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Differential colonization of microbial communities inhabiting Lede stone in the urban and rural environment

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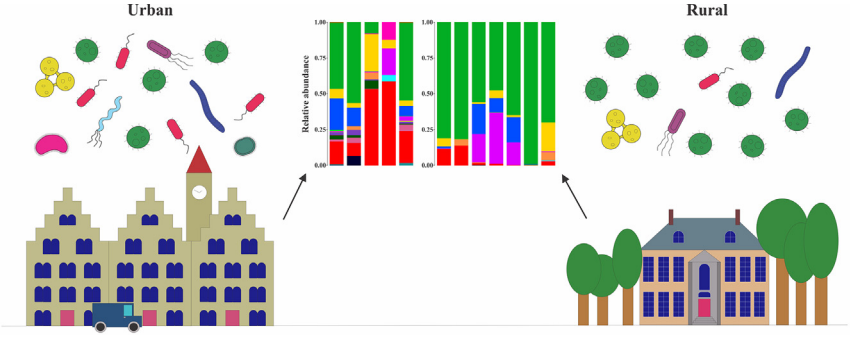
HIGHLIGHTS

- Structural difference in prokaryotes colonizing the urban and rural environment
- Successful 16S rRNA gene sequencing on sandy limestone was achieved.
- Isolation campaign reveals low bacterial acid production.
- Colour measurements reveal potential prokaryotic discolouration of building stones.
- Prokaryotic communities relate to soluble salt content and state of degradation.

GRAPHICAL ABSTRACT



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ABSTRACT

Air pollution is one of the main actors of stone deterioration. It influences not only the material itself but also prokaryotes colonizing rocks. Prokaryotes can affect rock substrates and biological colonization will most likely become relatively more important during the course of the 21st century. Therefore, it is necessary to understand the effects of air pollution on biological colonization and on the impact of this colonization on rock weathering. For this reason, we studied the prokaryotic community of Lede stone from two deteriorated monuments in Belgium: one in the urban and one in the rural environment. This research conducts 16S rRNA gene Next Generation Sequencing combined with an isolation campaign. It revealed diverse and complex prokaryotic communities with more specialized bacteria present in the urban environment, while archaea were barely detected. Some genera could cause biodeterioration but the isolates did not produce a significant amount of acid. Soluble salts analysis revealed an important effect of salts on the prokaryotic community. Colour measurements at least indicate that a main effect of prokaryotes might be on the aesthetics: In the countryside prokaryotic communities seemed to discolour Lede stone, while pollution most likely blackened building stones in the urban environment.

1. Introduction

Natural stones are essential parts of our built heritage. They are a fundamental component of our cultural identity and history that should be preserved in the best condition. Even though stone is expected to be

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durable and permanent, it slowly deteriorates through its interaction with the environment, through physical, chemical and biological action (Siegesmund et al., 2002). This results in erosional and depositional alteration of the material, such as crust formation, one of the prominent forms of deterioration of carbonate rocks. They occur as sulphate encrustations, prominently composed of gypsum crystals with small percentages of airborne dust and particulate matter, often giving it a black appearance (Camuffo et al., 1983). A strong correlation exists between this black crust formation and air pollution (Bonazza et al., 2007). Other than gypsum crust formation, microbial colonization significantly alters the rocks (Miller et al., 2012) for which prokaryotes take main credit because of their versatile metabolism (Gorbushina, 2007; Warscheid and Braams, 2000). As air quality improves along with the evident effects of climate change, microbial growth will presumable only become more important (Viles, 2012).

Prior research on prokaryotes colonizing heritage stone, focused on the deteriorating effects of specific groups. Autotrophic prokaryotes have been considered as the first colonizers of natural building stones (Crispim and Gaylarde, 2005). Cyanobacteria cause aesthetic deterioration, physical damage and potential dissolution by organic acids and chelating agents (Crispim and Gaylarde, 2005; Macedo et al., 2009). Sulphur and nitrogen oxidizing prokaryotes produce corrosive sulphuric and nitric acid (Mansch and Bock, 1998; Sand and Bock, 1991; Warscheid and Braams, 2000). Heterotrophic bacteria affect the stones by producing organic acids and pigments (Warscheid and Braams, 2000). Halophilic microorganisms furthermore are known to cause rosy discolouration, also on mural paintings (Piñar et al., 2014). Besides these negative effects, it is crucial to understand that bacteria and other microorganisms can protect rocks. Biofilms can help stabilize the surface, prevent further deterioration by shielding it from erosion or even by binding the rock surface (Gadd, 2017; Llop et al., 2013). They can absorb aggressive chemicals, protect the material from wind and wind-blown particles or keep the temperature and moisture of the surface more constant (Carter and Viles, 2005). Removal of such protecting biofilms can accelerate deterioration (de la Rosa et al., 2013). Some bacteria are actively used to remediate construction materials with microbial induced precipitation (De Muynck et al., 2010; Jimenez-Lopez et al., 2008; Montaño-Salazar et al., 2018).

Individual groups of prokaryotes can affect natural building stones but they are part of a complex community. It is still uncertain how these communities relate to the environment and how they affect natural building stones and degradation phenomena such as crust formation or discolouration. Air quality is one of the major parameters that strongly affects gypsum crust formation but also colonization. For example, the surface deposition of organic compounds as a result of incomplete combustion can sustain heterotrophic bacteria before initial autotrophic growth (Saiz-Jimenez, 1993; Zanardini et al., 2000).

The first step to understand the relationship of prokaryotic communities with the environment is to identify prokaryotes present in the stone. Prior research has used culture-dependent techniques and revealed a higher occurrence of nitrogen (Mansch and Bock, 1998) and sulphur oxidizers (Flores et al., 1997; Mitchell and Gu, 2000) in polluted, urban areas, just as the occurrence of hydrocarbon degraders (Mitchell and Gu, 2000; Ortega-Calvo and Saiz-Jimenez, 2006). Mitchell and Gu (2000) arguments that air pollution would harm microbial diversity. Culturing dependent techniques (growth) are necessary to understand the role of a specific species and to assess their effect on natural building stones. However, as most prokaryotes are non-culturable, they fall short to capture full microbial diversity (Overmann et al., 2017; Rappé and Giovannoni, 2003; Stewart, 2012). More recently culture-independent techniques can identify also non-culturable microorganisms. Initially, the most common techniques in conservation sciences included genetic fingerprinting, isolating nucleic acids and phylogenetic analysis (Dakal and Arora, 2012; Otlewska et al., 2014). Villa et al. (2015) for example, found specific functional genes for sulphur metabolism on gravestones in polluted urban areas. However, new techniques such as metagenomics and Next-Generation Sequencing (NGS) revolutionized our views on microbial ecology. This technology enables high-throughput sequencing, allowing in-depth studies of environmental samples (Marvasi et al., 2019). It described microbial communities on monuments across the world such as in China (Li et al., 2018, 2017, 2016), Cambodia (Zhang et al., 2018), Brazil (Gaylarde et al., 2017), but also in Poland (Adamiak et al., 2018; Dyda et al., 2018; Gutarowska et al., 2015) and Italy (Chimienti et al., 2016). Brewer and Fierer (2018) used it on limestone and granitic tombstones across three continents.

Within this research, 16S rRNA gene NGS was applied on the gypsum crust of deteriorated Lede stone in urban and rural environments. Lede stone is one of the most important natural and historical building stones in northwestern Belgium, used across Belgium and the Netherlands. This "Global Heritage Stone Resource" (De Kock et al., 2015) dominates the cityscape of historical cities as Ghent, Bruges and many more. 16S rRNA gene NGS should allow describing those prokaryotic communities in detail and will result in more insight into the relationship between urban/rural environment and those communities. 16S rRNA gene NGS can also give information about the function of the prokaryotes, although it is important to keep in mind that not all prokaryotes belonging to a certain group share the same functionality. Besides, it will provide us with a better understanding of the effect of air quality on the populations. It can show how those communities might change when air quality keeps improving in our cities. This work also incorporates an isolation campaign in an attempt to understand their potential role in stone deterioration by acid production. At least it includes colour measurements and soluble salt analysis to detect potential aesthetic changes on the sampled material and to reveal the effect of the crust on the prokaryotic population.

