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The interweaving roles of mineral and microbiome in shaping the antibacterial activity of archaeological medicinal clays

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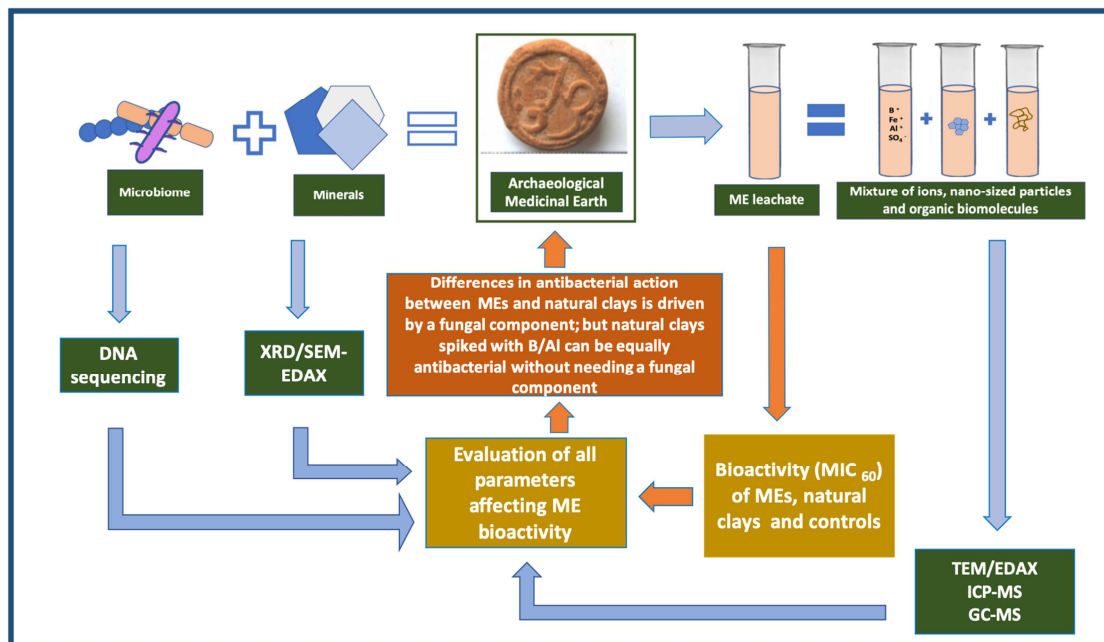
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Graphical abstract



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30 **The interweaving roles of mineral and microbiome**
 31 **in shaping the antibacterial activity of archaeological medicinal clays**

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47
 48
 49 **Abstract**

50 **Ethnopharmacological relevance:** Medicinal Earths (MEs), natural aluminosilicate-
 51 based substances (largely kaolinite and montmorillonite), have been part of the
 52 European *pharmacopoeia* for well over two millennia; they were used generically as
 53 ‘antidotes to poison’

54 **Aim of the study:** To test the antibacterial activity of three Lemnian and three
 55 Silesian Earths, medicinal earths in the collection of the Pharmacy Museum of the
 56 University of Basel, dating to 16th-18th century and following a prescribed
 57 methodology (see graphical abstract). To assess and prioritize the parameters which
 58 drive their antibacterial activity, if present.

59 **Materials and Methods:** The medicinal earths are characterised chemically (ICP-
 60 MS), mineralogically (both bulk (XRD) and at the nano-sized level (TEM-EDAX));
 61 their organic load (bacterial and fungal) is DNA-sequenced; their bioactivity (MIC₆₀)
 62 is tested against Gram-positive, *S. aureus* and Gram-negative, *P. aeruginosa*. The
 63 bioactivities (MIC₆₀) of natural clays from Lemnos, N Aegean, and Melos, SW
 64 Aegean, spiked with Al, Fe, Ti, and B are also tested against the same pathogens for
 65 purposes of comparison.

66 **Results.** Not all MEs are antibacterial. Of the three Lemnian Earths, only two are
 67 antibacterial against both pathogens; of the Silesian Earths only one is mildly
 68 antibacterial and against Gram-negative pathogen, only. The bioactivity of the two
 69 Lemnian Earths is driven by a fungal component, *Talaromyces spp*, a fungus of the
 70 family of *Trichocomaceae* (order Eurotiales), historically associated with *Penicillium*.
 71 This fungus was not found in the natural Lemnos clays examined here. Comparable
 72 bioactivity with that of the two Lemnian Earths can be obtained from
 73 kaolinitic/smectitic clays spiked with B or Al.

74 **Conclusions.** It is not known whether archaeological medicinal earths were used as
 75 antibacterials, over and above as absorbants of toxins. Nevertheless, some display
 76 antibacterial properties which appear to have their origins in an organic (fungal) load.

77
 78 **Keywords:** medicinal earths, Lemnian, Silesian, bioactivity, *Talaromyces spp*,
 79 mineral, nanoparticle, antimicrobial resistance

80 *1.Introduction*

81 Medicinal Earths (MEs) have been part of the European *pharmacopoeia* for well over
82 two millennia. Stamped medicinal earths or *terra sigillata* are natural aluminosilicate-
83 based substances (largely kaolinite and montmorillonite) with a well-recorded history
84 of use as ‘antidotes to poison’ and spanning over two and a half millennia
85 (Macgregor, 2013). Stamping the earth with a readily identifiable seal conferred
86 confidence on the product but also provided control of the trade in such substances
87 from antiquity to modern times (Nutton 2004). Lemnian Earth (LE), from the island
88 of Lemnos, N.E. Aegean, was the oldest and most established and with continuous
89 use until the early 20th century (Hasluck, 1910; Sealy, 1919). It is reported, amongst
90 others, in Theophrastus (4th c BC), Dioscorides (1st c AD) and Pliny (2nd c AD).
91 Galen (2nd c AD) visited the island and gave a detailed account of the various stages
92 in the process of extraction and ‘washing’ thereof, both activities purported to have
93 taken place once a year (Brock 1929, 185). Sometime in Late Antiquity and early
94 Byzantine times the practice appears to have waned or stopped completely but it was
95 certainly revived in the Ottoman period when its extraction and distribution was
96 tightly regulated (Hasluck and Hasluck, 1929; Tourptsoglou-Stephanidou, 1986). As a
97 result, many new MEs began to appear in the markets across the Mediterranean,
98 Northern Europe and the Middle East aiming to emulate (and rival) LE’s widely
99 acknowledged beneficial properties. Most carried the same tradition of being stamped,
100 hence their generic name, *terra sigillata*.

101 In the 16th c the most prominent medicinal earth emerging out of Central Europe was
102 Terra Silesia, in present day SW Poland (Dannenfeldt,1984). It was also a *terra*
103 *sigillata* since it bore the coat of arms of the city of Striga (Strigovia, Striegau, or
104 Strzegom) consisting of three mountain peaks (see Fig. 1f). Terra Silesia acquired
105 quite a reputation with doctors following the Paracelsus school who attributed its
106 healing properties to the gold within the local auriferous granite. Medicinal earths
107 from varying localities survive to this day in a number of museum collections as
108 collectors’ curiosities (Duffin, 2013). Only very few have been subjected to analysis
109 (Hardy and Rollinson 2016).

110 Over many years we have carried out research into some of the minerals discussed in
111 the Greco-Roman technical and medical texts, from the perspective of geo-
112 archaeological work and in an attempt to locate them in the field (Photos-Jones and
113 Hall, 2011; Photos-Jones et al., 2015, 2016). We have suggested reasons why LE
114 would have been efficacious, based on sampling of local sedimentary clays from the
115 purported area of its extraction (Kotsinas, N.E. Lemnos) (Hall and Photos-Jones,
116 2008). Recently we have analysed three samples of Lemnos *terra sigillata* (we will
117 refer to them as Lemnian Earth) in the collection of the Pharmacy Museum of the
118 University of Basel (Fig. 1a-c) and qualitatively assessed their antibacterial activity
119 (Photos-Jones et al., 2017).

