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Archaeological medicinal earths as antibacterial agents: the case of the Basel Lemnian sphragides

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Que les choses viles et de petite estime sont rendues precieuses par ceremonies (Belon 1553,65)

'Trivial things made precious by means of ceremonies':

Abstract

This paper presents the scientific investigation of three Lemnian sphragides (terra sigillata), a famed medicinal clay (earth) of antiquity, dated to the 16th-17th century, and presently in the collection of the Museum for the History of Pharmacy of the University of Basel. The three specimens are examined here against the backdrop of samples of sedimentary clays from the purported locality of its extraction, at Kotsinas, NE Lemnos, Greece. The study demonstrates, for the first time, that the three Lemnian sphragides displayed antibacterial properties against gram positive pathogens (staphelococcus aureus); the modern clays displayed none. Subsequent analysis with DPLC-MS of one of the three sphragides and one sample of clay revealed the presence of organic constituents in the sphragis which were absent from a sample of the modern clay. A fungal secondary metabolite is proposed here as the molecule responsible but other factors may have a role to play. The ongoing investigation for the bioactivity od some medicinal clays might aid in the re-evaluation of Belon's statement included at the start of this paper, namely, that the Lemnian Earth worked only because people in the past wished it to work.

Introduction

In the past ten years there has been a renewed interest in the study of Lemnian Earth, a medicinal clay extracted from the island of Lemnos in the NE Aegean (Fig. 1). It was celebrated in antiquity as an antidote to poison (Theophrastus, *On Stones 52*; Dioscorides *De Materia Medica* V.113; Pliny *Nat. Hist*. XXXV.14; XXVIII.24; XXIX.33; Galen *On Simple Drugs* IX, II). During the Ottoman occupation of the island (15th - early 20th century) it was used against various ailments and as a preventive against 'the plague' (Hasluck 1909-10; Sealy 1919; Hasluck and Hasluck 1929; Tourptsoglou-Stephanidou 1986; Jaronowski 2008; Hall and Photos-Jones 2008; Photos-Jones and Hall 2011; Macgregor 2012; Roumelioti 2013; Photos-Jones and Hall 2014). Throughout its centuries of use Lemnian Earth has been referred to by a number of names (Lemnian *miltos, Lemnia rubrica, terra Lemnia, terra sigillata, Lemnian Earth, Lemnian sphragis, tin-imathtum, ayiochoma).* It this paper we shall refer to it as Lemnian *sphragis* (seal, plural: *sphragides*) alluding to the fact that it has been stamped and thus prepared for a medicinal application. Chemical analysis of the product in the late 19th century, and while it was still being

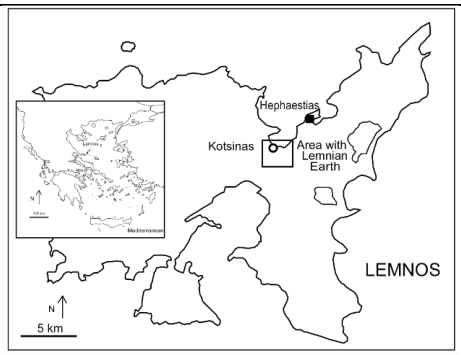
extracted, identified the presence of aluminosilicates with quartz and iron oxide (De Launay (1895); Tourptsoglou-Stephanidou 1986, 562). It was concluded that the medicinal clay bore no pharmacological properties, other than those attributed to it by those who believed in its efficacy. The earths of the Aegean and their potential antibacterial properties are currently the subject of investigation by our team and span not simply Lemnos but other islands in the Aegean as well (Photos-Jones *et al* 2015).

The Museum for the History of Pharmacy of the University of Basel, Switzerland, established in 1925, has in its collection 36 such *sphragides*, part of a larger collection consisting of c. 420 similar artefacts which can be identified as *terra sigillata* making it one of the most extensive collections of *terra sigillata* on display in the world (MacGregor 2012). In shape the *sphragides* from the various localities include spheroidal, triangular, square shapes ranging in size between 1 and 8cm in diameter. In colour they vary between white, beige, dark brown, light red, dark red violet and yellow.

