1 **RNA-based molecular survey of biodiversity and biogeochemical processes of limestone tombstone**
2 **microbiota in response to atmospheric sulfur pollution**
3
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12 **Significance and Impact of the Study:**

13 The structure of the active bacterial communities on tombstones located in a highly polluted urban
14 environment and in a less polluted location was analyzed using genes involved in different pathways of
15 sulfur metabolism (*soxB, aspA, dsrA*) as functional markers and the 16S rRNA gene as a phylogenetic
16 marker. For the first time, transcripts involved in important energy-yielding processes were investigated in
17 order to reveal the metabolic capabilities of the microflora inhabiting stone surfaces and its relationship
18 with sulfur-containing air pollutants. The results identifying specific target groups that are active, and
19 elucidated their physiological functions in the biofilm, providing important information about the ecology
20 of subaerial biofilms inhabiting artistic stone surfaces.

21

22

23 **Abstract**

24 Outdoor stoneworks sustain biofilm formation and are constantly at risk of deterioration by
25 microorganisms. We demonstrated that these biogenic phenomena at the stone/air interface are under the
26 direct influence of the atmospheric input, especially sulfur-containing air pollutants.

27 In this study, the biofilm microflora of historic limestone tombstones located in a highly polluted urban
28 environment (Cambridge, Massachussetts) and in a less polluted location (Lexington, Massachussetts),
29 were compared using comprehensive RNA-based molecular analyses of 16S rRNA gene sequences as well
30 as sequences of genes for different pathways of sulfur metabolism (*soxB, apsA, dsrA*).

31 The metabolically active microorganisms detected by DGGE analysis of 16S rRNA fragments were
32 predominantly represented by cyanobacteria (belonging to the family Nostocaceae and to the genus
33 *Chroococcidiopsis*) in both polluted and unpolluted environments.

34 The investigation of *soxB, apsA, dsrA* transcripts reflected the abundance and the diversity of sulfur-
35 oxidizing and sulfate-reducing bacteria in the Cambridge samples in comparison with the Lexington
36 samples. The investigation revealed that in addition to phototrophic sulfur bacteria belonging to the genera
37 *Thiocapsa*, *Halochromatium*, *Allochromatium*, *Thiococcus* and *Thermochromatium*, other sulfate-oxidizing

38 prokaryotes (e.g. the genus *Thiobacillus*) as well as sequences of Deltaproteobacteria from the genus
39 *Desulfovibrio* occurred at the polluted urban site. The interactions among the main functional groups
40 retrieved from the limestone tombstones were discussed.

41

42 **Keywords:** sub aerial biofilm; limestone tombstone; RNA-based molecular analysis; sulfur metabolism;
43 pollution

44 **Introduction**

45 Many important historic artifacts are embodied in tombstones that are exposed to the atmosphere.
46 The lithosphere-atmosphere interface is an ancient terrestrial niche exposed to extreme environmental
47 conditions (e.g. dissication, high or low temperature, low nutrient availability, intense solar radiation, pH)
48 that fluctuate widely, presenting a challenge to microbial colonists (Gorbushina and Broughton, 2009).
49 However, in spite of their hostile nature, the stone/air boundary sustains microbial colonization. The stone
50 serve as both a swelling and a source of mineral for microorganisms, while air-borne particles and volatile
51 compounds offer a source of inorganic and organic materials for chemolitotrophs and chemorganotrophs,
52 respectively.

53 In this sense, outdoor tombstones represent harsh habitats in direct contact with the atmosphere, that
54 trigger highly sophisticated multicellular behavioral responses, including the formation of sub aerial
55 biofilms (SABs) (Gorbushina 2007; Gorbushina and Broughton, 2009). SAB is a self-sufficient, microbial
56 ecosystem embedded in a self-produced polymeric matrix enabling microorganisms to develop coordinated
57 survival strategies, increasing microbial fitness and avoiding loss of energy and nutrients (Gorbushina
58 2007). Although the ability to colonize a surface and develop SABs is advantageous from the microbial point
59 of view, it causes severe decay, including discoloration and chemical and physical deterioration of the
60 surface (Berdoulay and Salvado 2009; Polo et al. 2012).

61 By intercepting compounds carried at the air, SABs and their activity are under the direct influence of the
62 atmospheric input. However, only few reports showed that the drastic changes in biological diversity
63 observed on rock surfaces in urban and rural environments are directly connected to differences in air
64 quality, especially regarding anthropogenic input of organic substances (Sterflinger and Prillinger, 2001;
65 Nuhoglu et al., 2006).