120 This paper revisits the same three samples of Lemnian Earth (Fig. 1a-c: 700.4, 700.17,
121 700.18) in an attempt to assess the same activity, quantitatively, (MIC₆₀), against two
122 pathogens (Gram-negative *P. aeruginosa* and Gram-positive *S. aureus*). These
123 specific bacterial strains were chosen because of their relation to public health issues,
124 and their use as valuable bacterial indicators. Further, it compares their efficacies as
125 antibacterials with a contemporary set of Silesian Earths (Fig. 1d-f 703.1, 703.2,
126 703.3), also dating to 16th-18th century AD. Although all six MEs were purported to

127 be medicinal, only two were found to be demonstrably antibacterial, and against both
128 of the above pathogens. Suffice it to say that clays can still be considered ‘medicinal’
129 even though they might display no antibacterial action. Antibacterial action is a
130 property that we are interested in because it is easily measurable and quantifiable. We
131 therefore asked the question: which parameters drive differences in antibacterial
132 action between different samples of medicinal earths? Is it their mineralogy, at both
133 bulk and nanosized level? or is it their elemental composition? or other factors?

134

135 By other factors we refer to the MEs’, natural or acquired organic load, their bacterial
136 and fungal microbiome. Bacteria and fungi, naturally present within soils, can
137 potentially have the inadvertent effect of rendering the clays medicinal (as
138 antioxidants, antibacterials or metal chelators) on account of their production of
139 secondary metabolites (e.g., Keller, 2019; Pettit, 2011). This is the result of either
140 intra- and inter-specific interactions (Tyc et al., 2016), or toxicological conditions, the
141 presence of metals (Locatelli et al., 2016) or salts (Medina et al., 2015). It can also be
142 the result of their growth conditions. Ecologically, the production of secondary
143 metabolites is advantageous to the microorganism since it increases its
144 competitiveness or survival in the environment (Macheleidt et al., 2016).

145

146 We therefore propose a step-by-step investigation of the MEs, from the perspective of
147 bulk mineralogy, chemistry of the leachate, and nanoparticle characterization,
148 followed by DNA sequencing of their microbiome, and MIC₆₀ testing, against specific
149 pathogens in order to shed light into the contribution of individual components to the
150 MEs’ bioactivity. The graphical abstract at the start of the paper illustrates our
151 proposed method which consists of the undertaking of a number of analytical
152 techniques aimed to evaluate: a. the bulk mineral (XRD); b. the mineral leachate
153 (TEM/EDAX/ICP-MS); c. the organic constituent (DNA sequencing of biome/GC-
154 MS of secondary metabolites); d. the testing of bioactivity against select pathogens. In
155 this study we have not undertaken the investigation of secondary metabolites (via GC-
156 MS)but refer to them in previous work (Photos-Jones et al., 2017).

157

158 Having outlined our approach, (graphical abstract), we shall demonstrate that some
159 clay samples can be rendered antibacterial on account of specific elements and/or
160 nanosized particles, while others, with similar mineralogy, can be rendered
161 antibacterial on account of their microbiome, bacterial or other. Demonstrating the
162 idea that there might be multiple drivers to antibacterial activity within the same type
163 of clay could potentially have far reaching implications.

164

165 Resistance against effective antibiotics has emerged as a serious and growing
166 phenomenon in contemporary medicine, making the growth inhibition of virulent
167 pathogens for humans and the environment quite a challenge (Manaia et al., 2016;
168 Venieri et al., 2017a, 2017b). Clays have the potential to exhibit bactericidal effect in
169 both Gram-negative and Gram-positive strains through the exchange of components
170 between them and bacteria and the ultimate prevention of the latter’s metabolic
171 functions (Haydel et al., 2008). Clay nanoparticles- based techniques have already
172 been introduced to induce antibacterial action within aquatic environments and during
173 water treatment (Unuabonah and Taubert, 2014). Although bacteria are considered
174 very adaptive to hostile conditions, up until now no resistance mechanism similar to
175 that against antibiotics has been recorded in clays (i.e. induction of antibiotic
176 resistance genes).

177 Archaeological medicinal earths are clay-based and have an uninterrupted history of
 178 use (in the case of LE) of over two millennia. It is not clear whether they were
 179 intended as antibacterials, and not merely as absorbants of toxins. Nevertheless, with
 180 this paper and the one preceding it (Photos-Jones et al., 2017) we have demonstrated
 181 that some *can* be antibacterial. What we seek to understand is the parameters driving
 182 this antibacterial behaviour in a small number of archaeological medicinal clays.

183

184 **INSERT FIG. 1**

185



Fig. 1a 700.4
Lemnian Earth
Mus. No 01432

Fig. 1b 700.17
Lemnian Earth
Mus. No 01422

Fig. 1c 700.18
Lemnian Earth
Mus. No 01424

Fig. 1d 703.1
Terra Silesia
Mus. No 01114

Fig. 1e 703.2
Terra Silesia
Mus. No 01133

Fig. 1f 703.3
Terra Silesia.
Mus. No 01137

186

187 *2. Materials and Methods*

188

189 *2.1 Materials*

190 A total of eighteen samples were examined in this study; they fall into three groups:
 191 a. six MEs consisting of three from Lemnos (LEs) (700.4, 700.17 and 700.18) and
 192 three from Silesia (SEs) (703.1, 703.2, 703.3). This group of samples derive, as
 193 mentioned earlier, from the collection of the Museum of Pharmacy, University of
 194 Basel (museum accession numbers are given in Fig. 1).

195 b. four natural clays consisting of two from Lemnos, N.E. Aegean (700.19 and
 196 700.20) from the area of Kotsinas, NE Lemnos, the purported area of extraction of
 197 LE and another two from Melos, S.W. Cyclades. The Melos bentonite sample (933)
 198 originates from the Angeria mine, N.W. Melos, and the Melos kaolin sample (900.9).
 199 from the abandoned kaolin mine at Loulos, 2 km north of the Paleochori Bay, S.E.
 200 Melos. The Melos samples are introduced here as ‘good’ basic clays with which to
 201 build synthetics. c. eight synthetic samples prepared from Melos bentonite and kaolin
 202 and spiked with four different elements (i.e. Ti, Al, Fe, and B). These include Melos
 203 smectite and kaolinite treated with aluminum sulfate ($\text{Al}_2(\text{SO}_4)_3 \cdot 16\text{H}_2\text{O}$), (samples 6
 204 and 7 respectively); smectite and kaolinite treated with boric acid (H_3BO_3) (samples
 205 4 and 5, respectively); smectite and kaolinite treated with natural fine iron oxides
 206 collected from the island of Kea, N Cyclades, (Photos-Jones et al., 2018)(samples 14
 207 and 15, respectively); and finally, smectite and kaolinite treated with analytical grade
 208 TiO_2 (anatase, Merck) (samples 10 and 9 respectively). The Kea samples have been
 209 chosen on account of the purity/finesse of their iron oxides and their recorded use
 210 from the 4th c BC.

211 The synthetic aluminium sulfate- and boron- treated samples were prepared as
 212 follows. 1g of clay (kaolin or bentonite) were placed in 50ml polyethylene centrifuge
 213 tubes. 15 ml of 1N H_3BO_3 or 0.5 N $\text{Al}_2(\text{SO}_4)_3$ (both of Sigma Aldrich analytical
 214 grade) solution were added, the clays were dispersed in an ultrasonic probe for 20s,
 215 and the tubes were covered with a stopper and left overnight. Subsequently, the
 216 suspensions were centrifuged, the clear supernatant solutions were decanted and the

217 whole procedure was repeated. In the following day the suspensions were centrifuged
218 and the tubes with the clay were dried at 60°C and the dry clay powders were
219 transferred in glass vials and stored. The synthetic samples with addition of iron
220 oxides and TiO₂ were prepared by adding 0.1 g to 0.4 g of bentonite or kaolin. The
221 materials were ground with an agate pestle and mortar using acetone to obtain fine
222 grained homogeneous mixtures.

223

224 2.2 Methods

225 2.2.1 Mineralogy - XRD

226 The mineralogical composition of all samples was determined with X-ray diffraction
227 (XRD), at the School of Mineral Resources Engineering, Technical University of
228 Crete, on a Bruker D8 Advance Diffractometer equipped with a Lynx Eye strip silicon
229 detector, using Ni-filtered CuK α radiation (35 kV, 35mA). Data were collected in the
230 2 θ range 3-70° 2 θ with a step size of 0.02° and counting time 1 s per strip step (total
231 time 63.6 s per step). The XRD traces were analyzed and interpreted with the Diffrac
232 Plus software package from Bruker and the Powder Diffraction Files (PDF). The
233 quantitative analysis was performed on random powder samples (side loading
234 mounting) by the Rietveld method using the BMGN code (Autoquan© software
235 package version 2.8).

