coasts of the archaeological Phlegraean Fields (Campania region, Italy). The remains of the ancient Roman city of Baia and its thermal complexes now lie on the sea bed, between 1 and 15 m depth, because of the effects of bradyseism (D'Arms, 2003; Miniero and Zevi, 2008). However, it is not yet completely clear whether Baia constituted a grandiose thermal complex surrounded by public spaces and luxurious residences or rather a vast residential area in which houses and public spaces have been built to exploit the healing waters coming out from the soil (Cairoli Giuliani, 1979; Di Luca, 2009). The two nymphaea object of this study are located in the "sector of Mercury", an area of the park that takes its name from the homonymous temple and is composed of two buildings, partially buried or submerged by water, with thermal functions (Veronese, 2018). Microbial communities colonising these two nymphaea were characterised for purposes of restoration. Specifically, we aimed at describing the prokaryotic and eukaryotic community of these two adjacent but different sites assessing: i) the structure of prokaryotic and eukaryotic community within each site; ii) a possible correlation between the colour of patinas and microbial profile; iii) the influence of groundwater in site 2 on the microbial diversity.

2. Materials and methods

2.1. Site description

The area investigated in this study is located in the "Mercury Sector", in the northeast area of the Archaeological Park of Baia. This denomination derives from a building that was initially believed to be a temple dedicated to Mercury, but then it turned out to be a *frigidarium*, a cold-water pool. The rooms, probably a second floor of a building for thermal use, currently appear as a partially emerged basement (Fig. 1A and B), equipped with a barrel roof made of *opus caementicium*. As regards the decorative apparatus, which can be attributed to the II-III century AD, there is a succession of white background murals, in full IV century style, and some painted stucco frames. These sites were probably part of a thermal complex and, despite their proximity, they are very dissimilar. One environment (hereby called "site 1" but officially known as SB-E0-R07) has been recently excavated and appears now as a hypogeum with frescoes on the back wall; the other environment (hereby called "site 2"), has only been partially excavated, and it appears as an arched vault which is subject to partial flooding events caused by infiltrations of underground water.

2.2. Biofilm sampling and environmental parameters collection

Seventeen biofilm samples were collected during spring 2021 across the two adjacent sites (Fig. 1A and B). The sampling points were chosen in view of restoration purposes and, therefore, taking into account the presence of relevant biofilms and the different types of substrata (Table 1). In the site 1, samples were collected both from the outer part of the nymphaeum ("external" samples) and the inner part ("internal" samples), which was partially covered with frescoes on the back wall; in the site 2, biofilm samples were collected from points at contact with water, nearby saline incrustations and in other portions of the nymphaeum where the microbial colonization was marked. Biofilms were collected by scraping the substrata with a sterile scalpel and gathering

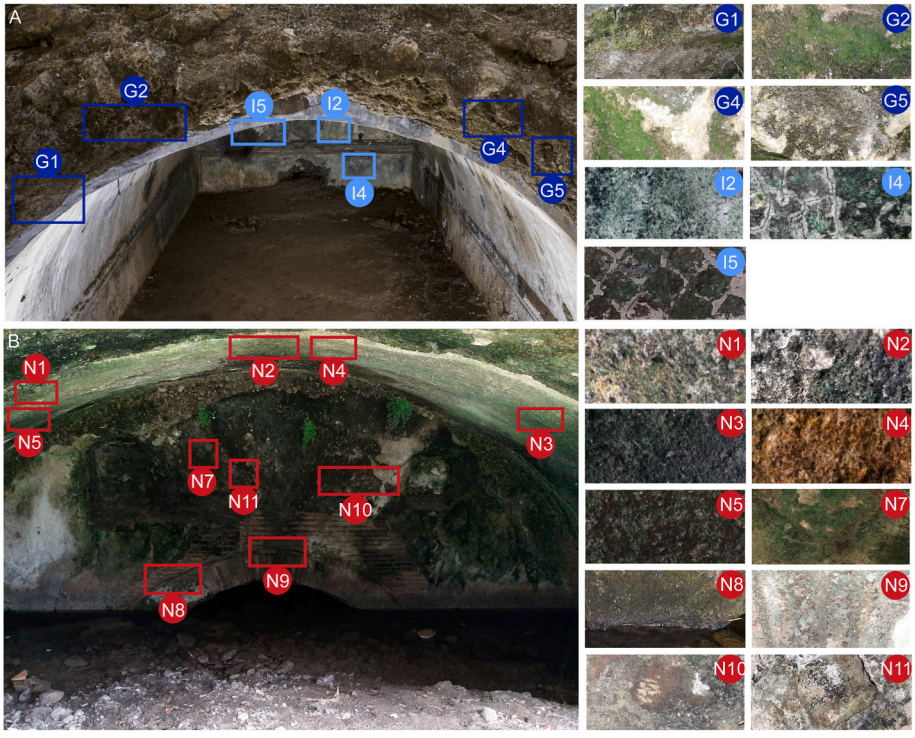

Fig. 1. Sampling points with details of biofilms. A) site 1; B) site 2 (samples N). Within site 1, samples have been collected on the external side (G) and on the internal side (I).

Table 1
Location, texture and characteristics of the substratum from which biofilms were collected.

Site	Sample	Substratum
1 (external)	G1	bricks
1 (external)	G2	bricks
1 (external)	G4	bricks
1 (external)	G5	bricks
1 (internal)	I2	*intonachino*
1 (internal)	I4	*intonachino*
1 (internal)	I5	*tufello*
2	N1	*intonaco*
2	N2	*intonaco*
2	N3	*arriccio*
2	N4	*intonaco*
2	N5	*arriccio*
2	N7	*opus caementicium* (repeated drafts of Pozzolanic mortars)
2	N8	Pozzolanic mortar similar to *opus caementicium*
2	N9	Pozzolanic mortar similar to *opus caementicium*
2	N10	*tufello*
2	N11	*tufello*

the material into sterile tubes. The samples were then transferred to the laboratory for analyses. Temperature (°C) and relative humidity (%) were using the data logger TESTO 177 H-1 (Testo Middle East FZCO, Dubai, United Arab Emirates); minimum, maximum and average values were reported. For samples collected in the site 2 and labelled as "N", measurements could not be taken because of the impossibility of fixing the data logger to a substrate, also in spite of the presence of rising water levels.