2. Materials and methods

2.1. Sampling

Samples were collected at the beginning of April 2019 from two monuments in northwestern Belgium: The City Hall of Ghent (51°03′16.3″N 3°43′31.0″E) and the Castle of Berlare (51°01′26.3″N 4°00′09.7″E). The current City Hall of Ghent dates back to the 15th Century, but the samples were retrieved from a part built during the 17th and 18th Century (Cnudde et al., 2009). The Castle of Berlare was built during the 18th Century (Agentschap Onroerend Erfgoed, 2020). The City Hall of Ghent lies within an urban environment, while the Castle of Berlare is located within a park with ponds in a small village. Fig. 1 illustrates both locations in Belgium with the imperviousness. According to The Royal Meteorological Institute (RMI), the climate is mild with an average temperature in Berlare of 10.6 °C, ranging from average 3.4 °C in January until 18.3 °C in July. The annual precipitation is about 830 mm, evenly spread along the year. Climate statistics of Ghent are almost identical but recently the data retrieved by the MOCCA project (Caluwaerts et al., 2016) revealed that the city centre of Ghent is significantly warmer compared to its countryside. Between 01/07/2016 and 30/06/2017 an average temperature of 12.6 °C has been measured at St. Bavo in Ghent (about 200 m from the City Hall) compared to 11.5 °C in the rural Melle (Guilbert et al., 2019). The City Hall of Ghent is furthermore exposed to significant air pollution compared to Berlare. This includes higher concentrations of NO_x, fine particles, black carbon, CO and slightly higher SO_2. The air in Berlare is cleaner but contains higher concentrations of ammonia and ozone. This can be explained by the rural character of the village with more agriculture resulting in higher ammonia emissions and less traffic inducing lower NO concentrations that degrade ozone (Vlaamse Milieumaatschappij, 2019). Overall air quality improved significantly over time: SO_2 concentrations decreased with about 90% since the 80ties. This resulted in minor differences between two locations with current average values below 4 $\mu g/m^3$. NO_x emissions decreased as well but overall a larger difference remains

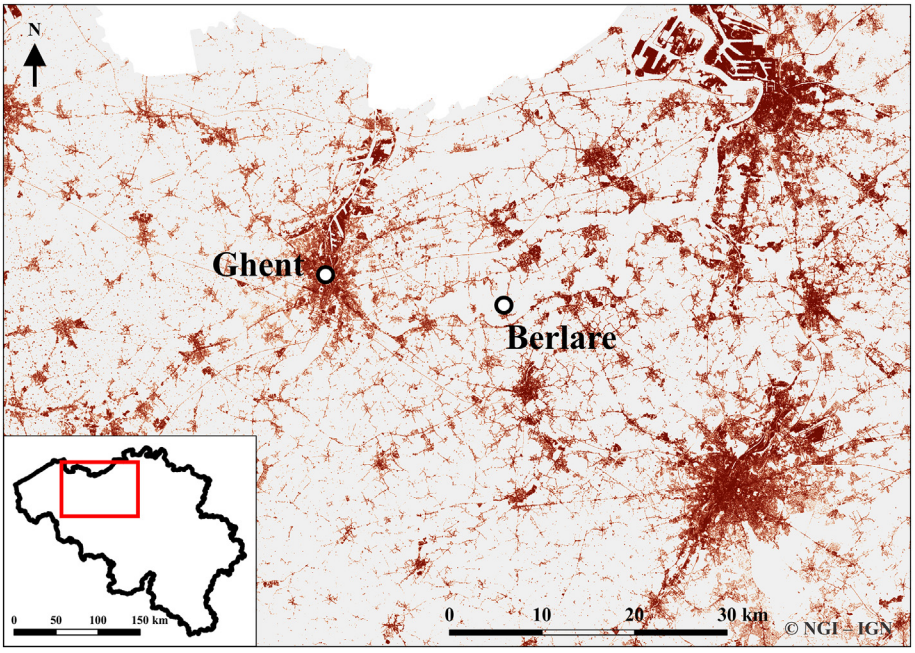

Fig. 1. Sampling locations plotted on a modified map derived from the CORINE High Resolution Layers-Belgium 2015 dataset, showing the imperviousness in red. It illustrates the highly urbanized area of Ghent compared to the rural Berlare.

between the urban (±45 μg/m^3) and rural (±20 μg/m^3) environment. These values are highly variable depending on the local traffic (Vlaamse Milieumaatschappij, 2019).

At both locations, the outer crusts of deteriorated Lede stone have been sampled, mainly around window frames and corners. It is a sandy limestone from the Eocene Lede Formation which contains glauconite (De Kock et al., 2015). The iron from those glauconite grains can oxidize causing discolouration (De Kock et al., 2017). For NGS about 0.5 g material has been collected using a small drill with flame-sterilized head, powdering the crust and the upper part of the Lede stone until a depth of about 8 mm. A flame sterilized chisel scrapped additional loose crust material (±10–±25 g) around every drill hole to perform the isolation campaign and to measure the soluble salt content. Respectively six (G1–G6) samples from Ghent and seven (B1–B7) from Berlare have been retrieved. They originated from walls facing several orientations: G1 until G4 (SSW); G5 and G6 (SEE); B1, B3 and B7 (S); B2 (E), B4, B5 (W) and B6 (N).

2.2. Material characterization: Colour and soluble salt content

A sterilized mortar and pestle powdered and homogenized the samples. Konica Minolta CM-600d spectrophotometer determined its colours. Furthermore, the soluble salt concentration of 4 g from each sample was acquired after 1:5 dilution in Milli-Q water. The samples were shaken for two hours, after which everything settled down for the next two hours. From the solution, the pH was determined with a consort C3020 pH electrode according to Greenberg et al. (1992). A 930 Compact Ion Chromatograph (IC) (Metrohm, Switzerland) equipped with conductivity detector determined the soluble anions: Cl^-, NO_2^-, NO_3^-, PO_4^{3-} and SO_4^{2-} and the cations: Na^+, NH_4^+, Ca^{2+}, K^+ and Mg^{2+}.

2.3. Isolation, cultivation and identification

0.25 g of the powdered stone material from Ghent (sample G1) and Berlare (sample B1) was added to freshwater basal mineral medium (per litre: 1.0 g NaCl, 0.4 g $MgCl_2.6H_2O$, 0.15 g $CaCl_2.2H_2O$, 0.2 g KH_2PO_4, 0.5 g KCl and 0.25 g NH_4Cl) and vortexed for three minutes to detach the cells. The bacteria were isolated after a serial dilution on two types of growth media: R2A agar for heterotrophic bacteria (per litre): 0.5 g Protease peptone, 0.5 g casamino acids, 0.5 g yeast extract, 0.5 g glucose, 0.5 g soluble starch, 0.3 g K_2HPO_4, 0.05 g $MgSO_4.7H_2O$, 0.3 g $C_3H_3NaO_3$ and 15 g agar and thiosulphate plates for sulphur oxidizers (per litre): 980 mL freshwater basal mineral medium, 9.7 g Na_2SO_4, 6 g $Na_2S_2O_3$, 0.02 g bromothymol blue, 10 mL 1 M $NaHCO_3$, 10 mL 1 M MOPS (pH 7.2), 1 mL 7-vitamin solution, 1 mL SL-10 trace element solution and 10 g agar. The plates were incubated until six weeks at room temperature both aerobic and anaerobic (with 2 g/L $NaNO_3$ added to the media). At different moments, colonies were transferred and isolated in triplicate based on their appearance to capture the highest diversity.

To identify the isolates, DNA was extracted by phenol/chloroform after bead beating with a PowerLyzer instrument (Qiagen, Venlo, Netherlands). 27F and 1492R LGC Primers amplified the 16S rRNA gene after which the obtained PCR products were purified using the innuPREP PCRpure Kit (Analytik Jena, Jena, Germany). These were sent to LGC Genomics (LGC Genomics GMbH, Berlin, Germany) to analyse with Sanger. The resulting sequences were identified using the National Center for Biotechnology Information (NCBI) BLAST and the Ribosomal Database Project (RDP). All sequences have been submitted to GenBank (Accession numbers: MT397137-MT397271).