66 Sterflinger and Prillinger (2001) reported that, in addition to the well-known rock inhabiting genera, such as
67 *Alternaria*, *Aureobasidium*, *Coniosporium* and *Sarcinomyces*, the hyphomycete genera *Coniothyrium*,
68 *Epicoccum* and *Phoma* were found on all rock surfaces in Vienna. The biodiversity of the fungi in the urban
69 environment of Vienna was much higher than on the same rock type in a rural environment, and this

70 difference was attributed to the elevated organic pollution in the city. Nuhoglu and colleagues (2006)
71 showed that a heterotrophic microflora, composed by fungi, actinomycetes and bacteria dominates the
72 stone surfaces in the historical buildings during the winter months in Erzurum. These findings confirmed
73 that heterotrophs are the dominant microflora of the stone materials exposed to atmospheric pollution,
74 because of their ability to grow utilizing organic pollutants (Nuhoglu et al., 2006). Thus, the continuous
75 interaction between the external environment and microorganisms strongly impact biofilm structure and
76 activities (Gorbushina, 2007).
77 Although the microflora inhabiting stone surfaces are known to respond to the input of organic pollutants,
78 there is a lack of knowledge about the selecting effects of inorganic air pollutants on SAB communities.
79 Until now, the efforts were focused on studying the direct effects of these contaminants on stoneworks
80 (Agelakopoulou et al., 2009; Tzanis et al., 2011; Tidblad et al., 2012), underestimating or neglecting their
81 influence in shaping the structure, composition and activity of SABs. In almost every instance, energy
82 supplied to SAB communities comes from the atmosphere rather than the rock, and this observation
83 strengthens further the correlation between inorganic air pollutants and SABs (Gorbushina and Broughton,
84 2009).
85 Among the commonly found inorganic air pollutants, sulfur-containing compounds are the most important
86 transboundary pollutants that cause serious problems for the global environment (Watt et al., 2009). Sulfur
87 dioxide is a major pollutant in most cities. Concentrations range from 20 to 200 ppb per hour depositing on
88 surfaces in urban environments, compared to less than 10 ppb in suburban areas (Mitchell and Gu 2000). A
89 previous investigation carried out by Mitchell and Gu (2000) on the biofilm microflora of two sets of historic
90 limestone gravestones located in a highly polluted urban environment and a less polluted rural location in
91 Massachussetts, revealed that the sulfur oxidizing microflora were enriched in the biofilms exposed to
92 sulfur pollution. In addition, a laboratory study demonstrated that low concentrations of sulfur stimulated
93 the growth of sulfur-oxidizing bacteria, resulting in a rapid acid production and biomass penetration into
94 the limestone.

To complicate this picture further, little is known about metabolically active members of SABs, as only few papers reported the use of RNA-based molecular analyses to investigate the key active members of biofilm communities inhabiting artistic surfaces (Gonzales et al. 2006; Portillo et al. 2008 and 2009a). Comparative DGGE analyses of the total (16S rDNA) and metabolically active bacterial communities (16S rRNA) from the walls of Altamira Cave showed the existence of a high proportion of the microbial community that remained undetected in the DGGE profiles from RNA-based molecular analyses (Gonzales et al. 2006; Portillo et al. 2008 and 2009a). Metabolically active microorganisms were assumed to directly participate in the development of alterations in the studied cave, making these results essential for understanding the development, physiology and potential of microbial communities.

The complexity of SAB communities, together with the gap of information available on a number of specific target group with a determinate metabolism detected in those environments, suggest the need to identify active microorganisms with specific physiological functions.

Within the scenario described by Michell and Gu (2000), the present work aimed at characterizing the SAB active communities inhabiting tombstones located at two different locations and their correlation to the amount of sulfur compounds, with particular attention to the ecology of microorganisms involved in the sulfur-cycling. The sampling sites are historic limestone gravestones located in two cemeteries in the North-east of the United States previously described by Mitchell and Gu (2000). One cemetery is located in the urban center of Cambridge where there is heavy continuous traffic, while the other is located in Lexington in a green area with minimal exposure to urban pollution.