2.3. X-ray diffraction (XRD)

X-ray diffraction analysis was performed to determine the inorganic components of the substratum colonized by the biofilms. XRD patterns were collected in the 3–90° 2θ range, according to the step scanning procedure with Co radiation on a Miniflex Diffractometer (Rigaku, Japan). The tube operated at 30 kV and 15 mA, and the counting time was 3600 s. The identification of mineralogical phases was achieved with a search/match on the Joint Committee on Powder Diffraction Standards.

2.4. Metabarcoding analyses

Total DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol and visualised after 0.8% agarose electrophoresis stained with the SafeView™ Classic (ABM, Vancouver, Canada) and including a high molecular weight marker (HyperLadder™ 1 kb; Bioline, Meridian Bioscience, Cincinnati, OH, USA) for a gross determination of molecular weight and concentration. DNA was then finely quantified with the Qubit 4.0 fluorometer using the Qubit™ dsDNA HS Assay Kit (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) and stored at −20 °C until further analyses.

Metabarcoding analyses were carried out using the V3–V4 hypervariable region of the 16S rRNA gene for the characterisation of bacteria and the V4 region of the 18S rRNA gene for eukaryotes. Amplicon library preparation and sequencing was carried out by Integrated Microbiome Resource (IMR; Halifax, Canada). For library preparation, the Illumina primers 41F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013) were used for amplification of bacteria, while the primer pair E572F (5′-CYGCGGTAATTCCAGCTC-3′) and E1009R (5′-AYGGTATCTRATCRTCTTYG-3′) (Comeau et al., 2011) to specifically target the eukaryotes. High-throughput sequencing was carried out on 300bp PE Illumina MiSeq platform (San Diego, CA, USA). Sequences were deposited in the Sequence Read Archive (SRA) as BioProject PRJNA917271.

Illumina paired-end raw reads (FASTQ format) were pre-processed to generate Amplicon Sequence Variants (ASVs) using the dada2 (Callahan et al., 2016) R package (R Core Team, 2020). In brief, primer sequences were removed and forward and reverse reads were first trimmed based on the Quality score. Subsequently, filtered reads were used to train the error model from the data using a machine learning approach. After this step, forward and reverse sequences were dereplicated to generate unique (non-redundant) sequences applying the trained error model. Finally, forward and reverse reads were merged and checked for chimeras. As result of this pipeline, we obtained a table with the list of a representative sequence for each ASV with its abundance across samples. Singletons were removed and, to account for differences in the number of ASVs across samples, data were normalised at the median value (14,152 for 16S dataset, 2972 for 18S dataset) using the function "rrarefy" of the vegan R package (Oksanen et al., 2020).

Taxonomy was assigned to sequence variants using the naive Bayesian classifier method (Wang et al., 2007) against the Silva reference database v138.1 (McLaren et al., 2021; https://zenodo.org/record/4587955#.Ylqor9NBw2w) for prokaryotes and the PR2 database v4.14.0 (ref; https://github.com/pr2database/pr2database/releases) for eukaryotes. Furthermore, taxonomic assignation at species level was carried out using the "assignSpecies" function implemented in dada2 (Callahan et al., 2016) for prokaryotes; we set the argument "allowMultiple" to the value of 5 in order to check that five exact matches actually belonged to the same species which, in that case, were retained. For eukaryotes, we used a Lowest Common Ancestor (LCA) approach (Huson et al., 2007) to identify ASVs at genus level when matches with different species occurred at the same similarity percentage.

Alpha-diversity (i.e., richness and Shannon index), beta-diversity (non-metric multidimensional scaling, NMDS) and taxonomic composition analyses were conducted separately for 16S or 18S markers using the package phyloseq (McMurdie and Holmes, 2013) and plotted using ggplot2 (Wickham, 2016). Taxonomic composition and alpha-/beta-diversity analyses were carried out each marker separately (16S or 18S). Venn diagrams and petal-plots were built using the file2meco (Liu et al., 2022) and microeco (Liu et al., 2021) R packages to assess the number of shared and exclusive ASVs: 1) among all samples; 2) between site 1 and site 2; 3) between the external (G) and internal (I) samples of site 1. Finally, a linear discriminant analysis (LDA) effect size (LEfSe) analysis (Segata et al., 2011) was carried out on the Galaxy web platform (Afgan et al., 2016; https://huttenhower.sph.harvard.edu/galaxy/) to identify the prokaryotic and eukaryotic taxa that are significantly different between site 1 and site 2 and the external and internal samples of site 1; we used a stringent threshold LDA score of 3.3.

3. Results

3.1. Environmental parameters and XRD analysis

In the area corresponding to the back wall of the grotto with frescoes (I), the mean values of temperature and relative humidity during the period of sampling were 14.9 °C and 95.7%, respectively (Table S1). Over the year 2021–2022, min and max values of temperature were 7.31 and 24 °C; for relative humidity, 77.4 and 100%, respectively, resulting into a cool and humid environment. Samples taken outside site 1 (samples G) experienced a different temperature regime. Regarding the mean values of temperature and relative humidity during the period of sampling, they were 15.3 °C and 90% respectively (Table S1), while over the year of sampling min and max temperatures were 5.1 and 25.6 °C and relative humidity 59.8 and 99.9%. As expected, the inner part of site 1 was drier and slightly warmer than the outside.