236

237 2.2.2 Bioactivity testing

238 2.2.2.1 Bacterial strains and antimicrobial tests

239 The bacterial indicators used for the assessment of antimicrobial properties of the
240 samples were *Pseudomonas aeruginosa* NCTC 10662 (Gram-negative) and
241 *Staphylococcus aureus* NCTC 12493 (Gram-positive). Both bacteria were cultured on
242 LB agar (LABM) and LB broth (LABM) and the desired bacterial concentration in
243 each experimental run was adjusted based on the McFarland scale, according to
244 which, an inoculum absorbance of 0.132 measured at 600 nm corresponds
245 approximately to a cell density of 1.5×10⁸CFU/mL. Our goal in this study was to
246 employ both a Gram-negative and a Gram-positive species, considering their
247 structural differences and physiology, which impose adverse behaviour in stressed
248 environmental conditions. Both bacteria are often reported for their notable antibiotic
249 resistance and their adaptability in hostile surroundings (Swetha et al. 2010; Venieri et
250 al. 2017b).

251

252 2.2.2.2 Sample preparation and antimicrobial tests

253 The antibacterial activity of the samples was assessed over both bacterial indicators
254 using their aqueous leachates. All leachates were prepared at a concentration of 600
255 mg/mL, mixing samples with sterile deionized water, followed by ultrasonication
256 (Julabo ultrasonic bath) for 30 min at 25 °C and centrifugation at 10000 g for 15 min
257 to remove all solids from the solution. The leachate was decanted, sterilized in the
258 autoclave (20 min, 120 °C), and tested against bacteria.

259

260 In order to compare the difference in activity of samples with and without organic
261 content, chemical oxidation was performed to breakdown organic matter with sodium
262 hypochlorite (NaOCl) as the oxidizing agent (Anderson, 1963). An aliquot 4 mL of a
263 NaOCl (6%) solution was mixed with of 2g of each sample into a centrifuge tube,
264 which was then placed in a boiling water bath for 15 min. Then, the sample was
265 centrifuged at 800 g for 10 min and the solution was decanted. The procedure was
266 repeated 3 times, after which the solid was washed with sterilized water, dried and

267 processed for further biological analysis. For reference to this protocol, see Andrews
268 (2001).

269

270 Antimicrobial activity of all samples (prior to and post chemical oxidation) was
271 studied using the broth microdilution method and estimating the Minimum Inhibitory
272 Concentration that inactivated 60% of the bacterial population (MIC_{60}). MICs were
273 measured labeling 96-well sterile microtiter trays with dilutions of each sample. The
274 bacterial inoculum in each case was adjusted to 10^5 CFU/mL. Microtiter trays were
275 incubated at 37°C for 18-24 h, followed by optical density measurement at 630 nm,
276 using a microplate reader (Labtech LT-4000 Plate Reader) and Manta LML software.

277

278

279 *2.2.3 Chemical analyses of leachates-ICP-MS*

280 The aqueous leachates were produced by adding 0.2 g of the samples in 5 ml distilled
281 water, dispersing with ultrasonic probe for 20 s, allowing standing for 1 h and
282 subsequent centrifugation. The supernatant was stored in polyethylene bottles for
283 ICP-MS analysis (7500CX coupled with Autosampler Series 3000, both by Agilent
284 Technologies) for major and trace elements. The precision of the analyses was tested
285 using elemental standards (1000mg/L) by Merck . The relative standard deviation of
286 the analyses varied according to the concentration, typically 7% for the major
287 elements, less for the trace elements.

288

289

290 *2.2.4 TEM-EDAX*

291 For Transmission Electron Microscopy (TEM) with EDAX approximately 10mg of
292 powder were suspended in 10ml of ultrapure water. The suspension was vortexed for
293 1 minute at full power (Rotamixer Hook and Tucker Ltd.) The sample was processed
294 in the ultrasonic bath for 5 minutes (Branson 1510 ultrasonic bath) and centrifuged in
295 15ml tubes in the Eppendorf centrifuge 5804R, at 4,000rpm for 10 minutes for clay
296 samples, and at 2,500rpm for 11 minutes for iron oxide samples, to remove particles
297 above 450nm. A drop of 35 microliters of the supernatant was deposited onto
298 200mesh copper grids with carbon film and left there without drying for one hour.
299 The excess sample was wicked from the grid, and the grid was washed 4x in water to
300 remove any salts. The grid was dried for 16 hours before use. TEM images and EDX
301 measurements were performed by the Birmingham University Centre for Electron
302 Microscopy, using a Jeol 2100 microscope.

303

304 *2.2.5 Particle size analysis*

305 Particle size for the samples were measured by DLS using a Malvern Instruments
306 Zetasizer nano ZS with a red (366 nm) laser. A method was developed to remove the
307 larger particles and provide stable suspensions of the smaller particles for DLS
308 analysis. The powders were dispersed in a 0.2% suspension of Novachem surfactant
309 (Postnova Analytics UK Ltd.) in ultrapure water. The suspension was shaken
310 thoroughly, vortexed (Rotamixer, Hook and Tucker Ltd.) at full power for 30 s and
311 treated in the ultrasonic bath (Branson, 1510) for 5 min. The samples were centrifuged
312 at 3000 rpm, for 5 min in 15 ml tubes using an Eppendorf 5804R centrifuge. This
313 removed the larger particles from the samples. Stable suspensions were obtained
314 under these conditions.

315 The zeta potential measurements of these suspensions were negative, and between
316 -40 and -50 mV, due to the effect of the Novachem surfactant which produced a high
317 surface charge. It follows that the zeta potential was altered and so is not
318 representative of the original material. However, this enabled us to stabilise the
319 suspensions and allowed reproducible size measurements to be made for the smallest
320 particulate size fraction.

321 2.2.6 DNA sequencing

322 DNA were extracted using MoBio PowerSoil Extraction kits (Qiagen) according to
323 manufacturer's procedures, except sample materials were agitated using a FastPrep24
324 cell homogenizer (MP Biomedicals; 6.0 speed, 2 x 20 seconds). Additionally, samples
325 were initially incubated at 70C for 10 minutes to facilitate the DNA extraction from
326 Gram-positive microorganisms.

327
328 Purity and quantity of extracted DNA were measured using UV-micro-
329 spectrophotometry. Extracts are stored at -80°C and further handled under UV-
330 irradiated biological cabinets with HEPA-filter laminar-flow air flow. Samples were
331 routinely diluted 1:50 with molecular-grade water to minimize inhibitors and improve
332 reaction efficiency of downstream processes.

333
334 Polymerase chain reaction (PCR) was used to selectively target the hypervariable V4
335 region of the 16S-rRNA gene. Primers were forward (AYTGGGYDTAAAGNG;
336 position 563-577) and combined set of reverse (TACNVGGGTATCTAATCC,
337 TACCRGGGTHTCTAATCC, TACCAGAGTATCTAATTC, and
338 CTACDSRGGTMTCTAATC; position 907-924). To minimize cost, primers were
339 further 'bar-coded' with a short 8-base genetic sequence to allow multiple samples to
340 be simultaneously sequenced and sorted post-analytically using RDP initial pipeline
341 bioinformatics tool (Cole et al., 2014;
342 <http://pyro.cme.msu.edu/><<https://mail.campus.gla.ac.uk/owa/redir.aspx?C=QAAlSmAq6PZR-ZWaTX0sUwu9AOPrSgI-UF8DmdH3dbzOhsDWft3UCA..&URL=http%3a%2f%2fpyro.cme.msu.edu%2f>>).

345
346 The presence of fungal and chloroplast DNA were tested by PCR with primers
347 targeting the 18S-rRNA gene (Hadziavdic et al., 2014) and 16S-rRNA gene of
348 chloroplasts, using aforementioned bacterial forward primer (position 563-577) and
349 the CYAN-786-a probe modified to become a reverse primer (Knapp and Graham,
350 2004), respectively.

351
352 Subsequent analysis found previous universal primers for detecting fungus failed to
353 detect members *Talaromyces spp.* As such, additional de novo primers were designed
354 (in this study) for the detection of *Talaromyces sp.* (via intergenic spacer region)
355 based on Genbank accession (JN899375) using NCBI's Primer-BLAST online design
356 tool: TTGAGGGCAGAAATGACGCT (forward, 5'-3') and
357 TGAAGAACGCAGCGAAATGC (reverse, 5'-3'). In silico analysis of primer
358 specificity via BLASTn predict detection of *Talaromyces spp.* and *Penicillium spp.*,
359 both of the Trichocomaceae family of Eurotiales order.