Bacterial sulfur oxidation and sulfate reduction pathways have been studied in a variety of microrganisms over the last few years in many different environments (*Inter alia* Karr et al., 2005; Headd et a., 2013; Xia et al., 2014). The biochemistry behind these pathways is quite complex and has been the focus of several excellent reviews (Muyzer and Stams 2008; Frigaard and Dahl 2008).

To the best of our knowledge, no reports are available regarding the sulfur-oxidizing bacteria (SOB) and sulfate-reducing bacteria (SRB) community structure inhabiting stone monuments based on functional genes. In this study, comprehensive RNA-based molecular analyses of the biofilm microflora of historic

121 limestone located in a highly polluted urban environment and in a less polluted location, were performed
122 using 16S rRNA gene sequences as well as sequences of genes for different pathways of sulfur metabolism
123 (*soxB, apsA, dsrA*). Finally, the ecological interactions appear to occur among the main functional groups
124 retrieved in the SAB communities inhabiting tombstones are discussed.
125

126 **Results and Discussion**

127 To quantitatively examine the relative similarities between the total and metabolically active bacterial
128 communities inhabiting stone surfaces in an urban and rural environment, the DGGE band profiles of
129 amplified 16 rDNA and reversely transcribed 16S rRNA fragments were analyzed by peak fitting (Figure 1a).
130 The analysis showed that a consistent proportion of the bacterial community present in these colonizations
131 remained undetected through RNA-based molecular analyses. The metabolically active members of the
132 bacterial community represented roughly 69% of the total number of bands detected by DGGE from biofilm
133 microflora of historic limestone located in a highly polluted environment. Similarly, in the less polluted
134 location, the metabolically active members of the bacterial community represented approximately 68% of
135 the bands detected. Portillo and colleagues (2009a) showed that less than 42% of the bands detected in
136 DNA profiles of the total bacterial community found in Altamira Cave were represented in PCR-DGGE
137 analyses based on RNA.
138 The composition of metabolically active bacterial communities detected by DGGE analysis of 16S rRNA
139 fragments, is reported in Table 1.
140 The phylum Cyanobacteria was the largest bacterial group retrieved from polluted and unpolluted
141 environments. Within the list of the most typical stone colonizers, Cyanobacteria have received particular
142 attention as they adapt to extremes of environmental stress and are able to readily colonize a wide variety
143 of terrestrial habitats, including modern and ancient buildings (Cappitelli et al. 2012; Hallmann et al. 2013).
144 Apart from the evident aesthetic damage to the surfaces due to biogenic pigments, cyanobacteria
145 contribute to the decay of stone-built artworks by exerting pressure on the stone as a result of water
146 uptake, expansion of the exopolymeric matrix (extracellular polymeric substances, EPS), and by dissolution

147 of stone by the release of organic acid and chelating agents and subsequent precipitation of calcium salts
148 around the cells (Macedo et al. 2009; Albertano 2012; Cappitelli et al. 2012).
149 In both urban and rural areas, the highest proportion of RNA within cyanobacteria was identified as
150 belonging to the order Nostocales. Worth noting was the identification of *Anabaena* sp. (97% similarity) in
151 samples collected from the unpolluted environment. *Anabaena cylindrica* was the dominant organism
152 found on undeteriorated stone objects (Mitchell et al. 2008). In this work, the molecular analysis of the
153 metabolically active cyanobacterial community showed the presence of *Petalomena* sp. (98% similarity)
154 that has been previously retrieved from limestone substrate (Uher 2010). In addition, the extremophile
155 *Chroococcidiopsis* sp. (99% similarity) has been detected in the biofilm samples collected in the urban area
156 of Cambridge. This microrganism occurs in hypolithic and endolithic communities worldwide, as in the Dry
157 Valleys in Antarctica or the Atacama Desert, where it persist in a dry ametabolic state for prolonged periods
158 (Billi et al. 2013).
159 The genetic potential of microbial sulfur metabolism was analyzed by amplifying key genes of known sulfur
160 oxidation and sulfate reducing pathways. A gene encoding an essential component of the Sox enzyme (*soxB*
161 gene), was chosen along with the gene encoding the dissimilatory sulfate reductase (*drsA* gene) and the
162 adenosine-5′-phosphosulfate (*apsA* gene). The DGGE profiles of *soxB*, *dsrA* and *apsA* transcripts in samples
163 C1, C4, L1 and L2 revealed overall high molecular diversity (Figure 1b-d). Sequences were obtained from the
164 study of the microbial community by BLAST in the NCBI database and are reported in Table 1.
165 Sequences detected from DGGE of PCR-amplified 16S rRNA genes were not ascribable to sulfur oxidizing
166 bacteria (SOB) and sulfate reducing bacteria (SRB). This is not surprising, as the analysis of 16S rRNA genes
167 cannot provide an unambiguous link between the genetic identity of an uncultured microorganism and its
168 physiological or metabolic capacity. The molecular detection of specific microbial communities by DGGE
169 may become difficult if they are present in low numbers, even more so in the case of SABs, which consist of
170 a complex mixtures of microorganisms (Gorbushina 2007; Gorbushina and Broughton, 2009). The DGGE
171 with bacterial primers mainly detects the major constituents of the analyzed community overlooking the
172 less abundant but potentially very important species. Analyses of functional genes circumvent these