2.4. 16S rRNA gene sequencing

From all samples, DNA was extracted out of 0.25 g drill powder by the DNeasy PowerSoil Kit (Qiagen, Venlo, Netherlands), according to the manufacturer's instructions. 20 μl genomic DNA extract was send out to LGC Genomics for sequencing on an Illumina MiSeq platform and library preparation. For bacterial 16S rRNA genes, it followed the same procedure as De Paepe et al. (2017) except using 35 PCR cycles. The analysis included two blanks retrieved after DNA extraction and sequencing without inoculum, following the same procedure. Furthermore, three samples of each location (G1, G4, G6; B1, B4, B6) were selected to amplify archaeal 16S rRNA genes. Nested PCR was used with 340F 1000R for the first run and U341F U806R for the second run (De Vrieze et al., 2018). Read assembly and clean-up was largely derived from the MiSeq SOP described by the Schloss lab (Kozich et al., 2013; Schloss et al., 2011) with Mothur (v.1.42.0) (De Paepe et al., 2017).

The sequence data has been submitted to the Sequence Read Archive (SRA) of the NCBI database (Accession numbers: SRR11277871 - SRR11277891) under BioProject number PRJNA611556.

The data were analysed in R using the Vegan (Oksanen et al., 2019) and Phyloseq (McMurdie and Holmes, 2013) package. The data was normalized by rarefaction (lowest sequence depth = 11894). Rarefication curves have been acquired. The alpha diversity has been identified after 1000 times bootstrapping the rarefied data, with the Richness, Inverse Simpson, Shannon and Chao 1 index. Beta-diversity has been established using Bray-Curtis index and plotted with PCoA. A PERMANOVA test determined the similarity between the two locations.

2.5. Acid production of the isolates

Every different species of the isolates (according to NCBI or RDP) has been tested for its acid production with biological triplicates. The isolates grew on R2A plates, after which a colony was inoculated R2A broth. After three days incubation, 100 μL was transferred to fresh 10 mL R2A broth. Depending on its requirements it grew at 20 or 28 °C and after 48 h: the pH of the broth was measured with a consort C3020 pH electrode according to Greenberg et al. (1992), together with the C2-C8 volatile fatty acids (VFA) according to Andersen et al. (2014).

3. Results

Both buildings contained gypsum crusts: these were thick botryoidal with black colour in the City Hall of Ghent, while in Berlare these were thinner, laminar with a rusty colour. The soluble salt content between the rock samples varied and is presented in Table 1. The concentrations were overall higher on the City Hall of Ghent with mean values of about 17 mg soluble salts per gram rock compared to 7 mg in Berlare. SO_4^{2-} was the most abundant soluble anion (1.2–10 mg/g_{rock}) and Ca^{2+} (0.5-–5 mg/g_{rock}) the most abundant cation. Cl^- and NO_3^- were also detected and the concentration in G2 and especially G4 from Ghent was high where it reached 5.3 mg Cl^-/g_{rock} and 12.5 mg NO_3^-/g_{rock}. B6 was the only sample of Berlare with higher soluble Cl^- (0.8 mg/g_{rock}) and NO_3^- (1.2 mg/g_{rock}). The important accompanying cations were Na^+, K^+, Mg^{2+}. Their abundance followed the same trends as the anions with high concentrations of Na^+ in sample G2 and G4. No significant amount of PO_4^{3-}, NO_2^- or NH_4^+ has been detected. The pH of those solutions was slightly basic and similar for the samples of Berlare (pH 7.20–7.45), while they ranged between slightly acidic (pH 6.5) or basic (pH 8.22) in the samples of Ghent.

Not only the soluble salt content differed significantly but also its colour. Fig. 2 shows the spectral reflectance graph of the powdered stone. This spectrum is relatively flat for the samples of Ghent (except G4), while in Berlare there is a steep gradient in reflectance between 400 and 550 nm. This confirms red/rusty colour of the Castle of Berlare but also in sample G4 from Ghent.

Furthermore, bacterial 16S rRNA genes of all samples were successfully sequenced except G1 (omitted for further analysis). The retained samples were distinctive from the blanks and contained at least six times more reads. The rarefaction curves of the bacterial 16S rRNA gene sequencing are included in the Supplementary information Figs. S1 and S2. The nested approach for archaeal 16S rRNA genes only detected archaea in sample G6. 16S rRNA gene NGS detected a diverse and variable bacterial community colonizing the two monuments. Despite this variation, Beta-diversity analysis with PCoA indicated clustering between the different samples based on location (Fig. 3). A PERMANOVA test confirmed this clustering, rejecting the null hypothesis. Establishing a link to the orientation was not possible. The alpha diversity (Table 2) varied, even within one location, but was on average higher in samples of Ghent compared to Berlare. This was especially the case for the Inverse Simpson and the Shannon index.

As shown in the beta-diversity, the composition of the bacterial community differed. Fig. 4 illustrates the distribution on the phylum level: Actinobacteria were dominant in Berlare with 71% of the OTUs, while Proteobacteria represented here 4%. In Ghent, the Actinobacteria were predominant together with the Proteobacteria, representing respectively 33 and 32% of the OTUs. Other abundant phyla belonged to Bacteroidetes, Chloroflexi and Deinococcus-Thermus, while the residual phyla including Cyanobacteria and Firmicutes formed a small part. Only 1.4% percent of the OTUs in Ghent and only 0.1% in Berlare could not be classified until the phylum level.

At the genus level, the main genera in Ghent were *Rubrobacter* (5.3%), *Ellin6055* (5.1%), *Halomonas* (4.6%). *Ellin6055* belongs according to NCBI Blast to the genus *Sphingomonas*. *Rubrobacter* was also the main occurring genus in Berlare but with 23%, followed by *Blastococcus* (11%) and *Truepera* (10%). More detailed information is shown in Fig. 5 showing respectively the composition of the genus level within the phylum Actinobacteria in Berlare (Fig. 5A), Ghent (Fig. 5B) and the Proteobacteria in Ghent (Fig. 5C). Both locations shared several of the most abundant genera within the Actinobacteria (e.g. *Agrococcus, Arthrobacter*, *Blastococcus*, *Cellulomonas*, *Conexibacter*, *Marmoricola*, *Nocardioides*, *Pseudonocardia*, and *Rubrobacter*).

Overall there was some similarity within Actinobacteria (Fig. 5A, B), but there was a lot of variation between the different samples, especially within the genus Proteobacteria (Fig. 5C). As shown in Fig. 4: the samples from Ghent: G2, G3 and G6 were mainly composed out of Actinobacteria (±50%), and contained a significant amount of Chloroflexi, Proteobacteria and Bacteroidetes. G4 and G5 were in particular different. There, Proteobacteria was the most abundant group and lacked Chloroflexi. Actinobacteria occurred slightly in G4 and was

Table 1
Amount Soluble ions (mg) per gram rock material in every sample of Ghent (G) and Berlare (B) and the pH of the solution. PO_4^{3-}, NO_2^- were not detected except in B1 with 0.001 mg/g_{rock} NO_2^- and in B4 with 0.002 mg/g_{rock} PO_4^{3-}. <BDL indicates a measurement below the detection limit.