360
361 Each 100 μL PCR reaction mixture consisted of 10 μL of diluted DNA sample, 50 μL
362 Taq PCR Master Mix kit (Qiagen; consisting of 1.5 mM MgCl_2 , 2.5 units of Taq
363 DNA polymerase, 1x proprietary PCR buffer, and 200 mM of each dNTP), 10 μL 10x-

364 primer mixture (0.2 μ M final concentration of each primer). Reaction conditions were
 365 as follows, on a BioRad iCycler5 (BioRad, Hercules, CA USA) instrument: 3-min
 366 initial denaturation (94°C); 30 cycles of: denaturation (30s at 94°C), primer annealing
 367 (30s at temperatures specific for each assay: 58°C for 16S-rRNA and chloroplast, and
 368 60°C for *Talaromyces*), and product extension (1 min at 72°C); and a final extension
 369 (10 min at 72°C). When completed, the instrument maintained the samples at 8°C.

370

371 To remove excess primers and un-polymerised nucleotides for bacterial DNA
 372 sequencing, PCR product were purified using QiaQuick PCR Purification kit
 373 (Qiagen). Quantities of PCR product were quantified by UV micro-
 374 spectrophotometrically, combined, and condensed to 30mL, > 20 ng/mL. Library
 375 preparation (e.g., adapter ligation) and MiSeq high-throughput sequencing (Illumina)
 376 were conducted by GATC-Biotech (Konstanz, Germany) with full quality-control and
 377 quality-assurance. The number of MiSeq reads per sample varied. Phylogenetic
 378 identity of each sequence was determined based on alignments with the “Classifier”
 379 function (Wang et al., 2007) of the RDPpipeline, which maintains databases for 16S-
 380 (and 18S-) rRNA sequences (Cole et al., 2014). The bootstrap cut-off was
 381 predetermined to be 70% based on sequence length.

382

383 3. Results

384 3.1 The mineralogy of MEs and natural clays

385 Table 1 shows the results of XRD analysis of six MEs and the four natural clays from
 386 Melos and Lemnos. LE 700.4 and 700.17 consist of kaolinite, illite and quartz, with
 387 dolomite being the dominant phase in 700.4 and hematite being a minor phase in
 388 700.17. 700.18 consists of smectite, quartz, illite and albite. The Silesian Earths (SEs)
 389 are primarily kaolinite with illite with varying amounts of quartz and small quantities
 390 of anatase. Melos 900.9 and 933 are natural kaolinitic and smectitic clays
 391 respectively, while chlorite and alunite are present in natural Lemnos clays (700.19
 392 and 700.20). Varying amounts of iron oxide are present in the three red samples
 393 (703.2, 700.17, 700.19).

394

395 INSERT TABLE 1

396 **Table 1** XRD analyses of Silesian and Lemnian Earths; also, geological samples from
 397 Kotsinas, Lemnos, 700.19 and 700.20 and geological samples from Melos 933 and
 398 900.9 (see discussion) included here for purposes of comparison; n.d. = not detected.
 399 Samples with asterisk (700.4, 700.17, 700.18, 700.19 and 700.20) were first published
 400 by Photos-Jones et al (2017).

401

Mineralogical Composition	703.1 Terra Silesia	703.2 Terra Silesia	703.3 Terra Silesia	700.4* Terra Lemnia	700.17* Terra Lemnia (after Photos-Jones et al 2017)*	700.18* Terra Lemnia	700.19* Natural red clay from Kotsinas, Lemnos	700.20* Natural clay from Kotsinas, Lemnos	933 natural smectite (Melos)	900.9 natural Kaolin (Melos)
Dolomite	n.d.	n.d.	n.d.	65.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Kaolinite	31.2	87.9	67.4	17.3	37.4	n.d.	69.3	1	6.3	48.6
Smectite/ montmorillonite	n.d.	n.d.	n.d.	n.d.	n.d.	66	n.d.	35.1	71.7	n.d.
Quartz	32.8	n.d.	25.8	7.6	17.7	6.9	n.d.	21	0.2	28.9

Opal-CT/ cristoballite	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.5	n.d.	n.d.	5.9
Illite	28.1	n.d.	6.5	9.9	41	18.1	n.d.	13.3	n.d.	n.d.
Anatase	1.2	4.6	0.3	n.d.	n.d.	n.d.	n.d.	n.d.	1.3	0.1
Albite	n.d.	n.d.	n.d.	n.d.	n.d.	9	n.d.	12.7	n.d.	n.d.
Alunite	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	22.5	n.d.	n.d.	3.3
Biotite	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Calcite	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.1	5.2	n.d.
Chlorite	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.9	n.d.	n.d.
Halite	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6.2
Hematite	n.d.	7.5	n.d.	n.d.	3.8	n.d.	1.8	n.d.	n.d.	n.d.
K-Feldspar	6.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	14.8	n.d.
Natroalunite	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7
Pyrite	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.5	n.d.
Tridymite	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.9	n.d.	n.d.	n.d.

402

403 In summary, the six archaeological and the four natural clay samples are broadly
 404 classified as either kaolinitic (700.17, all SEs, 700.19 and 900.9) or as smectitic
 405 (700.18, 700.20 and 933). 700.4 is primarily dolomitic with some kaolinite. In the
 406 section that follows we proceed to establish which of the above are bioactive.

407

408 3.2. Antibacterial activity of MEs, natural and synthetic clays

409

410 We first investigate the antibacterial activity of the six archaeological samples and the
 411 two Lemnos natural clays (700.19, 700.20) (Fig. 2 and Suppl file 1). The MIC₆₀ of the
 412 LEs is significantly lower than the MIC₆₀ of the SEs. The ranges of MIC₆₀ values of
 413 LE were 50-90 mg/mL and 12.5-45 mg/mL for *P. aeruginosa* and *S. aureus*,
 414 respectively, with the leachate of 700.17 being more active than the others. The
 415 respective MIC₆₀ values for SE were 66-264 mg/mL for *P. aeruginosa* and 132
 416 mg/mL for *S. aureus*, respectively. The order of bioactivity of the original samples
 417 towards the Gram-negative *P. aeruginosa* is 700.17 and 700.18 > 703.1 > 700.4 >
 418 703.2 > 703.3; The order of bioactivity of samples towards the Gram-positive *S.*
 419 *aureus* is 700.17 > 700.18 > 700.4 > all 703 series samples. However, the aqueous
 420 leachates of 703.1; 703.2 and 703.3 can hardly be described as “antimicrobial”,
 421 because the obtained MIC₆₀ values were considerably over 50 natural mg/mL. Natural
 422 clay samples 700.19 and 700.20 show low to no bioactivity. A sample of natural near
 423 pure alunogen (Al₂(SO₄)₃·17H₂O) from the solfatara at Fyriplaka, SE Melos (sample
 424 3), displayed here for purposes of comparison, is the most bioactive.

425

426 In summary, only two LEs (700.17, 700.18) are bioactive; 700.4, a dolomitic clay
 427 with small amounts of kaolinite, is not bioactive against *P. aeruginosa*. Lemnos
 428 natural clays 700.19, 700.20 and all the SEs (703.1, 703.2 and 703.3) are not bioactive
 429 following the criteria implemented here (MIC₆₀ < 50mg/ml). Given that kaolinite-
 430 rich clays can be *both* bioactive (700.17) *and* non-bioactive (700.19, 703.2) we
 431 suggest that mineralogy is not a key factor driving bioactivity. The same conclusion
 432 applies for the smectitic clays (bioactive: 700.18; non-bioactive 700.20).