Of these medicinal earths the majority of more than 150 specimens originate from Silesia, the region between Poland, Germany and the Czech Republic and known in the texts as *terra Silesia.* The remainder derive from Laubach in Germany, Bohemia, today in the Czech Republic; also from Malta and Cyprus (Häfliger 1931). Most of the Lemnian *sphragides* in the Basel Collection are of unknown provenance. It is known, however that half of the Lemnos specimens came into the possession of the museum via the pharmaceutical institute of the Swiss Federal Institute of Technology in Zurich (ETHZ) in the 1930s. The *sphragides* formed part of the pharmacognostic collection started by Prof. Eduard Schär between 1873 and 1892 and continued by Prof. Carl Hartwich until 1917 (Sticher-Levi 1996). The rest came from the private collection of Dr Joseph Anton Haefliger, a Basel apothecary, who bequeathed his collection to the university in 1925 and formed the basis for the Museum for the History of Pharmacy. However, a detailed history of the collection is yet to be written.

The Lemnian *sphragides* examined here *(*Figs. 3b-d*)* display a script on their surface which can be attributed to the 16th century (Richard Todd, *pers comm*). The grey *sphragis* (no 01424) reads *tin-Imakhtum* in a style of script that is very similar to the Belon's illustrations of Lemnian *sphragides* in circulation at the time of his visit to Lemnos, in the middle of the 16th century and reproduced from his book here (Fig. 3a). We can conclude that the grey *sphragis* is probably dated to the same period. The other two *sphragides* have a somewhat different style of script. Although less clear, they too probably, just say *tin-Imakhtum* (Richard Todd, *pers comm*).

The locality of the extraction of the raw material for the Lemnian *sphragis* (Figs. 1,2) near Kotsinas, NE Lemnos has been well documented in the sources mentioned above, as has the ritual of extraction and its subsequent processing. About fourty travellers' accounts written between the late 15th and early 20th century have been scholarly translated and edited by the historian Tourptsoglou-Stephanidou (1986). Information about the Lemnian Earth formed an integral part of any description of the island, together with its flora, fauna or customs of its inhabitants, during that period. Most of the sources agree that the raw material was extracted/dug out of a pit once a year, during the course of a single day (August 6th) and subsequently 'washed'. There were different grades of Lemnian Earth. Only the finest variety was stamped. Apart from the pit, there have also been suggestions that the water of nearby natural spring(s) may have been involved in the process of enrichment of the medicinal variety. For example Belon (1553) referred to two springs, Albacario, to three springs and finally Covel, to one (Tourptsoglou-Stephanidou 1986, 162). There are today three natural springs in the locality under the names Phthelidia, Strongyle and Kokala (Roumelioti 2013).



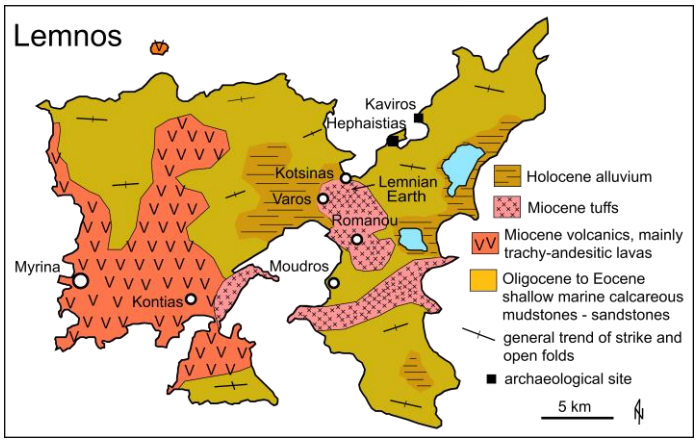


Fig. 1(left) Map of Greece and Lemnos with area of interest shown in the square. Fig. 2 (right) Geological map of lemnos (after Photos-Jones and Hall 2011, Fig. XX.) the area of the extraction of the Lemnian earth is in an area of miocene tuffs.