Sample	Na^+	NH_4^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	NO_3^-	SO_4^{2-}	Total	pH
G1	0.213	0.003	0.163	3.257	0.074	0.351	1.626	6.795	12.481	6.91
G2	0.537	0.005	< BDL	4.088	0.215	1.014	5.404	6.858	18.120	6.50
G3	0.090	0.009	0.216	3.010	0.031	0.103	0.261	7.337	11.057	7.20
G4	4.988	0.004	1.380	5.025	1.153	5.348	12.537	9.651	40.088	8.22
G5	0.064	0.006	0.064	2.955	0.016	0.112	0.238	6.948	10.404	7.75
G6	0.040	0.002	0.024	3.002	0.006	0.087	0.306	6.884	10.351	7.29
Mean (G)	0.989	0.005	0.369	3.556	0.249	1.169	3.395	7.412	17.084	7.31
B1	0.004	0.004	0.018	1.924	< BDL	0.004	0.006	4.000	5.959	7.20
B2	0.097	0.007	0.069	3.038	0.044	0.207	0.129	7.156	10.747	7.25
B3	0.007	0.001	0.058	2.196	0.003	0.005	0.004	4.952	7.225	7.24
B4	0.005	0.002	0.031	0.579	0.003	0.004	0.003	1.261	1.889	7.45
B5	0.010	0.001	0.061	2.038	0.006	0.005	0.005	4.632	6.758	7.35
B6	0.265	0.005	0.181	3.332	0.123	0.801	1.150	7.221	13.078	7.34
B7	0.044	0.004	0.036	1.270	0.005	0.008	0.005	2.851	4.222	7.29
Mean (B)	0.062	0.003	0.065	2.054	0.031	0.148	0.186	4.582	7.126	7.30

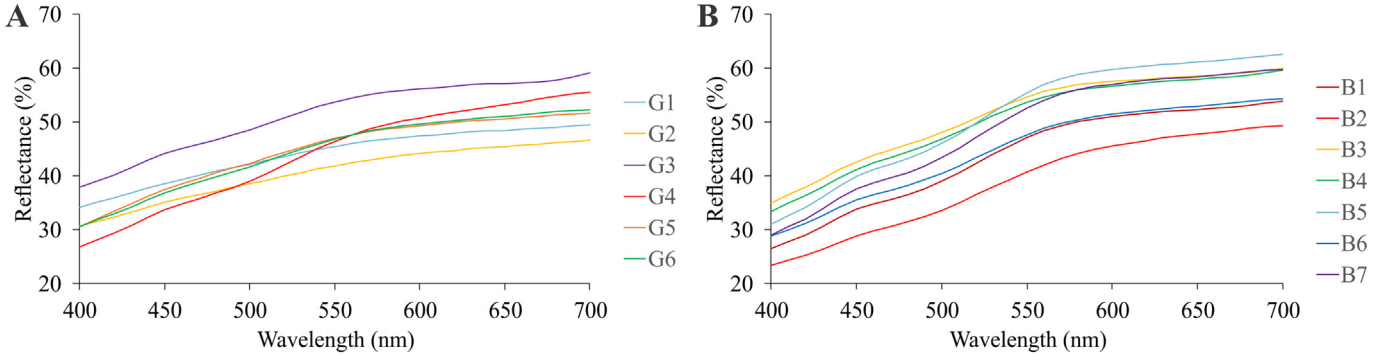

Fig. 2. Spectral reflectance graph of the samples from Ghent (A) and Berlare (B). Steeper gradient between 400 and 550 nm in G4 and the samples of Berlare confirm red discolouration.

completely absent in G5. G4 contained furthermore an important fraction of Bacteroidetes (26%). The most abundant genera were *Halomonas* (23%) and *Salinimicrobium* (15%). G5 contained a high abundance of *Fusobacterium* (6%), *Rhodoplanes* (16%), *Geoalkalibacter* (4%), *Acidibacter* (6%), *Salinimicrobium* (6%), Armatimonadetes (12%) and *Deinococcus* (18%).

In Berlare samples B3, B4 and B5 were very similar with Actinobacteria as leading phylum and a significant contribution of Deionococcus-Thermus and Chloroflexi (Fig. 4). Actinobacteria dominated samples B1, B2, B6 and B7. B1 and B2 contained relatively more Proteobacteria and B2 consisted of 73% out of *Rubrobacter* as shown in Fig. 5A. Sample B6 was almost completely composed out of Actinobacteria and especially an unclassified genus of *Propionibacteriaceae* (42%) and *Crossiella* (39%) (Fig. 5A). B7 had a relatively high amount of Bacteroidetes (especially *Pontibacter* (13%)) and Firmicutes (Fig. 4).

Apart from the dominating heterotrophic bacteria some genera containing lithoautrophic prokaryotes have been detected, mostly in a low abundance (<0.1%, except stated otherwise). They belong to ammonia oxidizing bacteria (AOB) or archaea (AOA), nitrite-oxidizing bacteria (NOB) but also to sulphur oxidizers and reducers. AOB have been found in Ghent and Berlare belonging to the family *Nitrosomonadaceae* with *Nitrosospira* (G4, G6 (0.7%), B1) and *Nitrosomonas* (G6). AOA, on the other hand, have only been detected in Ghent (G6) and belong to *Nitrososphaeraceae*. NOB at least were present in Ghent (G2) namely: *Nitrolancea* and *Nitrospira*. Some genera related to sulphur oxidizers have been retrieved as well, but only to purple non-sulphur bacteria. They were abundant in G5 that contained 16% of *Rhodoplanes*. Furthermore, there might be more present in some samples as the order *Rhodospirillales* was found in G4 and G6 with an unclassified genus of *Magnetospiraceae* (0.2% in G4) and with an unclassified genus of the *Rhodospirillaceae* (0.3% in G4). *Rhodobacteraceae* were represented as well, but again unclassified (0.5% in G2, G4, 0.4% in G6 and B1). Bacteria related to sulphur reduction have been detected at both locations with *Desulfuromonadales* within G3 but especially within G5 with 4% *Geoalkalibacter*, just as *Desulfobacteraceae* (G5, G6, B4, B6), with *Desulfofrigus* (G5, B4) and *Desulforhopalus* (B4).

Besides NGS, the isolation campaign retrieved in total 135 isolates from the two monuments: 48 from Ghent (G1) and 87 from Berlare (B1). They belong to 20 different genera, 14 families and four phyla. The sample of Ghent contained six isolated genera, while Berlare contained 17 genera. NCBI blast and RDP revealed respectively 31 and 30 closely related species. See the supplementary information (Table S1) for the list with the different species. Fig. 6 illustrates for B1 the isolated genera and their relationship with the Illumina 16S rRNA gene amplicon. Seven isolated genera have not been detected by 16S rRNA gene sequencing: *Curtobacterium, Pseudomonas, Psychrobacillus* and *Tardiphaga* (isolated from R2A), *Knoellia* and *Paenibacillus* (isolated

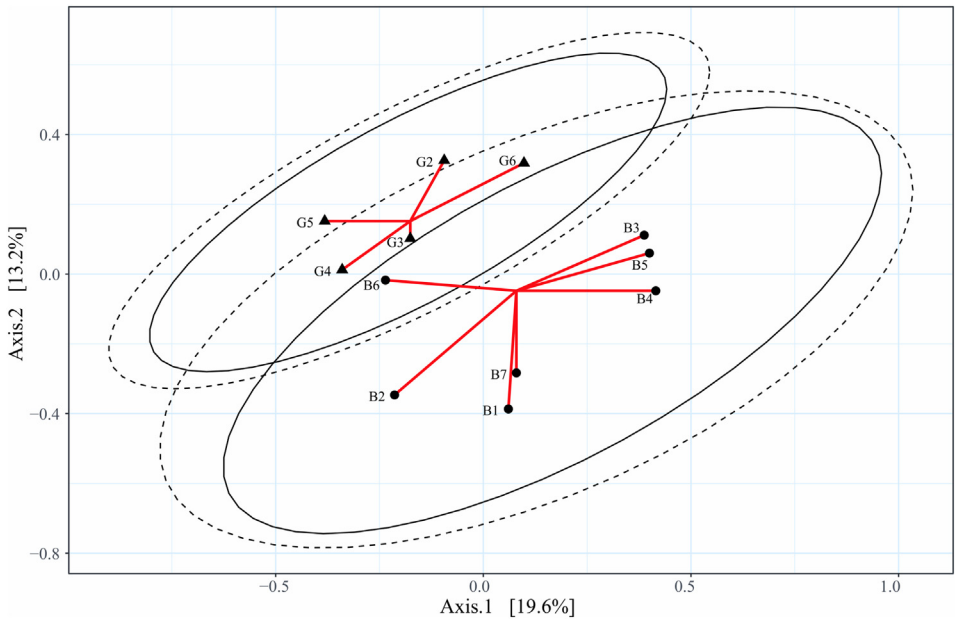

Fig. 3. Beta diversity – PCoA plot showing the variability between the different samples of Ghent (G1 – G6) and Berlare (B1 – B7) with confidential ellipses created based on the location.