The XRD analysis indicated that calcite and dolomite were the principal components of most of substrata, with feldspars and quartz constituting another important but less abundant component (Table S2). The arriccio, intonachino, and opus reticulatum samples contained similar quantity of calcite but different of dolomite; the mineralogical

composition of the tufello sample from site 1 (I5) was different from the tufello samples from site 2 (N10 and N11) in terms of calcite (not detected vs. abundant, respectively) and feldspars (abundant vs. low quantities, respectively). Samples of intonaco (site 2: N1, N2, and N4) contained the lowest quantity of calcite and the highest of feldspars in respect to all the other substratum samples (Table S2). We did not consider for XRD analysis the substrata from which samples G1, G2, G4, G5, N8 and N9 have been collected because their composition was already known. Indeed, the above-mentioned samples "G" grew on bricks (*latericium*), which are composed of iron oxides, calcium, aluminium and silicon, essential elements of clays, the main constituent of terracotta; samples N8 and N9 grew on rubble of Pozzolanic mortar, which has a mineralogical composition similar to the *opus caementicium* analysed in site 2.

3.2. Dataset characteristics

The V3–V4 16S rRNA dataset contained 745,451 raw sequences distributed across 17 samples. The clean, annotated dataset contained 360,348 sequences corresponding to 3314 ASVs; after the normalization procedure, 186,774 sequences and 3080 ASVs remained (Table S3). The raw eukaryotic dataset based on the 18S–V4 region included 292,423 sequences across 17 samples. The clean dataset contained 143,782 sequences corresponding to 329 ASVs; after normalization, 38,368 sequences and 305 ASVs remained (Table S4). All the details regarding the number of sequences discarded in each sample after pre-processing are provided in Table S5.

3.3. Taxonomic composition and diversity

We found 13 phyla of prokaryotes occurring at least once with relative abundance above the 3%. Details of prokaryotes at phylum level are in plotted in Fig. 2A. Samples from the external side of site 1 (G) were quite homogenous, with Actinobacteriota and Alphaproteobacteria constituting about the 50% of prokaryotic taxa, and followed by Cyanobacteria, Acidobacteria (only in 2 samples), Chloroflexi, Gammaproteobacteria, Planctomycetota and other phyla (Fig. 2A). Firmicutes and Bacteriota were only found in two different samples in G. The following 17 phyla occurred at relative abundance <3%: Armatimonadota, Bdellovibrionota, Calditrichota, Campylobacterota, Dadabacteria, Dependentiae, Desulfobacterota, Elusimicrobiota, Entotheonellaeota, Fibrobacterota, Halobacterota, Hydrogenedentes, Methylomirabilota, Myxococcota, Nitrospirota, Patescibacteria and Sumerlaeota. We identified 43 taxa at species level; most of them belonged to

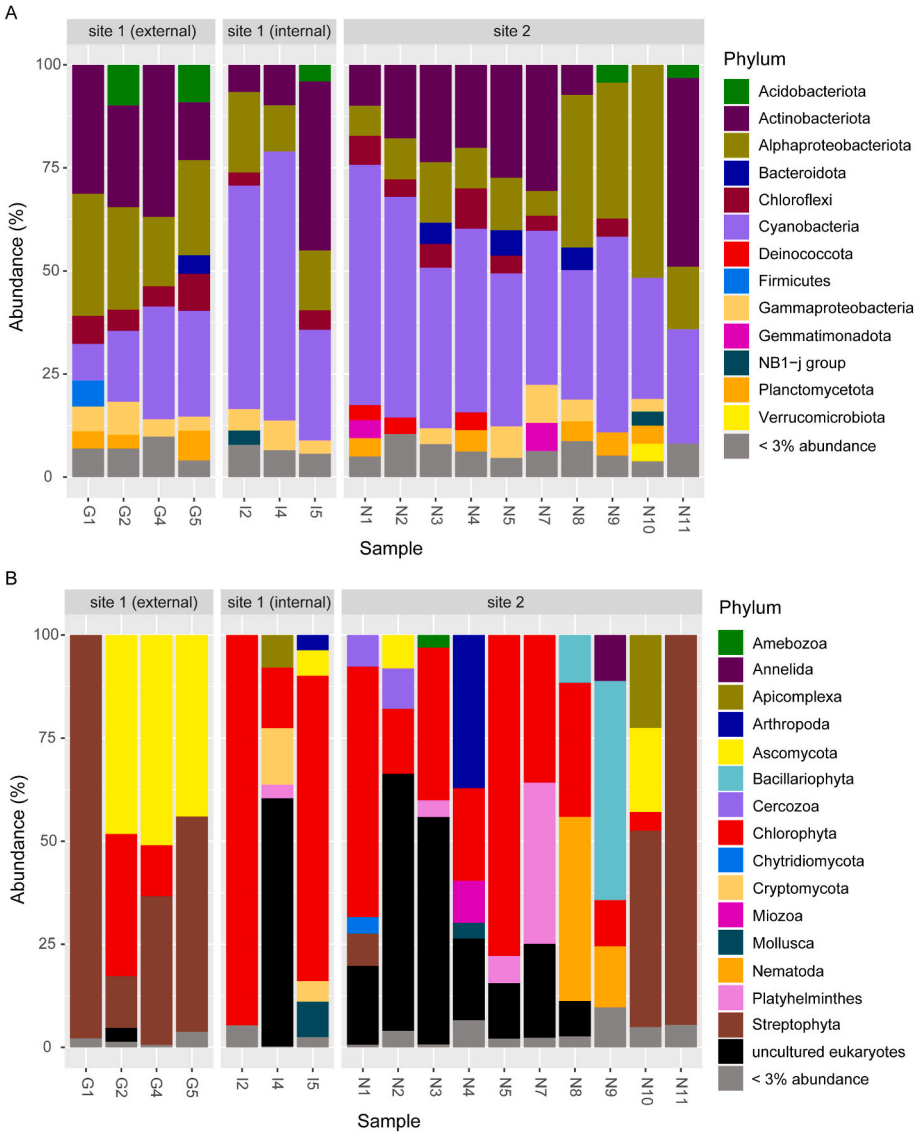

Fig. 2. Taxonomic composition at phylum level of microbial communities within each site. A) prokaryotes; B) eukaryotes.