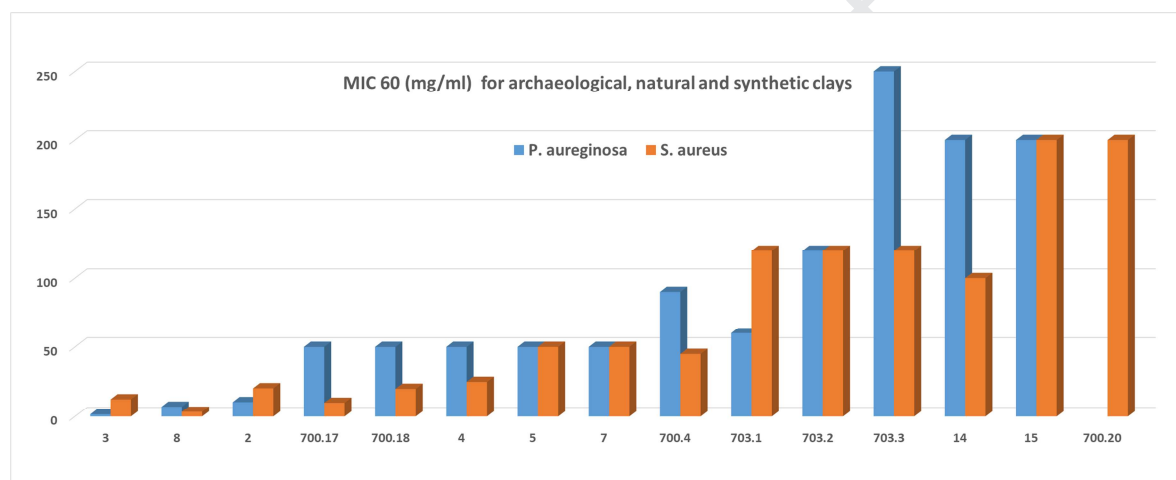
433

434 Turning now to the bioactivity of the synthetic samples (Suppl file 1 and Fig. 2),
 435 Melos smectite + B (sample 4) and kaolinite + B (sample 5) are the most effective
 436 synthetics against both *P. aeruginosa* and against *S. aureus*, with (4) being better than
 437 (5) re the latter. Melos kaolinite + Al (7) is equally effective. Melos smectite + Fe
 438 (15) and Melos kaolinite + Fe (14) are not bioactive against either Gram-negative
 439 and Gram-positive bacteria. Melos smectite + Ti (10) and kaolinite + Ti (9) are also
 440 non-active and neither is Al-spiked Melos smectite (6). Solutions of reagent-grade
 441 boric acid (2) and reagent grade aluminium sulphate (8) are also included here for
 442 comparison. As already mentioned the most effective antibacterial is sample 3, natural
 443 alunogen, from SE Melos, which is not a clay.

444

445 **INSERT FIG.2**

446



447

448

449 Fig. 2 Illustration of relative bioactivity between archaeological MEs, natural
 450 (700.19, 700.20) and synthetic samples consisting of kaolinitic/smectitic clays
 451 spiked with Al, B, Ti and Fe. The absence of a bar indicates lack of bioactivity. Blue
 452 denotes *P. aeruginosa* and Red *S. aureus*.

453

454 In summary, the most active samples against *both* pathogens are the two LEs, 700.17,
 455 700.18, as well as the two boron-rich synthetic samples (4 and 5) and the aluminium-
 456 rich kaolinite (7). It is not clear why this sample is bioactive while the aluminium-rich
 457 smectite (6) is not. As for clays spiked with Ti (9 and 10) or Fe (14 or 15) they were
 458 inactive. What transpires from the above results is that smectitic clays can be *both*
 459 bioactive (700.18) *and* non bioactive (700.20). Equally, kaolinitic clays can be *both*
 460 bioactive (700.17) *and* non bioactive (700.19). It follows that bulk mineralogy does
 461 *not* drive bioactivity. Referring back to the graphical abstract, the next step is to
 462 investigate in detail the chemical make-up of the leachate of the bioactive MEs and
 463 synthetics and the range of trace elements associated with each group. Suffice it to say
 464 that the graphical abstract has no prescribed sequence in the analysis. It is the
 465 combined results from all techniques and how one feeds into the other that help shape
 466 our understanding of the antibacterial efficacy of these clays.

467

468

3.2. The chemical composition of the leachates of the bioactive MEs

469

470 Table 2 compares the chemical composition of the leachates of the six MEs, the two
 471 natural clays from Lemnos (700.19 and 700.20) and the two from Melos (900.9 and
 472 933) and three synthetics (4, 5 and 7). The range of parameters appears at first
 473 bewildering and comparisons on an element by element basis seems to confuse rather
 474 simplify the picture.

475 The chemical composition of the six archaeological MEs, the four naturals (700.19,
 476 700.20, 900.9 and 933) and the bioactive synthetics (4, 5 and 7) is shown in Table 2.
 477 Focusing on the three bioactive synthetic samples above and the two bioactive
 478 archaeological LEs (700.17 and 700.18) we show that 700.17 is more abundant in Al,
 479 Ti, V, Cr, Cu, Sr and Ba than, for example 900.9. The latter is deficient in Al and thus
 480 it is not expected to be bioactive. It is only with enhanced amounts of (spiking with) B
 481 and Al that Melos kaolinite can match the bioactivity of archaeological LE 700.17.
 482 On the other hand, comparison of smectitic LE 700.18 with the natural Melos
 483 smectite 933, suggests that this latter clay cannot be bioactive, either, unless spiked
 484 with Al given the small concentrations in that element as well as, Ti, V, Cr and Cu.
 485 Regarding the natural Lemnos clays, 700.19 and 700.20, although rich in Ti, V, Mn
 486 and Ba, it has been demonstrated that they are not bioactive (Fig. 2).

487
 488 Turning now to the SEs, the B content (in ppb) in the leachates is higher than that of
 489 the LEs and yet the SE with the highest boron (703.2) is not antibacterial. In the case
 490 of 703.1, the Al content of the leachate is very low and yet this particular ME is
 491 antibacterial (against *P. aeruginosa*). Ti concentration is the highest in 703.2 and
 492 700.19 and yet none of these two samples are bioactive. Interestingly sample 703.2,
 493 the richest in iron oxide (Table 1) has a very low Fe content in the leachate, compared
 494 to 700.17 and 700.18. Finally, Melos smectite spiked with Al (6) is non-bioactive
 495 despite having similar or near-similar Al contents as the bioactive MEs. This may be
 496 due to the fact that Al is precipitated due to the buffering capacity of smectite.

497
 498 In summary, there is no obvious correlation between elemental composition of the
 499 leachate and bioactivity, and in reference to the elements investigated in detail here,
 500 namely Ti, Al, B, Fe. Samples with the above elements, whether MEs, naturals or
 501 synthetics, can be *either* bioactive or non-bioactive.

502

503 INSERT TABLE 2

504 **Table 2** ICP-MS data for the leachates of MEs, natural Lemnos clays (700.19 and
 505 700.20) and synthetic clays (4, 5, 7): bdl= below detection ; adl= above detection
 506 limit. Concentraions of the major elements (Si, Al, Mg, Fe, Ca, Na and K) are in
 507 ppm. The remaining elements are in ppb.

508

	703.1	703.2	703.3	700.4	700.17	700.18	4	5	7	900.9	933	700.19	700.20
Na	6	6	6	1	2	3	adl	9	5	adl	17	nd	nd
Mg	12	2	4	59	1	10	22	14	3	22	1	nd	nd
Al	2	24	2	4	4	4	28	33	adl	0	1	nd	nd
Si	2	11	1	1	2	1	1	3	bdl	1	7	nd	nd
K	10	5	3	2	9	4	13	8	1	14	3	nd	nd
Ca	8	4	7	89	3	6	7	532	5	8	2	nd	nd
Fe	1	1	0	1	18	14	0	5	0	6	0	nd	nd

Li	40	42	20	33	24	2	4	18	7	1	8	6	76
B	35	52	14	7	16	12	bdl	6	2	35	20	5	12
Ti	41	1260	448	115	216	827	1	47	5	1	48	1478	485
V	bdl	8	2	26	51	71	bdl	bdl	bdl	bdl	3	95	49
Cr	2	1	1	13	38	7	bdl	bdl	4	bdl	1	32	62
Mn	1099	72	14	43	65	258	4	2	27	5	bdl	979	291
Co	3	1	0	bdl	1	4	bdl	2	bdl	bdl	bdl	14	9
Ni	34	38	14	bdl	4	9	4	19	24	5	9	13	82
Cu	15	18	20	176	17	31	8	2	15	8	7	28	14
Zn	324	543	119	3	14	29	26	83	452	36	6	34	35
As	bdl	bdl	12	3	77	5	bdl	bdl	bdl	bdl	bdl	5	0
Se	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	18	nd	nd
Rb	5	8	5	16	52	25	4	103	1	4	8	29	38
Sr	32	51	45	323	52	25	209	706	32	214	10	301	92
Y	1	bdl	1	bdl	bdl	bdl	bdl	1	bdl	bdl	bdl	nd	nd
Mo	4	6	2	bdl	bdl	bdl	4	2	1	4	1	nd	nd
Cd	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	nd	nd
Sn	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	nd	nd
Sb	2	2	2	bdl	bdl	bdl	0	2	2	0	bdl	nd	nd
Cs	5	11	6	2	12	4	4	22	3	4	5	1	2
Ba	31	27	60	53	136	629	71	13	51	73	bdl	865	78
Hg	11	10	0	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	nd	nd
Pb	bdl	1	7	17	10	40	7	bdl	0	7	1	33	8
U	0	0	0	bdl	bdl	bdl	8	3	0	0	0	nd	nd