Table 2
Alpha diversity indices based on rarefied data. Those values represent the mean value after 1000 times bootstrapping. The relative standard deviation lies between 2.3 and 7.8% for the richness, between 0.9 and 2.4%, for the Inverse Simpson index, between 0.3 and 0.9% for the Shannon index and between 7.6 and 27.4% for the Chao1 index.

Sample	Richness	Inverse Simpson	Shannon	Chao1
G2	291.87	55.26	4.62	495.63
G3	81.99	13.87	2.98	129.61
G4	199.67	20.46	3.77	365.68
G5	58.45	9.11	2.40	80.32
G6	742.09	22.98	4.40	1428.81
B1	320.94	7.33	2.78	1046.65
B2	59.37	3.98	1.94	101.40
B3	129.59	14.28	3.15	257.08
B4	189.28	6.62	2.69	459.83
B5	109.29	9.17	2.78	148.63
B6	105.51	3.19	1.70	234.44
B7	104.73	13.52	3.18	149.10

for thiosulphate agar) and *Microbacterium* (isolated from both media). However, their order/families have been detected and they all contain (except *Pseudomonas*), unidentified genera. Such a correlation was not possible for G1, here; *Arthrobacter* and *Microbacterium* were isolated on both media, *Cellulosimicrobium* by thiosulphate agar and *Bacillus* and *Isoptericola* by R2A. Both locations shared *Arthrobacter*, *Microbacterium* and *Sphingomonas*. These three genera were among the most diverse and abundant isolates. Almost no clear growth occurred on the anaerobic plates with 2 g/L $NaNO_3$ except sample B1, where two *Pseudomonas* colonies grew. Eleven genera belonged to the Actinobacteria, four to the Proteobacteria and Firmicutes and one to the Bacteroidetes. All isolates were capable to grow aerobically, 15 of the 20 genera were Gram positive, four genera were endospore forming. Many isolates, certainly from Ghent contained red/pink (e.g. *Blastococcus*, *Arthrobacter*), orange (e.g. *Arthrobacter*) or yellow (e.g. *Sphingomonas)* pigments. The Colony-forming unit (CFU) was for both samples and both aerobic media in the order of 10^5 per gram crust/rock. Sulphur oxidizers were not isolated.

Fig. 7 illustrates at least the VFA production and the induced pH of the isolates. Overall, they produced little VFA with quantities around the detection limit of the method, which influenced the average values. Acetic acid was mainly produced, occasionally accompanied with isobutyric, isolvaleric and caprionic acid. The pH of R2A remained after 48 h mostly between 6.5 and 7.5, while the blank had a pH of 6.95 (Fig. 7B). Growth of *Isoptericola sp.*, *Cellulosimicrobium* sp. and *Paenibacillus* sp. decreased the pH significantly to respectively 6.14, 5.58 and 5.03.

4. Discussion

The Lede stone of the City Hall of Ghent and the Castle of Berlare both contained crusts. High soluble Ca^{2+} and SO_4^{2-} concentrations in every sample confirmed the gypsum. The overall higher concentrations of soluble salts in Ghent confirm the visual observation of stronger gypsum crust formation in the samples of the urban environment. As nitrate salts are very soluble (Steiger, 2016), the high NO_3^- concentrations in sample G2 and G4 from Ghent indicate a stable gypsum crust, less exposed to rain, compared to the other sampling locations. The variation in soluble salt content emphasises the heterogeneity on the outer surface of one building, even if constructed with the same building stone.

The prokaryotic community revealed by NGS existed mainly out of aerobic chemoorganotrophs, while archaea were barely detected. It included taxa, found on other monuments around the world: such as *Blastococcus, Arthrobacter*, *Rubrobacter*, *Sphingomonas*, Deinococcus-Thermus etc. (Chimienti et al., 2016; Li et al., 2016). This is confirmed by the isolates which are closest related to several species previously isolated from monuments (e.g. *Arthrobacter parietis*, *Arthrobacter tecti*, *Arthrobacter tumbae* (Heyrman, 2005)) or stones and sediment (e.g. *Agrococcus jenensis* (Groth et al., 1996), *Blastococcus aggregatus* (Urzi, 2004), *Nocardioides cavernae* (Han et al., 2017)). For sample B1 of Berlare, the isolates also corresponded to the NGS data as three out of four most abundant OTUs have also been isolated.

Photoautotrophic bacteria, seen as the pioneers in colonization were rarely detected. Cyanobacteria occured in several samples but reach never >1%. This contrasts with the findings of Li et al. (2016) and Zhang et al. (2018) where Cyanobacteria made up an important fraction of the community. This could relate to the sampling material as in this study the prokaryotic communities were determined on and

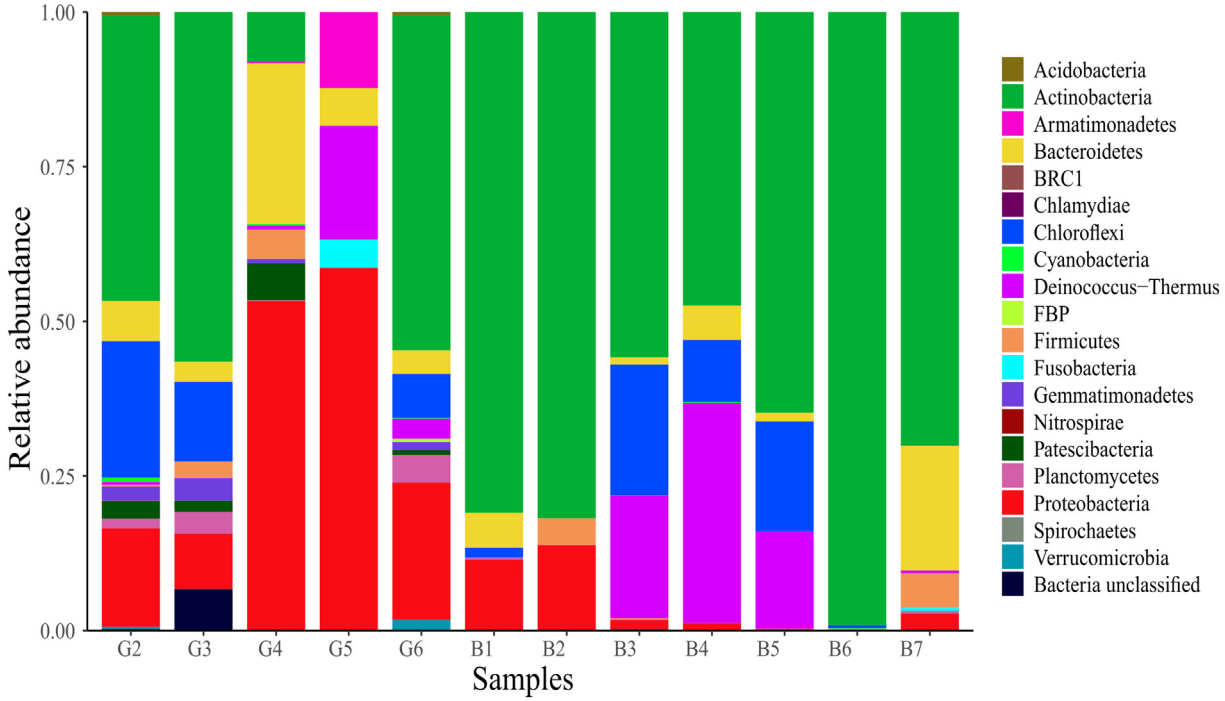

Fig. 4. Distribution bacterial phyla within each sample of Ghent (G2–G6) and Berlare (B1–B7).