Alphaproteobacteria, followed by Actinobacteriota, Gammaproteobacteria, and Firmicutes (Fig. S1). None of the species was equally distributed across samples; instead, samples G from site 1 contained the most of species reported, while the sample N8 (site 2) contained several, almost exclusive, species (Fig. S1). *Roseococcus suduntuyensis*, *Tabrizicola aquatica*, and *Elioraea tepidiphila* were the species occurring across most of samples with high relative abundance.

The taxonomic profiles of the eukaryotic community in sites 1 and 2 are shown in Fig. 2B. We found 15 phyla with abundance above the 3% at least in one sample plus a group of unclassified eukaryotes. Differently from prokaryotic community, the eukaryotic one showed less homogeneity across samples from the same site (Fig. 2B). Samples from the external portion of site 1 (G) were mostly constituted by streptophytes (e.g., bryophytes), fungi (Ascomycota) and algae (Chlorophyta), while samples from the internal side (I) were dominated by green algae or uncultured eukaryotes. Samples from site 2 (N) were highly heterogeneous, with dominance of Bacillariophyta, Chlorophyta, Nematoda, Streptophyta or uncultured eukaryotes according to the case (Fig. 2B). The phyla Basidiomycota, Bigyra, Endomyxa, Mucoromycota, Ochrophyta, Oomycota, Rhodophyta and Rotifera did not reach the abundance >3% in any sample. Taxa identified at genus or species level mostly belonged to fungi and green algae (Chlorophyta); within the latter category, *Ctenocladus circinnalis*, *Jenufa* sp., and *Pseudopleurococcus printzii* were particularly abundant across all samples analysed (Fig. 3). Mosses of the genus *Pottia* were particular abundant in samples collected at the entrance of site 1 (G) and in sample N11 of site 2.

Alpha-diversity of prokaryotes expressed as richness showed no differences across samples from the two sites and within site 1; samples G1 (site 1) and N8 (site 2) behaved as outliers (Fig. S2A). According to the Shannon index, the G samples (site 1, external) presented the highest diversity, followed by samples N (site 2) and I (site 1, internal), respectively (Fig. S2A); some exceptions (e.g., samples N8 and I5) occurred also for this index. Samples grouped for substratum material showed no pattern for richness (Fig. S2B); on the contrary, the Shannon index was higher for brick samples and lower for intonaco and intonachino samples than all the other samples (Fig. S2B). For eukaryotes, no differences were found in both alpha-diversity estimates across samples grouped for site and substratum material (Figs. S3A and S3B).

The non-metric multidimensional scaling (NMDS) of prokaryotic (16S) dataset marked by site showed two distinct groups encompassing the samples from the external side of site 1 (G) and the samples from the internal side of the same site (I); samples collected from site 2 (N) were highly heterogeneous (Fig. 4A). The NMDS coloured by material (Fig. 4B) highlighted the occurrence of several prokaryotic groups reflecting samples from arriccio, brick, and intonaco; no particular signal was detected for the other substrata. Regarding the eukaryotic (18S) community, no particular pattern was found in the NMDS for samples coloured by site (Fig. 4C); on the contrary, marking the samples for substratum material, similarities were detected between samples of arriccio, intonaco, Pozzolanic mortar and, to some extent, of brick and intonachino too (Fig. 4D).

The petal plot of prokaryotic data (Fig. 5A) showed that no ASVs were common to all samples analysed and that the sample N8 contained the highest number of exclusive ASVs (626). Site 1 and site 2 shared the 8.9% of prokaryotic ASVs (Fig. 5B) and contained a comparable percentage of exclusive ASVs (47.4% vs. 44.2%, respectively); within site 1, the percentage of exclusive prokaryotic ASVs in the external side was almost three times bigger than in the internal side (68.7%, Fig. 5C), and the percentage of shared ASVs was of only 2.7%. Regarding eukaryotes, the petal plot in Fig. 5D indicated that no ASVs were shared among all samples, and that the number of exclusive ASVs was quite homogeneous, with the exception of samples I4 and I5 (site 1, internal), which

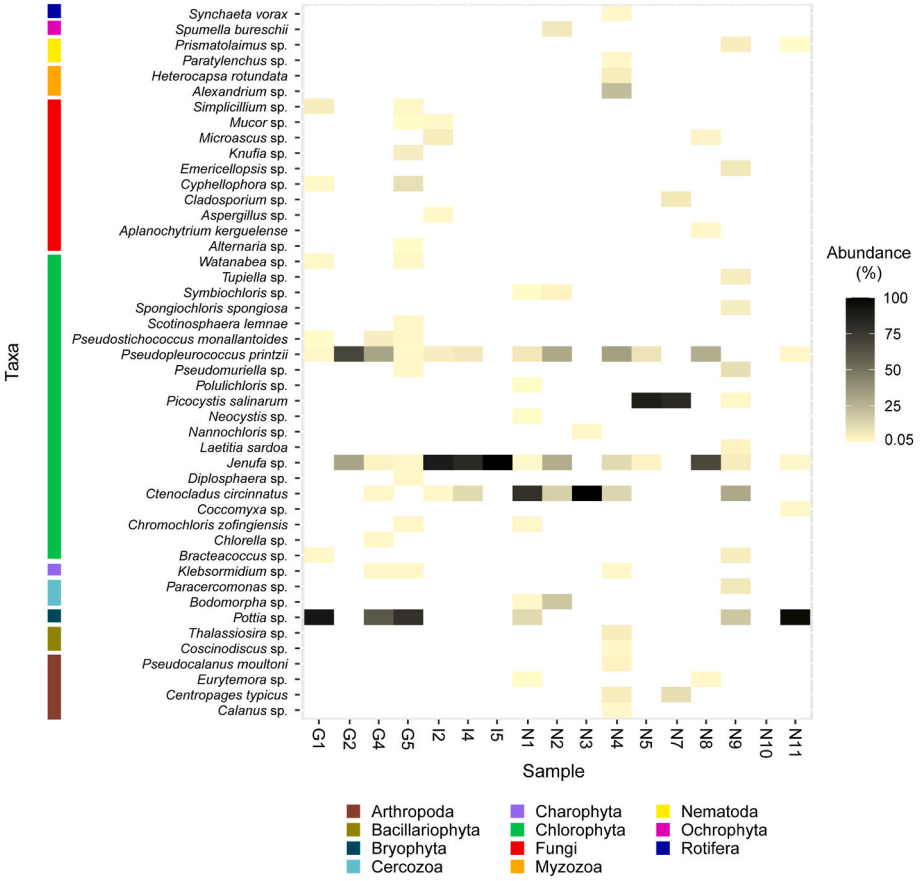

Fig. 3. Heatmap of relative abundance of eukaryotic taxa identified at species or genus level.