509

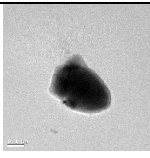
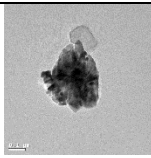
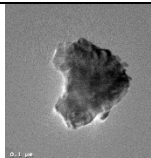
510 *3.5.Nanoparticle analysis*

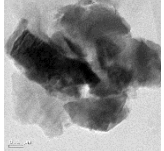
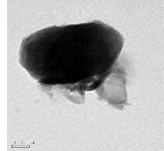
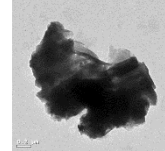
511 TEM/EDX data of the fine fractions of the bioactive samples (700.17, 700.18) are
512 shown in Fig. 3. For the rest of the non-bioactive samples see and suppl files 2a-b.
513 The particles of 700.17a and c are characterized by the presence of anatase (TiO₂
514 polymorph) even though it was not detected in the bulk sample (Table 1). The
515 analysis of 700.17b is consistent with silicates and clays containing Fe and low levels
516 of titanium oxide, and may represent a mixture of phases. The cobalt and vanadium
517 present are also seen in the ICP-MS data (Table 2) and may be associated with
518 titanium oxide minerals.

519

520 **INSERT FIG. 3**521 **Fig. 3** Nanoparticles of bioactive LEs

522

						
700.17a (400nm)	700.17b (400nm)	700.17c (465nm)				
			700.17a	700.17b	700.17c	700.18a
						700.18b
						700.18c
			Na			0.34
			Mg			0.52
			Al	0.91	1.27	0.25
			Si	2.85	38.75	0.37
			P			6.18
			S			6.85
			K		0.19	2.19
			Cl			0.13
			Ca			3.59
						0.09
						0.62
						0.62

			
700.18a (>1000nm)	700.18b 1240nm	700.18c (1560nm)	
			Analysis of nanosized particles for Lemnos medicinal earths (in %). Blanks denote absence

523

524 The particles from 700.18 are of three different compositions: the analysis of particle
525 700.18c is consistent with a mixture of clay minerals and anatase, with cobalt and
526 vanadium being present. The XRD analysis (Table 1) did not show any anatase in the
527 bulk material, but the leachate showed high Ti levels (Table 2). In contrast, the
528 composition of 700.18b is consistent with the presence of quartz and illite/mica and
529 low levels of clay minerals, in line with the XRD data for the bulk material (Table 1).
530 The composition of 700.18a is variable, with a wide range of elements present. The
531 levels of phosphorous and sulfur may indicate some organic material. There also
532 appears to be clay minerals present. Suppl files 2 show TEM images and EDAX table
533 of data for the non-bioactive samples, whether MEs (SEs) or naturals (Lemnos
534 natural clays).

535

536 In summary, the nanosized fractions of 700.17 and 700.18 reflect their bulk
537 compositions, but they are not informative of the difference in bioactivity between
538 these two samples and the non-bioactive samples.

539

540 Regarding the particle size analysis data, the average diameters of the two
541 archaeological samples is 200nm (700.17) and 309nm (700.18) (see suppl file 2c);
542 their large standard deviations indicate highly variable particle sizes. The size
543 variation in these samples is attributed to the coexistence of clay and non-clay mineral
544 nanoparticles (quartz, anatase, carbonates, Fe-oxyhydroxides) which are expected to
545 have different sizes.

546

547 3.6. The organic load: DNA community analysis

548

549 The quantities of extracted DNA from all samples were relatively low (< 2ng/ul), or <
550 1.0 ng/ul, of starting material (Table 3); for example, 703.2 and 703.3 yielded 1.2 and
551 1.5 ng/ul, of DNA material, respectively. Although these values fell below the range
552 for quantification (i.e. [DNA] are not significantly greater than zero), bacterial signals
553 were noticed following PCR amplifications. Similar PCR screens, using
554 cyanobacterial/chloroplast 16S-rRNA gene-specific primers and presumed universal
555 primers for fungus (Hadziavdic et al., 2014), did not reveal any strong signals (except
556 trace chlorophyll/chloroplast in 700.18). As such, metataxonomic analysis of the
557 microbial communities initially focused on the bacteria. The results are presented in
558 Table 3 and in more detail in Suppl file 3.

559

560 INSERT TABLE 3

561

562 **Table 3** Bacteria identified from DNA extracted from four samples, based on 16S-
 563 rRNA meta-taxonomic analysis; their relative abundances are denoted, as % of total.
 564 Superscripts refer to the bacterial genus present within each sample.
 565

Bacterial phylum	700.17 ¹	700.18 ²	700.19 ³	703.1 ⁴	Genus
α-proteobacteria	44.6	55.8	0	30	<i>Bradyrhizobium</i> ^{1,2} , <i>Sphingomonas</i> ¹ , <i>Acidiphilum</i> ⁴ , <i>Brevundimonas</i> ¹ , <i>Devosia</i> ¹ , <i>Microvirga</i> ⁴
β-proteobacteria	41.7	4.7	0	0	<i>Achromobacter</i> ¹ , <i>Comamonas</i> ²
γ-proteobacteria	4.4	2.3	5	0	<i>Acinetbacter</i> ¹ , <i>Pseudomonas</i> ² , <i>Aeromonas</i> ³
ε-proteobacteria	0	2.3	0	0	<i>Acrobacter</i> ²
Actinobacteria	0	2.3	0	0	<i>Gaiella</i> ²
Bacteroidetes	2.2	18.6	0	0	<i>Flavobacterium</i> ^{1,2}
Chlorobi	0	0	15	0	<i>Chlorobium</i> ³
Chloroflexi	0.7	0	5	0	<i>Anaerolinea</i> ^{1,3}
Cyanobacterium	0	2.3	0	0	<i>GpXIII</i> ²
Firmicutes	0	2.3	5	0	<i>Staphylococcus</i> ² , <i>Clostridium</i> ³
Fusobacteria	0	0	5	0	<i>Fusobacteria</i> ³
Thermotogae	2.2	0	10	0	<i>Mesoaciditoga</i> ^{1,3}
Verrucomicrobia	0	0	0	10	<i>Spartobacteria</i> ⁴
Unknown	4.2	9.4	55	60	

566
 567 However, subsequent PCR-primer development and confirmatory DNA sequencing
 568 successfully detected DNA signatures related to the fungal Trichocomaceae family
 569 (Ascomycota (division), Eurotiomycetes (class), and Eurotiales (order)) with
 570 *Talaromyces*, or related, DNA often showing closest resemblance. The following
 571 concentrations were found based on qPCR: 700.18 (approx. 10² gene copies/mg) and
 572 700.17 (approx. 10¹ gene copies/mg). 703.1, which appeared to have stronger DNA
 573 sequence bias towards *Aspergillus* sp. than *Talaromyces* sp., had approx. 10³ gene
 574 copies/mg; signals in 703.2 were below detection.

575
576 To confirm the quality of PCR detection of the *Talaromyces* and related fungus
577 products were sent for DNA sequencing (Eurofins Scientific) and compared via
578 BLASTn algorithms to GenBank (National Centre for Biotechnology). All results
579 were represented closely-related clades within the Eurotiales phylogenetic order,
580 particularly: *Talaromyces*, *Penicillium* and *Aspergillus*. While it remains difficult to
581 recognise specific microorganisms from short DNA sequence from a single locus,
582 there were specific patterns in that could be discerned. Sample 700.17 showed the
583 strongest evidences of specific *Talaromyces* species, with commonalities among the
584 bi-directional reads, while the possibility for *Penicillium* sp. and *Aspergillus* sp. is still
585 present. Sample 700.18 was clearly represented by either *Talaromyces* or *Penicillium*;
586 both genera are teleomorphs, and *Talaromyces* sp. have historically been included
587 within the *Penicillium* nomenclature; as such organisms in this clade may be
588 mentioned in the literature interchangeably (Yilmaz et al., 2014; Frisvad, 2015),
589 which further complicates recognition. Sample 703.1 showed greater alignments with
590 *Aspergillus* and different *Penicillium* sp., and sample 703.2 had minimal DNA and
591 had the least conclusive data (with greater mis-alignment of the sequences). It should
592 be noted that the presence of a genus does not suggest antibiotic production, rather the
593 possibility of micro-organisms that may produce exometabolites.