173 limitations and have been successfully applied for biodiversity studies (Meyer et al. 2008; Belila et al.,
174 2013).
175 Noteworthy was the observation that diverse genes of sulfur energy metabolism are present and actively
176 transcribed in SAB populations under different atmospheric chemistry. However, the utilization of multiple
177 sulfur oxidation pathways is not unusual among bacteria and is likely related to substrate availability. In
178 particular, the huge variety of gammaproteobacterial *apsA* transcripts found in this study indicated that
179 Gammaproteobacteria at the urban site mainly use the adenosine-5'-phosphosulfate (APS) reductase
180 pathway for sulfur oxidation.
181 Analyses of the functional genes revealed that in addition to anoxygenic phototrophic sulfur bacteria
182 belonging to the genera *Thiocapsa*, *Halochromatium*, *Allochromatium*, *Thiococcus* and *Thermochromatium*,
183 other sulfate-oxidizing prokaryotes occurred at the polluted urban site, including aerobic sulfur oxidizing
184 bacteria belonging to the genus *Thiobacillus*. In addition, a sequence of Deltaproteobacteria from the genus
185 *Desulfovibrio* was identified in samples collected from the urban polluted site. Thus, the active bacterial
186 communities inhabiting tombstones was mainly composed of cyanobacteria, anoxygenic phototrophic
187 sulfur bacteria, aerobic sulfur oxidizing bacteria and sulfate reducing bacteria. In contrast, the SAB
188 microflora retrieved from the unpolluted site was characterized by the presence of cyanobacteria and few
189 anoxygenic phototrophic sulfur bacteria.
190 Large numbers of strictly autotrophic *Thiobacillus* sp. have been found not only under the surface of highly
191 deteriorated stones, which presented a pulverizing aspect, but also in deeper layers where there was no
192 stone decay yet visible (Krumbein and Gorbushina 2009). Interestingly, *Thiobacillus thioparus* found in an
193 urban site is able to convert sulfur dioxide gas in the atmosphere to sulfuric acid, which attacks carbonates
194 and converts these to the soft and very soluble gypsum (Wisseman 1980). In addition, this bacterium can
195 oxidize volatile additional reduced sulfur compounds such as dimethyl sulfide, methyl mercaptan and
196 dimethyl disulfide (Cáceres et al. 2010). Thus, the presence of *Thiobacillus* sp. on stoneworks might be a
197 good indicator of both anthropogenic impact and potential biodeterioration risk.