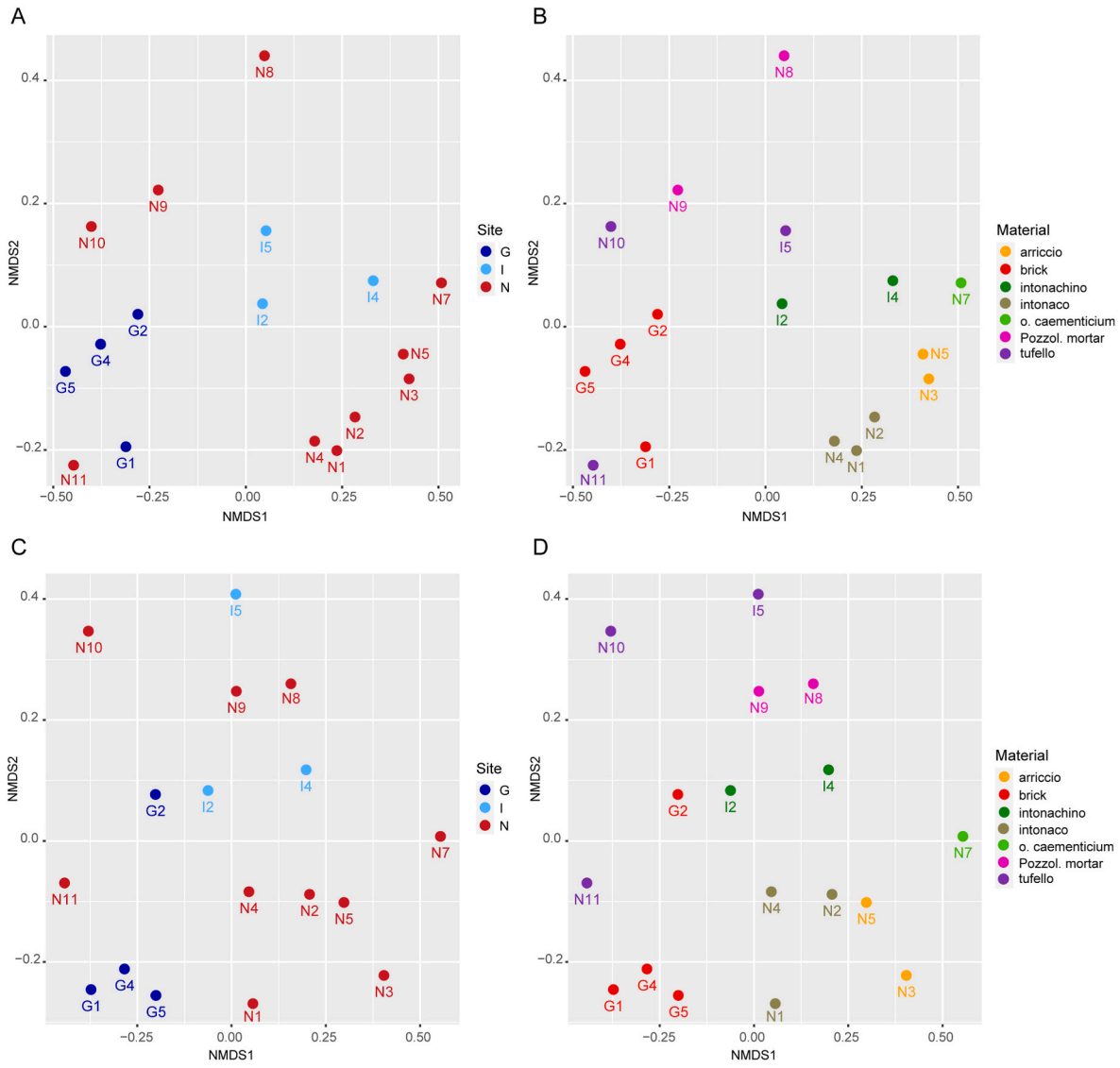

Fig. 4. Non-metric multidimensional scaling (NMDS) of microbial communities. A) prokaryotic community coloured by site; B) prokaryotic community coloured by substratum material; C) eukaryotic community coloured by site; D) eukaryotic community coloured by substratum material.

contained 3 and 2 exclusive ASVs, respectively. The venn diagram comparing eukaryotic ASVs between site 1 and site 2 (Fig. 5E) indicated that site 2 contains more than the double of exclusive ASVs than site 1 (60.3% vs. 27.6%, respectively), and the 12.1% of ASVs was shared between the two sites. Within site 1, similarly to prokaryotes, the percentage of exclusive eukaryotic ASVs in the external side was almost three times bigger than in the internal side (67.8% vs. 24.8%, respectively), and the percentage of shared ASVs was of only 7.4% (Fig. 5F).