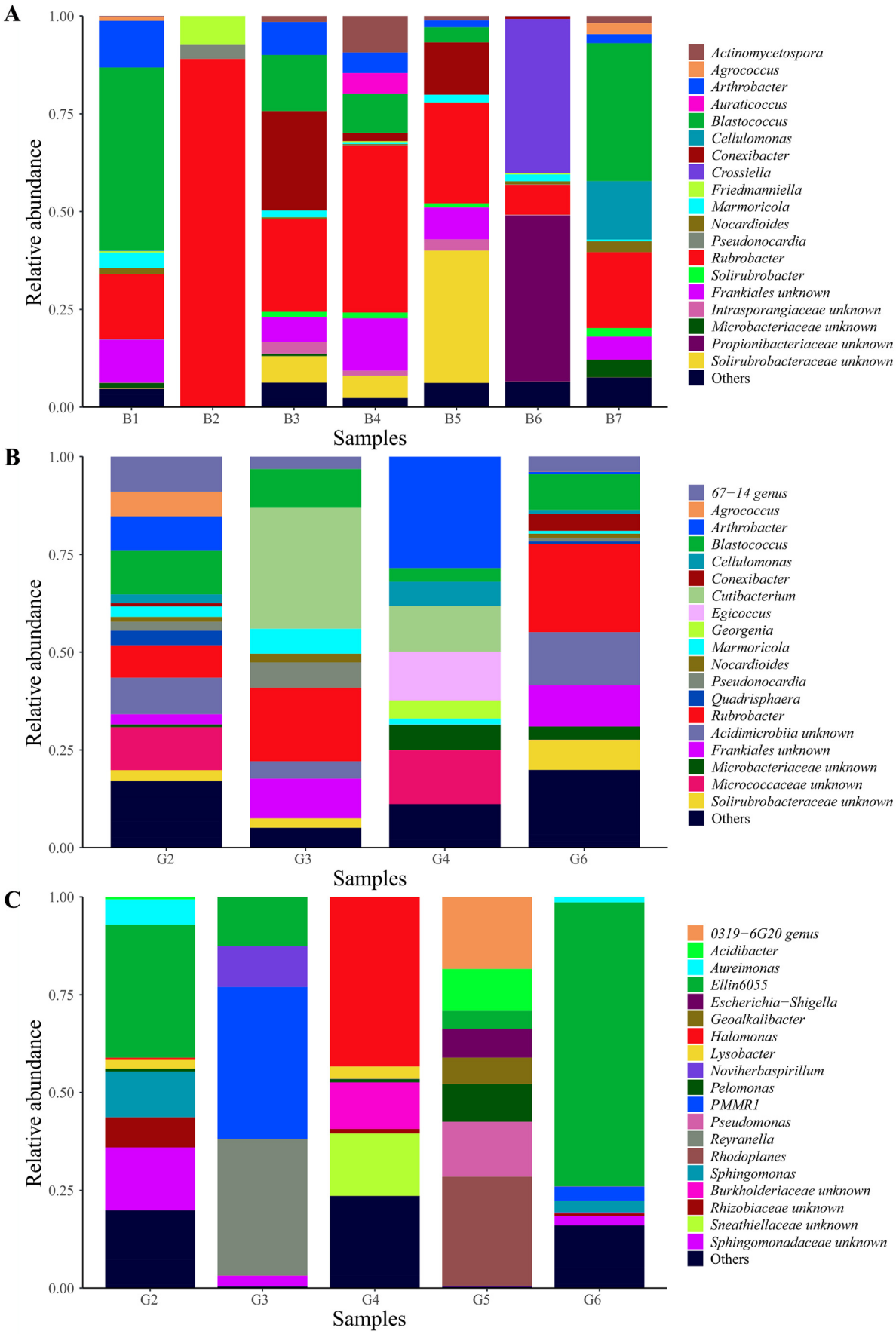

Fig. 5. Distribution genera within the phylum Actinobacteria from A) Berlare (B1 – B7), B) Ghent (G2, G3, G4, G6) and C) Distribution genera within the phylum Proteobacteria from Ghent (G2 – G6). Determination until a certain taxonomy level was not always possible and this has been illustrated with the lowest determined taxonomy level + "unknown".

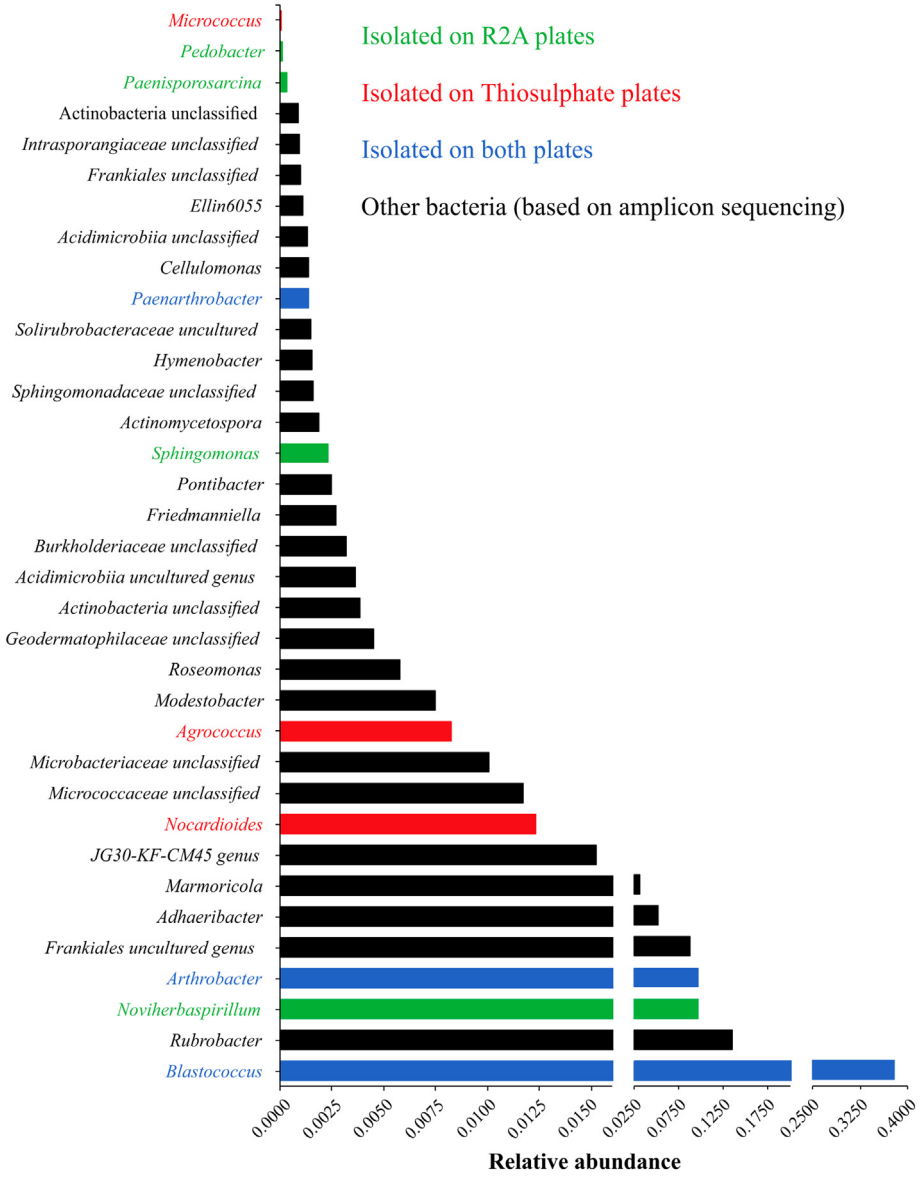

Fig. 6. Results of 16S rRNA gene sequencing of sample B1. The Y-axis shows the main sequenced genera, completed with isolated genera. The X-axis shows the relative abundance. *Pedobacter*, *Paenisporosarcina*, *Sphingomonas* and *Noviherbaspirillum* were isolated on R2A; *Agrococcus* and *Nocardioides* on the thiosulphate plates; *Paenarthrobacter*, *Arthrobacter* and *Blastococcus* by both media.

underneath gypsum crusts. Other possibilities could be the sampling conditions in the early spring, in rather sheltered locations. Chloroflexi was abundant in several samples mainly as Thermomicrobia, but this class is not known to contain photoautotrophs (Gupta et al., 2013). Only sample G5 from Ghent contained a high amount of facultative photoautotrophic *Rhodoplanes* (Hiraishi and Ueda, 1994).