198 Results from this study showed the presence of active members of the genus *Desulfovibrio.* Although SRB
199 have been generally considered obligatory anaerobic bacteria and they grow optimally in the absence of
200 oxygen, several studies have recognized a wide habitat range and oxygen tolerance. Members of the genus
201 *Desulfovibrio*, could actually survive beyond strictly anaerobic systems and develop under microaerophilic
202 conditions (Wildschut et al. 2006). The presence of oxygen-concentration gradients within a biofilm
203 explains, among other phenomena, how microaerophiles and strict anaerobes can thrive in biofilm that are
204 continuously exposed to air. Stewart and Franklin (2008) emphasized that the failure of oxygen to
205 penetrate throughout a biofilm is not a result of physical exclusion, but rather a result of the respiration by
206 cells in the upper layers of the biofilm. A molecular study carried out by Portillo and colleagues (2009) on a
207 painting has shown the presence of a diverse community of SRB in Altamira Cave and an unexpected high
208 level of microdiversity within the genus *Desulfovibrio,* suggesting potential negative effects, as some
209 species of SRB have been reported to produce exopolymeric substances (EPS), which might further
210 contribute to pH changes and crystal deposition on stone. In addition, a potentially aesthetic damaging
211 effect of SRB metabolism might be the result of reacting sulfides and metal ions leading to darkening of
212 specific zones where this phenomenon occurs (Portillo et al. 2009). However, even though the marble of
213 the Milan Cathedral contains traces of iron, darkening of the surface during the successful removal of black
214 crusts from stone employing the SRB *Desulfovibrio vulgaris* was not observed (Cappitelli et al. 2006;
215 Pedrazzani et al. 2006). In addition, SRB have been recognized as key players in the precipitation of calcium
216 carbonate leading to their use as stone consolidants (Baumgartner et al. 2006).
217 The coexistence of different bacterial communities showing genetic and physiological heterogeneity in a
218 single environment has been described as a consequence of exploiting distinct niches present within the
219 biofilm, since environmental conditions and physiological responses of the bacteria to their local
220 environment are not homogeneous throughout a biofilm (Stewart and Franklin 2008).
221 Here, we propose a close and interdependent cycling of nutrients that appears to occur among the main
222 functional groups retrieved from a tombstone located in the polluted environment. Cyanobacteria produce
223 organic carbon during photosynthesis that stimulates the growth of heterotrophs such as SRB, and to a

224 lesser extent SOB. However, the high O_2 production by cyanobacteria could partially inhibit the growth of
225 SRB, although it stimulates that of SOB. The SOB quickly remove the metabolic products of SRB, S^{2-}, that
226 could inhibit the cyanobacteria and at higher concentrations also SRB. The resulting proposed consortium
227 carefully balances the three metabolic processes. The synergy among the different microrganisms having
228 coordinated metabolic activity would allow the functional groups to thrive on the stone/air interface. The
229 same ecological interactions have been previously observed in microbial mat systems (Decho et al., 2010;
230 Whitton, 2012).
231 The differences between the active SAB microflora under a polluted and unpolluted environment were also
232 highlighted by Principal Component Analysis (PCA). The PCA model (figure 2a) explained 94.11% (77.89%
233 PC1 + 16.22% PC2) of the variance in the original data, illustrating PCA usefulness as qualitative pattern
234 recognition method. In fact, the PCA score plot showed that the microbial community structures from the
235 polluted (objects C1 and C4) and less polluted sites (objects L1 and L2) are different as they clustered in
236 different areas in the plots. The plot displayed that variation between the different biological groups was
237 more pronounced on the PC1, which counts for the highest variation in the models (77.89%). The
238 dendrogram obtained from HCA (figure 3b) revealed significant differences in the raw data and indicate the
239 existence of the two separate groups composed by the samples C1-C4 and L1-L2, exacerbating the results
240 of PCA.
241 Diversity indices calculated using band position and intensity of the DGGE profiles indicated samples
242 collected from the unpolluted environment showed the lowest genetic diversity of the microbial
243 community and samples collected from the polluted environment the highest (figure 2b). It is generally
244 accepted that a reduction of species diversity and richness will occur as a consequence of a pollution event
245 (Ager et al. 2010). In addition, the loss of species is accompanied by a change in community structure (Ager
246 et al. 2010). In contrast to these general assumptions, our findings showed that the biodiversity in the
247 polluted area was higher than that assigned to SABs collected in the less polluted rural area. The increase in
248 bacterial-community diversity in the polluted environment was assumed to be due to the presence of

249 highly specialized microorganisms adapted to take advantages or buffer the environmental stresses caused
250 by the atmospheric pollutants.
251 The SAB community represents a multi-component open ecosystem sensitively reacting to all
252 environmental factors including air pollutants. However, until now the bioindicative potential of the SAB
253 communities responding to air pollutants has been underestimated, as only lichenoindication was
254 previously employed (Landis et al. 2012; Llop et al. 2012).
255 Our findings suggest that by identifying specific target groups that are active, and elucidating functional
256 interaction network of the whole community able to buffer environmental perturbations, it will be possible
257 elucidate the role and behavior of microorganisms within the biofilm and their complex interaction with
258 the external environment.
259
260 **Materials and Methods**
261 **Sampling sites and methods**
262 Tombstones were selected in two cemeteries in Massachussetts as previously described by Mitchell and Gu
263 (2000). The polluted location was Harvard Square, Cambridge, MA. The cemetery is located close to the
264 urban center where there is heavy continuous traffic. The tombstones in this cemetery date from the 17th
265 century. For our study, we selected limestone gravestones dating from the mid-19th century (samples C1
266 and C4). For the less polluted location, a cemetery in Lexington, MA, was chosen. The cemetery,
267 approximately 15 km from Cambridge, is in an area with minimal exposure to urban pollution. We sampled
268 from limestone tombstones dating from the mid-19th century (samples L1 and L2). The four collected
269 samples came from multiple gravestones at each location used. Samples were obtained under aseptic
270 conditions by scraping off materials with a sterile scalpel and immediately preserved in a RNA stabilization
271 reagent (RNAlater, Quiagen, USA) on ice until arrival at the laboratory and then processed immediately.
272
273 **Nucleic acid extractions and amplification**