LEfSe analysis indicated 32 prokaryotic taxa as biomarkers for site 1 and 12 for site 2 (Fig. S4). Specifically, for the site 1 we found as such members of the genera *Chelativorans* (Alphaproteobacteria; ASV18), *Iamia* (Actinobacteriota; ASV25), and *Truepera* (Deinococcota; ASV29 and ASV31), or members of unclassified genera within the families Gemmatimonadaceae (ASV83), Nitriliruptoraceae (ASV50) and Phormidiaceae (ASV5). For the site 2, *Crossiella* (ASV28), *Cyanothece* (ASV114), and *Tabrizicola* (ASV138) were detected as biomarker genera, together other higher taxonomic categories (Fig. S4). Within site 1, 22 taxa were found as biomarkers for the internal side (Fig. S5), of which the following at genus level: *Elioraea* (ASV213), *Illumatobacter* (ASV68), *Pseudofulvimonas* (ASV131), *Pseudonocardia* (ASV15), and *Rhodobaculum* (ASV72); other taxa were within unclassified genera in specific families (e.g., ASV59 in Acetobacteraceae and ASV419 in Actinomarinales) or higher taxonomic categories (e.g., ASV1 in Cyanobacteria). For the external side of site 1, 31 biomarker taxa were identified (Fig. S5), from order (e.g., Leptolyngbyales, Micrococcales, and Rhizobiales) to genus (*Hyphomicrobium* for ASV204) level. Regarding eukaryotic taxa, LEfSe analysis indicated 25 biomarkers for site 1 and.

7 biomarkers for site 2 (Fig. S6); for the former, most were uncultured eukaryotes except Nematoda, while for the latter there were several undescribed species of fungi as *Dothideomycetes* (ASV26) and *Mastodia* (ASV6), green algae as *Polulichloris* (ASV35) and *Pseudostichococcus* (ASV9) or eukaryotes at higher taxonomic ranks. Within site 1, a few eukaryotic biomarker taxa were detected to distinguish the two sides (Fig. S7), specifically 9 for the external side (mostly green algae and mosses) and 7 for the internal side (Cryptomycota and an undescribed species of *Jenufa*).

4. Discussion

This study aimed at characterizing the taxonomic profile of prokaryotic and eukaryotic communities colonizing two adjacent but structurally different Roman nymphaea located in the archaeological site of Baia (Campania region, Italy) through a DNA metabarcoding approach. Compared to culture-dependent approach and gel-based

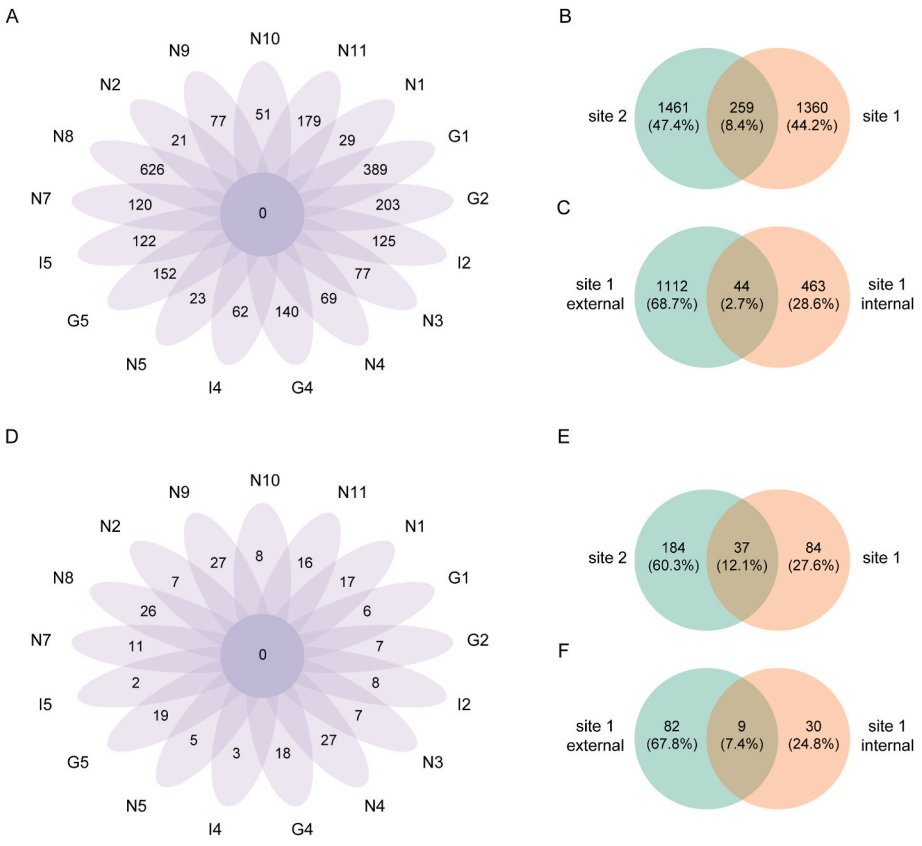

Fig. 5. Petal plots and Venn diagrams showing differences in ASVs across samples and sites. A) number of exclusive (petals) and shared (center) prokaryotic ASVs across samples; B) number and percentage of exclusive and shared prokaryotic ASVs between site 1 and site 2; C) number and percentage of exclusive and shared prokaryotic ASVs between the external and the internal side of site 1; D) number of exclusive (petals) and shared (center) eukaryotic ASVs across samples; E) number and percentage of exclusive and shared eukaryotic ASVs between site 1 and site 2; F) number and percentage of exclusive and shared eukaryotic ASVs between the external and the internal side of site 1.

techniques, the analysis of environmental biofilms through DNA metabarcoding provides more detailed information of taxonomic identity and relative abundance of microorganisms. We first described the microbial community of the two nymphaea and then searched for differentially abundant taxa between the two sites and within site 1 (external and internal side).