594

595 4. Discussion

596 4.1 Bioactivity - the contribution of the inorganic component (in reference to elements 597 Ti, Fe, Al and B as leachates or nanoparticles)

598

599 A total of 21 samples consisting of six archaeological medicinal clays (700.4, 700.17,
600 700.18, 703.1, 703.2, 703.3), four natural clays (700.19, 700.20, 900.9, 933), eight
601 synthetic clays (4,5,6,7,9,10,14,15) deriving from the spiking of two natural clays
602 (900.9, 933) with B, Al, Ti, Fe and three (non-clay) controls (3, 2, 8) were examined
603 mineralogically, in bulk and for their nanoparticle composition, chemically, and also
604 tested for bioactivity against *P aeruginosa* and *S aureus* following a method
605 illustrated in the graphical abstract.

606

607 Of a total of eighteen samples examined, only archaeological samples 700.17, 700.18
608 and synthetic samples 4 (smectite spiked with B), 5 (kaolinite spiked with B), and 7
609 (kaolinite spiked with Al) displayed antibacterial action against *both* Gram-positive
610 and Gram-negative pathogens. For the purposes of this discussion we consider
611 bioactive the samples which display $MIC_{60} < 50\text{mg/ml}$. Natural clays, while not
612 naturally bioactive, were rendered bioactive with the addition of B and or Al.
613 However the archaeological samples 700.17 and 700.18 did not contain any
614 meaningful concentrations of these two elements. Therefore the question arises: why
615 are these two samples bioactive? Before addressing this issue we turn to a brief
616 review of the literature regarding antimicrobial clays and the mechanisms proposed,
617 so far, for their antimicrobial activity.

618

619 Various researchers have attributed the bioactivity of the antimicrobial clays they
620 have investigated to different reasons: a) to the toxic influence of heavy metals such
621 as Cu, Zn and Ni (Otto et al., 2010); b) to the role of nanosized accessory Fe^{2+} -
622 bearing phases, that might generate reactive oxygen species (Morrison et al. 2016;
623 Williams 2017); c) to the presence of soluble Al^{3+} (Londono et al., 2016); finally, d)
624 to the existence of Fe^{2+} -atoms in active unsatisfied bonds at clay mineral edges which

625 might form hydroxide radicals upon oxidation (Wang et al., 2007). These studies have
626 focused on specific ions and heavy metals, but they did not necessarily offer definitive
627 answers for clay antimicrobial activity. For example, when these proposed
628 mechanisms were applied to clays other than those involved in the studies (for
629 example Fe-saponite), they yielded contradictory results (Zarate-Reyes et al., 2018).

630

631 Interestingly, none of the above studies has acknowledged or sought to investigate the
632 presence and contribution of an organic load. Indeed, all researchers have used clays
633 which have been sterilized in the autoclave, prior to any detailed investigation, thus
634 destroying any microorganisms within. Here we examine both the nature of the
635 microbiome and the contribution of each one of the elements already discussed in
636 the above studies i.e. Al, Fe, Ti and B; also the role of some transition metals in
637 influencing the bioactivity of the archaeological medicinal earths. Since the toxic
638 influence of transition metals has already been discussed (Otto et al., 2010; Otto &
639 Heydel, 2013) we note that, although largely present in the SE samples, these samples
640 displayed low or no bioactivity. Therefore we conclude that transition metals did not
641 have a role to play in driving the bioactivity of the two LEs.

642

643 *Titanium*

644 Recent work has shown that TiO₂ nanoparticles readily produce reactive oxygen
645 species (ROS) which are toxic to the membrane cells of bacteria, when exposed to
646 visible light, especially in arid environments (Georgiou et al., 2015). The generated
647 ROS will be rapidly converted to H₂O₂ upon contact with water by dismutation (i.e.
648 simultaneous oxidation and reduction) (Halliwell and Gutteridge, 2015). In this case,
649 the antibacterial activity will be controlled firstly by the abundance of TiO₂ reactive
650 nanoparticles in the leachate and secondly by the probability of contact and reaction
651 between the generated H₂O₂ and the bacterial cells. In the present study, although
652 titanium is present in both MEs and is considerably higher in SEs (Table 2). However,
653 TiO₂ nanoparticles were found primarily in 700.17 and 700.18, the bioactive LEs
654 (Fig. 3) and not in the SEs despite the presence of c.4% anatase (TiO₂ polymorph) in
655 703.2.

656 The two LE samples are indeed the more bioactive, thus corroborating the
657 contribution to bioactivity of TiO₂ nanoparticles rather than their ionic counterparts.
658 Equally the synthetic control samples of kaolinite and smectite (9 and 10) (Table 2)
659 with 20% anatase nanoparticles did not yield leachates with, as anticipated,
660 antibacterial properties. This may be due to aggregation of TiO₂ nanoparticles to
661 larger particles, which decreased their activity. Therefore, we suggest that the
662 potential antibacterial role of TiO₂ nanoparticles should be examined carefully. The
663 formation of ROS as mentioned above (Georgiou et al., 2015), would have
664 necessitated a photochemical reaction and therefore is not relevant to the present
665 study. We conclude that the TiO₂ nanoparticles in 700.17 and 700.18 may play a
666 small role in the samples' bioactivity but their overall effect would depend on the
667 amounts present.

668 *Fe-oxides*

669 The LE and the SE samples do not contain traceable amounts of Fe²⁺-bearing phases,
670 such as pyrite, which might have contributed to their antibacterial potential; this
671 would have taken place via generation of ROS, causing detrimental effect on bacteria

672 through oxidative stress, penetration of the cell wall and destruction of cellular
673 components (Cagnasso et al., 2010; Morrison et al., 2016; Williams, 2017). LEs
674 700.17 and 700.18 do contain Fe⁺ above the rest of the samples. However, the Fe-
675 content of the nanoparticles present in the leachates of the LEs is considerably lower
676 than that of their counterparts in the SEs, with the exception of 703.1; which showed
677 low bioactivity. When natural clays were spiked with Fe (oxides/ oxyhydroxides)
678 hematite/goethite, (samples 14, 15), they showed no bioactivity. We conclude that
679 Fe⁺ in 700.17 and 700.18 might play a small role in the samples' bioactivity.

680 *Aluminium and Boron*

681 Aluminium originating from the dissolution of clay minerals and/or aluminium
682 sulphates in the leachates is toxic to cells and might trigger antibacterial action (e.g.
683 Londono et al., 2016; Williams, 2017). Similarly, boron has been reported to be
684 antibacterial (Photos-Jones et al., 2015). However, Al and B concentrations in the
685 leachates of 700.17 and 700.18 were very low compared to the rest of samples which
686 were not bioactive (Table 2). By contrast the Melos kaolinite spiked with alum
687 (sample 7, Fig. 2) and especially the Melos alunogen (sample 3, Fig. 2) showed
688 antibacterial activity, particularly the latter. Melos kaolinite and smectite spiked with
689 Boron are also equally antibacterial (Fig. 2). We conclude that Al and B are not
690 driving the bioactivity of 700.17 and 700.18.

691

692 *Nanoparticle active edges*

693 The possible contribution of the active nanoparticle edges on the
694 antibacterial/bacteriostatic activity of the leachates should also be considered. All
695 leachates contain phyllosilicates, mainly illite, kaolinite and smectite along with
696 anatase and dolomite. Carbonates are not considered to have antibacterial properties
697 and the possible role of TiO₂-polymorphs such as anatase was considered previously.
698 Therefore, in this section we focus on the possible influence of the nanoparticle active
699 edges of clay minerals. Smectite edges have been shown to have oxidative capacity
700 due to formation of superoxide oxygen radical by chemisorption of oxygen atoms in
701 crystallite edges (Thompson and Moll, 1973), caused by simultaneous oxidation of
702 structural Fe²⁺, which have been shown to have antibacterial activity (Wang et al.,
703 2007).