274 DNA was extracted from 0.2 g of scrubbed stone material using the UltraClean Soil DNA kit (MoBio
275 Laboratories) according to the manufacturer's recommendations. DNA was eluted in 50 µl of 10 mM Tris
276 solution and stored at -20 °C.
277 For RNA extraction and handling, all glassware was treated with 0.1% diethylpyrocarbonate (DEPC), and
278 nondisposable plastic ware was cleaned with RNase Away (Gibco). Total RNA was extracted from 0.25 g of
279 scrubbed stone material using the RNeasy Mini kit (Qiagen). The protocol for total RNA extraction included
280 DNaseI treatment (Qiagen Inc., Italy) as per the manufacturer's instructions to remove any DNA remaining
281 in the final RNA extract.
282 Both DNA and RNA samples were quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo
283 Scientific, Wilmington, DE).
284 DNA and RNA extracts were subjected to PCR and reverse transcription-PCR (RT-PCR) targeting 16S rRNA
285 gene. Amplification of the functional genes *dsrA*, *apsA* and *soxB* was carried out from biofilm-extracted
286 RNA. Primer sets used in this study are reported in Table 2.
287 All PCR reactions were set in 50 µl reaction volume containing 3 µl of template DNA or RNA, 1X reaction
288 buffer with 2.0 mM $MgCl_2$, 200 µM of dNTPs, 0.5 µM of each primer and 2 unit of Taq DNA polymerase
289 (Qiagen). RT-PCRs were performed by using the OneStep RT-PCR Kit (Qiagen) as per manufacturer's
290 instructions. The amplification procedure from RNA samples included a reverse transcription step for 30
291 min at 50 °C, followed by an initial denaturation step for 15 min at 95 °C.
292 Bacterial 16S rDNA and 16S rRNA were amplified by nested-PCR as previously described (Polo et al. 2012).
293 The PCR program to amplify *dsrA* gene transcripts was as follows: denaturing step of 94 °C for 1 min,
294 followed by 35 cycles at 94°C for 30 s, 60 °C for 30 s and 72 °C for 1 min, with a final extension step of 72 °C
295 for 10 min. The following thermocycling program was used to amplify the *apsA* gene transcripts: denaturing
296 step of 94 °C for 2 min, followed by 35 cycles of denaturing for 45 s at 95 °C, annealing for 45 s at 55 °C, and
297 extension for 2 min at 72 °C. A final extension step of 72 °C for 10 min was performed. The conditions of the
298 RT-PCR for the *soxB* gene transcripts were: denaturing step of 94 °C for 2 min, followed by 10 cycles at 94°C