4.1. General overview of prokaryotic and eukaryotic taxa

Our analyses indicate that the two nymphaea (here indicated as site 1 and site 2) harbor different microbial communities. In particular, roughly the 45% of total prokaryotic community was exclusive of one of the two sites, and only the ~8% was shared. Furthermore, the internal side of site 1, which included samples collected on frescoes, shared ~2% of bacterial community with the external side. Despite a similar proportion of exclusive prokaryotic taxa between site 1 and 2, the former contains exclusive ASVs distributed across a bigger number of taxonomic categories (32) than the other (12), which may be due to structural complexity of site 1 in respect to site 2. For instance, the external side of site 1, which is similar to ground and colonized by plants, contains, among the others, bacterial taxa commonly found in soils as Rhizobiales, Burkhorderiales, and Frankiales (Valseth et al., 2017) which are not present in the internal side of site 1 and in the site 2. Cyanobacteria were the dominant phylum in almost all samples regardless the provenience, followed by Actinobacteriota and Alphaproteobacteria, which, on the contrary, occurred across samples in similar proportions or one dominated over the other. Cyanobacteria are well known colonizers of ancient and modern stone monuments, where they play an important role in the biodeterioration of these substrata (e.g., reviewed in Crispim and Gaylarde, 2005). Because of their capability of using low-light levels for photosynthesis (Gisriel et al., 2020) and of resisting to adverse conditions (especially high UV levels) thanks to a thick outer envelope, cyanobacteria are found in a large variety of environments. These high adaptability and low light requirements could explain their occurrence in internal side of site 1 that has been excavated in the last year and was covered of ground and debris for most of its extension. On the contrary, at the external portion of site 1 (samples G), where environmental conditions mimic an open ground and the substratum is made of brick, cyanobacteria compete, in terms of relative abundance, with Actinobacteriota and Alphaproteobacteria. These two phyla have been found to be the dominant component in many cultural heritage substrates as plaster, stone and limestone (Saarela et al., 2004; Coelho et al., 2021; Saridaki et al., 2022). Among prokaryotic taxa occurring at lower abundances, Planctomycetota are worthy of mention because they have been recently found to be important colonizers of concrete exposed to seawater (Karačić et al., 2022) but also, despite in minor quantities, in basalt sculptures close to the coast in the Leizhou Peninsula (Wang and Liu, 2021). Planctomycetota are ubiquitous bacteria but they are especially abundant in marine bacterial communities, often associated to macroalgae and sponges (Wiegand et al., 2018), as well as in soils (Xie et al., 2023). In our study, this bacterial phylum was present in many samples from site 2, (the one close to brackish water) but also in most of external samples of site 1 and not in the internal side of the same site. Chloroflexi, a phylum of highly heterogeneous bacteria including aerobic thermophiles, anoxygenic phototrophs, and anaerobic halorespirers, where present in almost all samples with relative abundance between 3 and 10%. These bacteria are commonly found in biofilms of stone monuments (Zhang et al., 2018; Dias et al., 2021).

We find that most of dominant bacteria colonizing our sites are extremophiles. For instance, we report the occurrence of: *Elioraea tepidiphila*, a slightly thermophile member of Alphaproteobacteria discovered into a hot spring in the in the Azores (Albuquerque et al., 2008), in all I samples and *Roseococcus suduntuyensis*, an aerobic bacteriochlorophyll A-containing bacterium isolated from a low-mineralized soda lake of Eastern Siberia (Boldareva et al., 2009) in samples N1–N4. Other extremophiles, not assigned at specific level, include bacteria of the

genera *Crossiella*, known to be involved in $CaCO_3$ precipitation and white crust formation in stone monuments (Li et al., 2018; Coelho et al., 2021). Some of these taxa were also biomarkers for specific sites, as *Crossiella* for site 1 and *Elioraea* and *Truepera* for site 2. In addition, we found bacteria known to degrade chemicals and by-products of industry activities as: *Parvibaculum lavamentivorans* (sample N8), a heterotrophic bacterium isolated from activated sludge in Germany capable of degrading commonly used surfectants as alkanesulfonates (Schleheck et al., 2004); *Porphyrobacter sanguineus* (sample N8), an aerobic bacteriochlorophyll-containing bacterium capable of degrading biphenyl and dibenzofuran (Hiraishi et al., 2002); and *Sphingopyxis chilensis* (samples I2, N1, and N2), a species able to degrade 2,4,6-trichlorophenol (Aranda et al., 2003).

Regarding eukaryotes, fungi were found as biomarkers for site 1 in respect to site 2. For instance, Ascomycota were almost exclusive to samples collected outside site 1 (G samples); in all the other samples they were absent or in low percentages (e.g., I5, N2, and N10). Other fungi as Cryptomycota were found only on biofilms on the internal side of site 1 (samples I). The low presence of fungi in all the samples except G can be likely explained with the paucity of organic matter of the substrates (mostly plasters and Pozzolanic mortars). Green microalgae of the genus *Jenufa* constitute almost the totality of eukaryotic microorganisms in samples I (internal side of site 1), which is characterized by high humidity levels. Indeed, they are reported in literature as common colonizers of archaeological sites and stone monuments exposed to water or high humidity (Hallmann et al., 2013; Zammit, 2019) and where organic matter is low (Jurado et al., 2022). This habitat preference is probably related to their ability of growing and secreting exopolymers for extended periods with minimal input of nutrients from outside sources (Zammit and Agius, 2022). The scarcity of fungi and the abundance of generalist autotrophs as *Jenufa* on these frescoes is probably the consequence of paucity of organic matter in this substratum. Another widely distributed microalga in our samples was *Pseudopleurococcus printzii*, a common colonizer of frescoes, hypogean environments, and stone monuments (Caiola et al., 1987; Albertano, 1993; Giaccone and Di Martino, 1999). Uncultured eukaryotes were a dominant component in many samples and principal biomarkers of site 2 in respect to site 1. Their considerable number suggests a lack of molecular reference libraries for this component and the need of dedicated studies on micro-eukaryotic taxa and their functional role in archaeological sites and stone monuments deterioration.

We did not find relevant differences in alpha- and beta-diversity estimates of both prokaryotic and eukaryotic communities in respect to the composition of the substratum. A slight signal was only detected for prokaryotic taxa colonizing bricks and intonaco/intonachino in terms of Shannon index. Alpha-diversity measured with this index was highest for samples from bricks, which can be considered as a bare stone, and lowest for artificial materials as plasters. Nonetheless, the sampling strategy of this study was not aimed at detecting such differences (e.g., the number of samples for each substratum is not homogeneous) but at prioritizing biofilms colonizing the two sites for restoration purposes; therefore, this may have hampered the detection of such signal. However, some studies have shown that the Mediterranean climate, with its months of high temperature and dry conditions, exerts a strong selective pressure on microorganisms, favouring extremophiles organisms adapted to desiccation and UV radiation regardless other environmental conditions (Ragon et al., 2012; Chimienti et al., 2016). Furthermore, other studies have demonstrated that even at short distances, each sampling spot may form an insulated niche with its own microbial community shaped by a plethora of environmental conditions instead of the simple mineralogical composition (De Wit and Bouvier, 2006; Scheerer et al., 2009; Chimienti et al., 2016).