Overall, Actinobacteria were most abundant, especially in the countryside. This phylum is frequently found on natural building stones (e.g. Chimienti et al., 2016; Zanardini et al., 2016). Several members can survive extreme conditions such as desiccation, high pH, etc. (Bull, 2011). Stone surfaces are exposed to solar radiation, low nutrients, extreme temperature and water fluctuations (Gorbushina, 2007). 16S rRNA gene sequencing revealed specific extremophilic genera, including abundant thermophilic bacteria such as Thermomicrobia (Hanada, 2014; Houghton et al., 2015), *Rubrobacter* (Chen et al., 2004) and Deinococcus-Thermus (Albuquerque et al., 2005; Garrity et al., 2001). Furthermore, the latter two are well known for their resistance against γ-radiation. Their occurrence is remarkable as the sampling occurred in a mid-latitude region at the beginning of the spring. It was not possible to link the communities with orientation; however, B6 from Berlare, the only sample facing north, lacked high amounts of thermophilic bacteria. It contained *Rubrobacter*, but with the lowest concentration of any sample in Berlare. Furthermore, the isolates at both locations are closely linked to species related to extreme cold conditions: e.g. *Arthrobacter agilis* (Fong et al., 2001), *Paenisporosarcina indica* (Reddy et al., 2013) and *Psychrobacillus psychrodurans* (Krishnamurthi et al., 2010). Thermophilic bacteria were not identified, mainly due to the isolation conditions at room temperature. This indicates a versatile community. Halophilic bacteria such as *Halomonas* and *Salimicrobium* were present as well, especially in samples with high soluble salt content (G4 in Ghent). Furthermore, drought-resistant groups were abundant with representatives of the three known genera of *Geodermatophilaceae*. *Blastococcus,* especially abundant in the samples of Berlare, also in the isolates, survives in low availability of water and nutrients (Montero-Calasanz et al., 2012). This is also the case for some Proteobacteria such as species of *Sphingomonas* like *Sphingomonas desiccabilis* (Reddy and Garcia-Pichel, 2007).

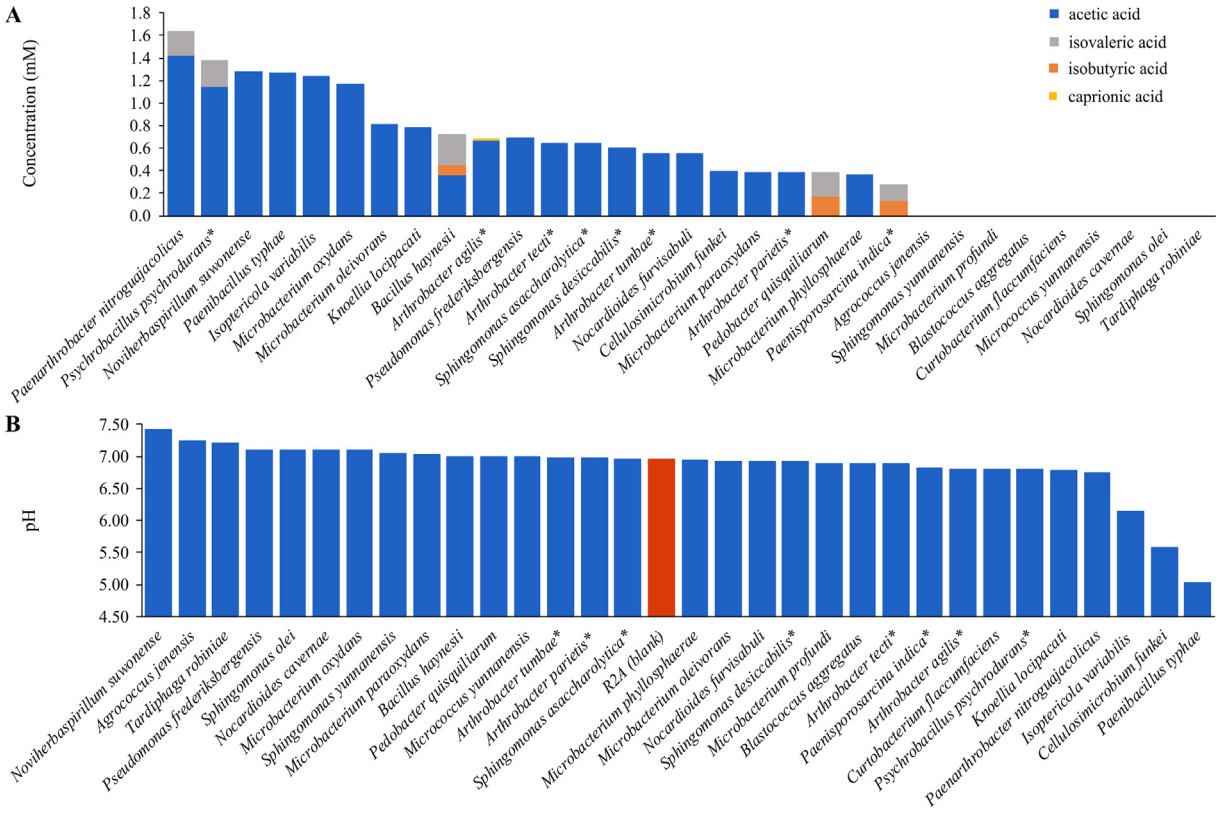

Fig. 7. A) Average VFA production and B) pH from triplicates of the isolates expressed after 48 h growth in R2A at 28 °C (* at 20 °C), zero measurements indicate value below detection limit. Most VFA measurements were around the detection limit and this influenced the average values. (The isolates are named after their closest related species according to NCBI blast).

Those resistant bacterial groups were present across all samples and localities; however, they were more abundant in the countryside. This is surprising as the Castle of Berlare stands in a park where less direct sunlight and higher humidity is expected. This should favour also Proteobacteria which are less known to survive harsh conditions (Gaylarde et al., 2017). A previous study by McNamara et al. (2006) however related an endolithic community with Actinobacteria while epilithic communities contained more Proteobacteria. This could explain the difference in the occurrence of Actinobacteria between our localities: Gypsum crust in Ghent where thicker and botryoidal and can potentially sustain more epilithic bacteria.

The isolates and sequence data has similarities with airborne bacteria. The most common airborne bacteria belong to Proteobacteria, Actinobacteria and Firmicutes (Després et al., 2012), which is comparable with the most common phyla on the monuments. Several isolated genera such as *Arthrobacter*, *Curtobacterium*, *Nocardioides* and *Sphingomonas* have been isolated out the atmosphere of Sweden (Fahlgren et al., 2010) and clouds from France (Amato et al., 2007). There are also some similarities with the airborne community of Milan (Bertolini et al., 2013). Even further away in China, many isolated airborne bacteria by Fang et al. (2016) are similar with the NGS data and the isolates from the two monuments. These similarities suggest air as a prime source for bacterial colonization. Overall the air in rural environments is regarded to contain less bacterial cells but a more diverse bacterial community (Després et al., 2012, 2007; Liu et al., 2019). This does not explain our communities as this is opposite of the bacterial diversity found on our natural building stones. Previous studies mainly related airborne bacteria with soils or leaf-surfaces (Després et al., 2012). However, our results indicate a strong influence of rock surfaces. Colonization could also have occurred during deposition or within the subsurface. This has been brought forward with the findings of halophilic archaea on monuments (e.g. Chimienti et al. (2016)) and was studied by Meier et al. (2017), but more research in this field is necessary.