299 for 45 s, 55 °C for 45 s and 72 °C for 2 min, 25 cycles at 94°C for 45 s, 50 °C for 45 s and 72 °C for 2 min, with
300 a final extension step of 72 °C for 10 min.
301 DNase-treated nucleic acids, without being subjected to RT, were used as controls in PCR to check for
302 residual DNA in RNA preparations.
303 PCR positive controls include DNA templates prepared from *Escherichia coli* ATCC 25404 (16S rRNA gene),
304 *Allochromatium vinosum* ATCC 17899 (*dsrA*), *Desulfovibrio vulgaris* ATCC 2957 (*apsA*) and *Paracoccus*
305 *pantotrophus* ATCC 35512 (*soxB*). PCR was routinely assessed against a negative control reaction set up
306 identically to the experimental PCR, but without template DNA (water blanks).
307 The amplification products were analyzed by electrophoresis on 1.5% (wt/vol) agarose gels, followed by a
308 15-min staining with ethidium bromide (0.5 mg/l).
309
310 **Denaturing gradient gel electrophoresis (DGGE) analysis**
311 Both phylogenetic and functional fingerprint analyses were obtained by denaturing gradient gel
312 electrophoresis (DGGE). DGGE was performed as reported by Polo et al. (2012) with a 40-60% denaturant
313 gradient for total (16S rDNA) and active (16S rRNA) bacterial community, as well as for the *soxB*, *dsrA* and
314 *apsA* transcripts (100% denaturant contains 7 M urea and 40% v/v formamide).
315 Dominant DGGE bands were excised and eluted by incubation in 50 µl of sterilized distilled water overnight
316 at 37°C at 450 rd/min. Five µl of the eluted DNA was used as a template to reamplify the band of interest
317 using the same primer sets described above without the GC-clamp. The amplified products were purified
318 with the QIAquick PCR Purification Kit (Qiagen) and sequenced by PRIMM (Milano, Italy). Sequence
319 similarity searches were conducted using BLAST network service of the GeneBank database to identify the
320 nearest relatives of the excised dominant bands (www.ncbi.nlm.nih.gov/BLAST). In particular, the similarity
321 analysis of the sequences was obtained by using the BLASTN and BLASTX programs for the comparison of a
322 nucleotide query sequence against a nucleotide sequence database and a nucleotide query sequence
323 translated in all reading frames against a protein sequence database, respectively. Sequences were
324 screened for chimeras by the CHECK-CHIMERA program of the Ribosomal Database Project

325 (http://rdp.cme.msu.edu/cgis/chimera.cgi?suSSU). Sequences yielding a bell-shaped histogram, matching
326 91% with sequences in the database, and showing different closest relatives from two sides of a breakpoint
327 were considered chimeras and were removed from the data set.
328

329 **Statistical analysis**

330 Using DGGE profiles the line plots were generated with ImageJ software (Rasband W, 2008), and then
331 imported into an Excel® file as x/y values. To examine the relative similarities among samples, the matrices
332 of x/y values of DGGE line profiles were analyzed by Principal Component Analysis (PCA) and Hierarchical
333 Cluster Analysis (HCA). PCA was carried out as reported by Polo et al. (2012). Multivariate investigations
334 were conducted with XLSTAT version 2009.4.07 (Addinsoft, NY) software using the Pearson correlation as
335 similarity index. The significance of the PCA model was tested by a cross-validation procedure.
336 The hierarchical clustering procedure was based on the 'complete linkage' method, applying the Euclidean
337 to evidence any natural grouping samples and using XLSTAT version 2009.4.07 (Addinsoft, NY) software.
338 Community profiles were subjected to peak fitting analyses (PeakFit, SPSS, Inc.) to quantify the band
339 position (peaks representing individual taxonomic units, differentiated by a reference fragment value, Rf)
340 and intensities (peak area representing the abundance of each taxonomic unit) as reported by Cappitelli et
341 al. (2012). The following indexes were calculated: Richness (S), the Simpson Diversity Index (1/D), the
342 Shannon-Weaver index (H') and Evenness (J) (Cappitelli et al. 2012).
343

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348 **Conflict of interest**

349 No conflict of interest declared.

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432 **Figures and tables**

433

434 **Figure 1:** Microbial community fingerprints of bacteria from different locations (C1 and C4 from polluted
435 urban area of Cambridge; L1 and L2 from the less polluted area of Lexington) as determined by PCR-DGGE
436 and line image profiles generated by image analysis. DGGE profiles of (a) 16S rDNA and 16S rRNA, (b) *apsA*
437 transcripts, (c) *soxB* transcripts, and (d) *drsA* transcripts. Panel (e) reports an example of peak fitting
438 analysis.

439

440 **Figure 2:** (a) PCA scores plot of DGGE profiles of SAB samples obtained from different locations (C1 and C4
441 from polluted area; L1 and L2 from the less polluted area). (b) Diversity indices based on DGGE data
442 analyses.

443

444 **Figure 3: sampling areas**

445

446

447 **Table 1:** Identification of 16S rRNA, *soxB, dsrA* and *apsA* transcript sequences from DGGE profiles obtained
448 by using the BlastN program.