4.2. Influence of brackish water on microbial communities

An interesting factor distinguishing the communities of site 1 and site 2 is the presence of brackish underground water in the latter site, which is responsible for the occurrence of several taxa in the site 2 typically of the marine/brackish environment. The biodeterioration caused by the action of marine organisms colonizing underwater cultural heritage (biofouling) has been investigated in different sites around the world (Bastida et al., 2004; Hughes et al., 2013; López Garrido et al., 2015), and also in the area of Baia. Indeed, a notable part of the ancient Roman city and its harbour lie on the sea bed and constitutes the site known as "Baia sommersa". A mid-term experiment, conducted in this area to assess the pace and rate of biological activity on submerged calcareous substrates, identified about 50 animal and algal taxa like polychaetes, molluscs, crustaceans, red algae and a few green and brown algae (Casoli et al., 2015). However, the putative occurrence and influence of marine taxa on archaeological sites or cultural heritage in general that lie in proximity of coastal waters has not been investigated in detail. In our study, the investigated areas are located around 120 m from the sea (harbour of Baia). As expected, we did not observe the same composition of taxa reported in the above-mentioned studies because our sites were not submerged; however, we found that the presence of infiltrations of underground brackish water in site 2 affect the microbial profile that colonizes this environment. This regards not only the samples closest to the water pond (e.g., N8 and N9), where saline formations are evident on the stone surface, but also the ones more distant. We found several eukaryotic taxa typical of saline waters encompassing green algae, diatoms, bivalves, and copepods. Some of these taxa have been reported as colonizers or biodeteriogens of cultural heritage, while others have not. Among dominant green algae in site 2, we found the filamentous alga *Ctenocladus circinnatus*, typical of aquatic and saline environments, but also found in archaeological sites close to the sea (Ariño and Saiz-Jimenez, 1996) and the spherical one *Picocystis salinarum*, to date not reported in cultural heritage studies.

Interestingly, sample N4, apparently distant from water and characterized by an amber patina, was the only one in which dinoflagellates, bivalves and copepods were found. For this sample we also report the occurrence of diatoms of the genera *Coscinodiscus* and *Thalassiosira*. The genus *Thalassiosira* includes mostly marine species (c. 100) but also few freshwater species (Hasle, 1978), while only marine species are currently known in *Coscinodiscus*. Taxonomic assignation for these taxa was limited to the genus level; therefore, we could not assess if these taxa are really marine or freshwater, but it is worth to note that diatoms of these genera have not been found in other samples from this site or in site 1, strengthening the hypothesis of their marine origin. Other diatoms common in marine (*Nitzschia*) or freshwaters (e.g., Sellaphoraceae) were observed in the sample N9, which was close to brackish water. Among metazoans, we report, in particular, the occurrence of *Centropages typicus*, one of the most abundant and best studied calanoid copepods in the Mediterranean Sea and North Atlantic Ocean (Carlotti and Harris, 2007). This small arthropod could reach the emerged part of the nymphaeum during occasional rise of water level in the pond and is likely to be an occasional visitor of such environment, despite it has the ability of producing resting eggs to survive harsh environmental conditions (Uye et al., 1979; Carlotti, 2001). Nevertheless, a possible role of marine spray in the colonization of marine taxa cannot be excluded.

Beside marine eukaryotic taxa, we also found several species of bacteria previously reported from marine (especially thermal vents) environment. For instance, *Glycocaulis abyssi*, a bacterium isolated from a deep-sea hydrothermal vent near the Vancouver Island in Canada (Abraham et al., 2013) was particularly abundant not only in samples at direct contact with water (e.g., N8), but also in samples that are not, both in site 2 and in site 1 (I4). This finding is not surprising at all because several salt incrustations have been found in both site 2 and the back-wall of site 1 (which is not in direct contact with water); these formations could be the result of infiltrations of the brackish water across rock and could create a saline environment suitable for the survival of this bacterial species. Other examples of marine prokaryotes

regard bacteria of the methylotroph genus *Methyloceanibacter* (sample N8), which play a key role in the global carbon cycle by metabolizing reduced one-carbon compounds that are found in high concentrations in marine waters (Vekeman et al., 2016) and *Luteimonas vadosa* (samples I2, I5, and N8), a microorganism isolated from sediments collected from the Sea of Japan seashore (Romanenko et al., 2013).

5. Conclusions

The advent of high-throughput sequencing techniques has revolutionised our ways of surveying the biodiversity of several environmental matrices. This is also true for biofilms colonizing cultural heritage. The taxonomic characterization of such microbial communities provides an important baseline information that can be used to develop adequate strategies of biofilm removal and restoration of cultural heritage. In this study, we have demonstrated that biofilms collected in two close but structurally dissimilar Roman nymphaea can have different prokaryotic and eukaryotic communities. Furthermore, differences can also be found among samples within the same site, with several taxa acting as biomarkers for each site. No specific patterns were found comparing microbial diversity and substratum composition, but the presence of brackish underground water resulted in the presence of several taxa typical of the marine environment in some samples.

Author contributions

Conceptualization: P.C.; methodology: D.D.L. and R.P.; resources: P.C.; formal analysis: D.D.L., G.T., R.P.; other analyses: G.T.; data curation: D.D.L. and R.P.; resources: P.C.; writing-original draft preparation: D.D.L.; writing, review and editing, D.D.L., G.T., P.C., R.P.; funding acquisition: P.C. All authors have read and agreed to the published version of the manuscript.

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This article does not include any studies of human participants or animals by the authors of this investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data not published alongside the article are available as Supplementary material. Raw metabarcoding data are available in NCBI under BioProject PRJNA917271.

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Appendix A. Supplementary data

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