704 The oxidative capacity of kaolinite and illite has not been evidenced so far. In the
705 present study the octahedral Fe in smectites is considered to be in Fe³⁺ form, which is
706 not known to contribute to bioactivity. However, the formation of superoxide oxygen
707 radicals in smectite edges might be controlled by particle size as well (Gournis et al.,
708 2002). In this aspect the smectite nanoparticles present in the leachates might also, to
709 some extent, contribute to the observed bioactivity of the LE samples. Nevertheless,
710 their importance should not be overemphasized. This because although illite, the main
711 phyllosilicate which might contain Fe²⁺, a potential source of superoxide oxygen
712 radicals nanoparticles, is present in both LE and SE earths, only the LE ones are
713 bioactive.

714 In conclusion, the clay nanoparticles present in the leachates of LE and SE samples do
715 not seem to be the dominant factors driving bioactivity. In the case of the synthetic
716 control samples bioactivity is controlled by the chemicals added, namely H₃BO₃ and
717 Al-sulfate and to a lesser degree by metals released such as Zn. The role of Fe²⁺-

718 bearing phases and active oxides such as TiO₂, which may produce superoxide
719 oxygen radicals during oxidation via Fenton-like reactions, seems also to be limited.

720

721 4.2 . Bioactivity – the contribution of the organic component

722 Most DNA signatures represented soil bacteria (Table 3); some species are recognised
723 as producers of antibacterial compounds. For example, *Bradyrhizobium* (alpha-
724 proteobacteria) found in bioactive 700.17 and 700.18 are bacteria commonly
725 associated with nitrogen-fixation in soils. They excrete porphyrins, which act as metal
726 (M⁺²) chelators and may become antibiotic precursors. 700.17 also contains abundant
727 *Achromobacter*, which are known hydrocarbon degraders that may produce
728 intermediary compounds with greater toxicity.

729

730 Further to the above, rhizobial bacteria, *Sphingomonas* and sulfur-related bacteria
731 (e.g. *Chlorobium*), naturally affect sulfur compounds which may increase solubility of
732 metals/metalloids potentially toxic to bacteria. However, since the concentrations of
733 these metals /metalloids in the SE and LE leachates (Table 3) are low, their
734 contribution to antibacterial activity must be considered to be limited. Moreover,
735 *Pseudomonas*, *Comamonadaceae*, *Arcobacteria*, *Aeromonas*, and *Achromobacter*
736 contain species related to pathogenesis although they may also be considered
737 environmental. It is concluded that both bioactive and non-bioactive MEs.

738

739 Apart from bacteria genetic analysis was also conducted on fungi (based on their
740 analogous 18S-rRNA gene). Of greatest interest was the presence, within samples
741 700.17 and 700.18, of Trichocomaceae (Eurotiales) fungi. Following genetic analysis
742 we discovered by in-silico analysis (via RDP and NCBI databases for genetic
743 sequences) that the “universal” primers for detecting fungus (e.g. Hadziavdic et al.,
744 2014), while able to capture many signatures for such communities, they would not
745 have recognized the 18S-rRNA from *Talaromyces*; as a result new genetic primers
746 were developed (see Methods section).

747

748 *Talaromyces* (and *Penicillium*) are saprotrophic organisms and contribute to the
749 spoilage of carbohydrate-rich foodstuff. But they are notorious producers of
750 exometabolites (Yilmaz et al., 2014; Frisvad, 2015), including antibiotics (e.g.
751 penicillin), and highly tolerant of extreme conditions (Samson, 2016). Both
752 *Talaromyces* are expected to form biofilms (on surface), when low on nutrients or
753 stressed, or be plankton-like (i.e., floating) when “feasting”. Being saprobes, living
754 off dead or decaying organic material, they will tend to remain at/near clay sediments;
755 the latter may help adsorb nutrients. They do not need light and will respire CO₂.

756

757 Another reason that the evidence for *Talaromyces* attracted our attention was the
758 recent publication by Pangging et al. (2019) who discovered that a new isolate,
759 *Talaromyces apiculatus* from Korean soil, produced bioanthracene. The detection of
760 bioanthracene has already been highlighted by Photos-Jones et al. (2017) and in
761 association with 700.18, the only one of the three LEs analysed at the time.

762

763 Although acknowledged, the specific mention of bioanthracene production by
764 *Talaromyces* remains, nevertheless, rather limited in literature (e.g. Yilmaz et al.,
765 2014; Panggling et al., 2019; Gao et al., 2013) with *T. apiculatus* being the one most
766 frequently mentioned. However, *Talaromyces* produce other bioactive compounds

767 summarized by Nicoletti and Trincone (2016) and Yilmaz et al., (2014) with
768 beneficial and detrimental health-related effects depending on exometabolite.
769 Bioanthracene has been found to be bioactive against the parasite *Plasmodium* and
770 potentially against bacteria as well (Saepua et al., 2018; Jaturapat et al., 2001).

771
772 Bioanthracene aside, *Talaromyces sp.* and some *Penicillium sp.* have also gained
773 their notoriety for their ability to produce polyketide-based pigments, many of which
774 also carry antibacterial properties (Caro et al., 2016; Rao et al., 2017). Conditions for
775 their production and excretion of exo-metabolites have been found to be
776 environmentally based, for example a source of carbohydrate, pH, temperature and
777 geochemical conditions in their surroundings (e.g. presence of potentially toxic
778 elements, which promote extra-cellular excretions) (Mendez et al., 2011; Santos-
779 Ebinuma et al., 2013); further, biotechnological efforts continue to research optimum
780 production for the food (e.g. Defosse, 2006) and textile industries as dye producers
781 (Chadni et al., 2017).

782

783 5. Concluding remarks

784 Over the last few years we have been testing the bioactivity of archaeological
785 medicinal earths first, because it is a relatively straight forward parameter to measure
786 (their reported use as ‘antidotes to poison’ is clearly too generic a description to begin
787 to address experimentally and in a meaningful way) and second, on the grounds that
788 they *might prove to be* useful antibacterials. This paper provides a quantitative
789 assessment of the bioactivity of six samples of medicinal earths from the collection of
790 the Pharmacy Museum of the University of Basel.

791

792 Of the six MEs only two LEs (700.17 and 700.18) are bioactive against one Gram-
793 positive and one Gram-negative bacteria, while the third (700.4) is bioactive against
794 Gram-positive only and one SE (703.1) is mildly antibacterial against Gram-negative
795 only. Bioactivity, under the conditions set out in this paper was defined as having an
796 $MIC_{60} < 50\text{mg/ml}$.

797

798 The bioactivities of the leachates of 700.17 and 700.18 are comparable with synthetic
799 Melos smectite and kaolinite spiked with Boron and also Melos kaolinite spiked with
800 Al. We note that 700.17 and 700.18 are Al and B deficient and so they cannot be
801 bioactive on account of these two elements.

802

803 Looking at other reasons for their bioactivity and more specifically into their
804 microbiomial load, we note that 700.17 and 700.18 contain, with certainty, the fungus
805 *Talaromyces spp.* A greater certainty for the fungus *Aspergillus* (another member of
806 the phylogenetic clade) and a different *Penicillium* were suggested for 703.1 which
807 was mildly antibacterial against *P. aeruginosa*, only. We conclude that the fungal,
808 rather than the bacterial load, is the key driver imparting bioactivity to the three MEs
809 (700.17, 700.18 and to a lesser extent in 703.1)

810

811 Based on a protocol of analysis (illustrated in the graphic abstract), we suggest that
812 antibacterial activity of archaeological MEs seems to derive primarily from the clays’
813 organic load; the contribution of TiO₂ nanoparticles, if in sufficient numbers might
814 have also a role to play. We do not know how the LEs examined here acquired their
815 specific organic load, although we acknowledge that *Talaromyces* and *Penicillium* are

816 ubiquitous. We conclude such that clays with a fungal or bacterial load might be
817 worth investigating further as potentially serious antibacterial agents.

818
819

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826
827

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832 Principal investigator: E. Photos-Jones.

833
834

835 **Authors' contributions**

836 GEC- responsible for mineralogical /elemental analysis and interpretation, author of
837 relevant sections.

838 CK- responsible for DNA sequencing and interpretation of ME microbiome, author of
839 relevant sections.

840 DV and IG - responsible for MIC measurements, interpretation of antibacterial
841 activity and authorship of relevant section.

842 CE and EVJ- responsible for nanoparticle analysis, interpretation and authorship of
843 relevant section.

844 EPJ - initiator/coordinator of project and responsible for overall preparation and
845 manuscript submission and resubmission after reviewing.

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849 **References**

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