449

BlastN reference strain (16S rRNA)							
Bands	**C1**	**C4**	**L1**	**L2**	**Closest relative strain**	**Closest homologue accession number**	**Similarity**
1	X		X	X	*Petalonema* sp.	HQ847568.1	98%
5	X				Uncultured cyanobacterium	JF929516.1	97%
12		X	X		Uncultured cyanobacterium	HM224428.1	100%
13	X	X			Uncultured bacterium	JF235029.1	95%
14	X	X			Uncultured bacterium	FJ466398.1	97%

23	X	X			Uncultured cyanobacterium	JF789037.1	98%
25			X	X	*Anabaena* sp.	EU078527.1	97%
26				X	*Sphaerospermopsis aphanizomenoides*	GU197654.1	94%
27	X	X			Uncultured *Deinococcales*	JQ627416.1	99%
28	X				Uncultured cyanobacterium	JQ627427.1	98%
29	X	X			Uncultured *Chroococcidiopsis* sp.	FJ805855.1	99%
30			X	X	Uncultured Nostocales	EU434908.1	95%

BlastN reference strain (*soxB* gene)

Bands	**C1**	**C4**	**L1**	**L2**	**Closest relative strain**	**Closest homologue Accession number**	**Similarity**
12	X	X			*Methylobacterium extorquens*	CP001510.1	96%
22	X	X	X	X	*Ralstonia solanacearum*	CP002819.1	92%
23	X	X			Uncultured bacterium	KC332957.1	91%

BlastN reference strain (*dsrA* gene)

Bands	**C1**	**C4**	**L1**	**L2**	**Closest relative strain**	**Closest homologue Accession number**	**Similarity**
12	X				*Rhodomicrobium vannielii*	CP002292.1	96%
22	X				Uncultured bacterium	AB544674.1	94%

BlastN reference strain (*apsA* gene)

Bands	**C1**	**C4**	**L1**	**L2**	**Closest relative strain**	**Closest homologue Accession number**	**Similarity**
3	X				*Thiocapsa roseopersicina*	EF641938.1	97%
4		X	X		Uncultured prokaryote	DQ995794.1	98%
5		X			Bacterium enrichment culture	EU722721.1	98%
6		X	X		Uncultured prokaryote	JN162589.1	98%
8	X				*Halochromatium salexigens*	EF641933.1	95%

9		X			*Allochromatium minutissimum*	EF641963.1	95%
33	X	X			*Thiobacillus thioparus*	EF641954.1	98%
37			X	X	Uncultured alpha proteobacterium	JN934445.1	95%
39	X				*Thiococcus pfennigii*	EF641942.1	96%
40	X	X			*Thiobacillus plumbophilus*	EF641956.1	95%
41	X	X			*Desulfovibrio* sp.	EF442891.1	96%
42		X	X		*Thermochromatium tepidum*	EF641936.1	96%
45				X	*Allochromatium vinosum*	CP001896.1	91%
49	X	X			Uncultured sulfate-reducing bacterium	DQ450398.1	97%

Table 2: PCR primer set used in this study.

Microorganism and primer	Sequence 5'-3'	Length	Reference
16S rRNA gene			
27f	GAGATTTGATCCTGGCTCAG	1500 bp	Lane (1991)[1]
1495r	CTACGGCTACCTTGTTACGA		
357f[a]	CCTACGGGAGGCAGCAG	600 bp	Muyzer et al. (1993)[2]
907r	CCGTCAATTCCTTTGAGTTT		Lane (1991)[1]
dsrA			
PGdsrAF[a]	CAYGGBCAGACCGGBRAYATYATG	490 bp	Mori et al. (2010)[3]
PGdsrAR	RCAGTGCATRCAKCGHACRCA		Mori et al. (2010)[3]
apsA			
APS-FW[a]	TGGCAGATMATGATYMACGG	390 bp	Friedrich (2002)[4]
APS-RV	GGGCCGTAACCGTCCTTGAA		Friedrich (2002)[4]
soxB			

soxB432F	GAYGGNGGNGAYACNTGG	1000 bp	Petri et al. (2001)[5]
soxB1446B	CATGTCNCCNCCRTGYTG		Petri et al. (2001)[5]
soxB693F[a]	ATCGGNCARGCNTTYCCNTA	750 bp	Petri et al. (2001)[5]
soxB1446B	CATGTCNCCNCCRTGYTG		Petri et al. (2001)[5]

452 [a]5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGCACGGGGGG-3'

453 [b]5'-CCGGCGCCGCGGCGGGCGGGGCGGGGGCACGGG-3'

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459 [5]FEMS Microbiol Lett 197: 171–178.