Neglecting the origin of the prokaryotic populations, both the Actinobacteria and Proteobacteria can disrupt natural building stones by acid production and siderophores (Gaylarde et al., 2017). The isolates of this study nonetheless did not produce a significant amount of VFA in R2A as they approached the detection limit of the method. Three isolates could potentially dissolve $CaCO_3$ as their growth lead to a pH decrease, most likely due to the production of CO_2. Several groups detected by 16S rRNA gene sequencing, e.g. *Modestobacter*, *Solirubrobacter*, *Rubrobacter* and isolates produce on the other hand pigments, causing discolouration, which will turn black when there is no dominating pigment (Gaylarde et al., 2017). *Rubrobacter*, previously related to rosy discolouration of monuments (Laiz et al., 2009; Schabereiter-Gurtner et al., 2001), dominated our samples especially in Berlare. Its dominance together with the red-pigmented *Blastococcus* from our isolates, could contribute to the red discolouration of the gypsum crust on the Lede stone in Berlare, additional to iron oxidation. Many red coloured isolates (such as *Arthrobacter agilis*) were present in the sample of Ghent as well but in here, airborne particles and black carbon present in the urban environment most likely caused the black colour of these samples. Halophilic prokaryotic communities have been linked to rosy discolouration (Piñar et al., 2014) and could explain the discolouration in sample G4. These results illustrate that improved air quality (possibly combined with climate change effects) leads to other discolourations than black. "Greening" and "yellowing" of natural building stones has previously been brought forward by respectively McCabe et al. (2011) and Grossi et al. (2007) but "reddening" or any other colour depending on the dominating pigment is possible as well.

Nitrogen oxidizing prokaryotes can oxidize air pollutants such as NH_3 (NH_4^+), NO_2 into HNO_3 and sulphur oxidizing prokaryotes SO_2

into H_2SO_4. They could potentially enhance gypsum crust formation (e.g. Mansch and Bock, 1996). Members of these groups were present in selected samples of especially Ghent and in a lesser amount at Berlare. Genera belonging to purple non-sulphur bacteria were the only sulphur oxidizers. Purple non-sulphur bacteria can oxidize sulphur but only at low amounts (Hunter et al., 2009). Members of rock damaging genera such as *Thiobacillus* (Krumbein and Gorbushina, 2009) have not been found. This contrasts with results of Villa et al. (2015) who did find those groups in the polluted urban environment but only after targeting specific genes. The absence of sulphur oxidizers can be explained by the currently very low SO_2 (<4 μg/m^3) concentrations (Vlaamse Milieumaatschappij, 2019). Overall, our results still suggest a positive correlation between air pollution from the urban environment and the occurrence of nitrogen and sulphur oxidizing prokaryotes. At the countryside in Berlare, no link was found between the slightly higher NH_3 emissions, the NH_4^+ concentration within the soluble salts and the occurrence of ammonia oxidizing prokaryotes. AOA were only present in G6 with a low diversity, while AOB occurred in several samples. This contrasts with findings of Meng et al. (2017, 2016) who detected a higher abundance and variability of AOA on monuments. They sequenced the amoA gene instead of the 16S rRNA gene; amoA might be a better biomarker to detect AOA. Overall, the abundance of nitrogen and sulphur oxidizing prokaryotes was very low, except for *Rhodoplanes* in G5 and many samples with thick gypsum crusts lack these groups. It shows that, combined with decreasing SO_2 pollution, gypsum crusts are mainly accumulations from the past. Although it cannot be excluded that prokaryotes played a significant role back then.

Besides the occurrence of deteriorating prokaryotes, some abundant genera within Ghent and Berlare can remediate the building stones. Some members of *Arthrobacter*, most abundantly present in Berlare, can induce calcium carbonate precipitation, consolidating the natural building stone (Cacchio et al., 2003; Jroundi et al., 2012). Li et al. (2018) suggested that *Crossiella*, abundant in sample B6 could induce calcium carbonate precipitation as well. Sulphate reducing bacteria, on the other hand, can besides further deterioration also reduce sulphate and precipitate calcium carbonate (Castanier et al., 1999; Krumbein and Gorbushina, 2009). However, their occurrence was very low except in sample G5.

Air pollution further explains the absence of photoautotrophic bacteria as the pollution sustains heterotrophic communities (Saiz-Jimenez, 1993; Zanardini et al., 2000). Although pollution is regarded to lower the bacterial diversity, our findings suggest the opposite with more highly specialized microorganisms in the city that are adapted to mitigate and degrade the atmospheric pollutants. This agrees with the previous research by Villa et al. (2015), is confirmed by the presence of more members of nitrogen oxidizers in Ghent, combined with sulphur reducers and by the isolation of potential oil degraders (closest related to *Microbacterium olei*, *Sphingomonas olei* and *Pseudomonas frederikbergensis*). Pollution still affected Berlare in some degree as the two latter oil-degrading isolates were retrieved from the Castle from Berlare. In Berlare autotrophic bacteria were missing as well and some nitrifiers and sulphur bacteria were present but in a much lower amount. The NGS results contradict earlier research by Mitchell and Gu (2000) and Zanardini et al. (2000) using culturing techniques. They suggested that pollution reduces biodiversity. It confirms the results of the isolates, where fewer genera and species were isolated from the sample of Ghent.

The indirect effect of air pollution on prokaryotic communities might be more important as it accelerates stone deterioration leading to gypsum crust formation and increased salt content (Doehne and Price, 2010; Graue et al., 2013). Those changes in the material can have a bigger effect compared to air pollution itself. Although based on the isolates, the high soluble NO_3^- in Ghent does not sustain denitrifiers; the data suggest a major effect of the soluble salt content in sample G4, from Ghent, sustaining a halophilic prokaryotic community. Furthermore, samples G4 and G2 with the highest amount of soluble NO_3^- contain both nitrifying bacteria.

5. Conclusions

This work provides an overview of prokaryotic communities on deteriorated building stones, a highly variable substrate, mainly colonized out of the atmosphere. It illustrates that weathered Lede stone contained both in the urban and in the rural environment, a diverse bacterial community, while archaea were mainly absent. Overall, the City Hall of Ghent was predominated by Actinobacteria and Proteobacteria and the Castle of Berlare only by Actinobacteria. Both localities contained potential damaging prokaryotes that produce acids or pigments. Based on the isolates the effect of acid production should be limited as most bacteria did not decrease the pH of R2A and produced low amounts of VFA. However, the prokaryotes could induce an aesthetic impact as many isolates produce pigments. *Rubrobacter* dominated furthermore, the samples of Berlare where it even could contribute to the red discolouration, normally attributed to iron oxidation. Contrary, beneficial genera linked to calcium carbonate precipitation such as *Arthrobacter* have been detected as well. Air quality differed between the two locations and explains the higher abundance of lithoautotrophic prokaryotes in selected samples of the urban environment. They can potentially induce gypsum crust formation, but mainly relates to accumulations from the past. Air quality also explains the higher diversity in the samples of the urban environment with more highly specialized prokaryotes. The effect of the soluble salt content on the prokaryotes could even be more important. This parameter varied significantly and is only one of the factors influencing the prokaryotic communities. It illustrates the huge complexity of this substratum. The main effect of the air pollution seems to be the discolouration potential: in regions with higher pollution such as Ghent, it turned buildings black. At the countryside, other actors such as prokaryotes might cause other discolouration. This study confirms the potential effect of air pollution on natural building stones. It can be the basis for further investigation to estimate the effect of improving air quality on our built heritage. Those studies should include interdisciplinary research on several buildings to unravel this complex substratum. Molecular techniques will be important, also with targets for specific genes such as amoA to describe the functionality in detail. Furthermore, future research should focus on the aesthetic damage not only caused by the material itself but also by pigmented microorganisms.

CRediT authorship contribution statement

Laurenz Schröer: Conceptualization, Methodology, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Tim De Kock:** Conceptualization, Resources, Writing - review & editing, Supervision. **Veerle Cnudde:** Conceptualization, Resources, Writing - review & editing, Supervision. **Nico Boon:** Conceptualization, Resources, Writing - review & editing, Supervision.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.139339.

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