The exact nature of the involvement of the springs is not clear, however, there are some tantalising suggestions. For example, Joos van Ghistele, a Dutchman who visited Lemnos in 1485, wrote that "(*Terra Sigillata) is produced in Lemnos in a pool which dries up every summer and is full of water in winter. When this pool begins to dry up, a thick scum, variegated in colour, forms on its surface. This is skimmed off and laid on clean planks as required, according to the method in use locally. When dry, it is made up into round pellets or flat cakes".* It has been pointed out that the Dutch/Flemish word for scum is equivalent to *"a scum on the surface of beer or wine caused by fungus"* (Hasluck and Hasluck 1929, 674). Another account suggests that '*this earth* (was collected) *from the mud of a spring*' (Carlier de Pinon (in the late 1500's) in Tourptsoglou-Stephanidou 1986, 111). Yet a third, pointed out that the presence of a water source kept the pit moist all year round. It follows that the pit may have acted as a settling tank throughout the year (Jacopo Soranzo (in 1581) in Tourptsoglou-Stephanidou 1986, 119*)*. Apart from van Ghistele, and in reference to the 'frothiness' associated with the pit, another writer reported that "*the 'sacred earth 'jumps*' and '*overflows*' (Sibthorp (1818) in Tourptsoglou-Stephanidou 1986, 451).

The coastal hamlet of Kotsinas which first emerges, by name, in Byzantine records became well known for its potting tradition and indeed there were, still in 2007, remains of several workshops/kilns in various stages of abandonment. Ethnographer Psaropoulou (1986, 235) who interviewed the local potters in the 1970's mentions that some of their forebears "*made cups from the earth taken from a locality known as Kokalas"*. The name Kokala(s) must have been associated with the third spring mentioned above (Tourptsoglou-Stephanidou 1986, 50).

The aim of this paper is to examine the Lemnian *sphragides* in the Basel Collection not simply from their mineralogical and chemical make-up but also from a microbiological perspective,

namely from the perspective of their potential antibacterial properties. There is no way of establishing for certain that the Basel Lemnian *sphragides* did indeed originate from Lemnos. Stamped Lemnian Earth was well sought after and so fakes were extensively circulating throughout central Europe and the bazaars of the Ottoman Empire (Macgregor 2012). Therefore, the Basel *sphragides* need to be examined from the perspective of clays from the purported area of their extraction. Thus a total of eight samples have been examined here to include the three Lemnian *sphragides* (Fig. 3b-d) and five samples of sedimentary clays retrieved

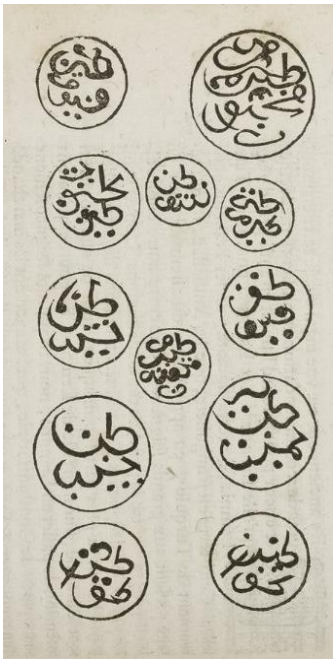


Fig. 3a Belon's illustration of the stamps on the Lemnian sphragides at the time of his visit (Belon 1553,55)

Fig. 3a Lemnian sphragis (red). MA no 01422/44.

Fig. 3b Lemnian sphragis (grey). MA no 01424

Fig. 3c Lemnian sphragis (white). MA no 01432

from various depths in the vicinity of the purported area of its extraction, in Kotsinas. The samples are taken from both surface and at depth within an area of weathered volcanic tuffs (Fig. 2) (Roumelioti 2013). There is no way of knowing what the boundaries of the area of extraction were over a period of 500 years or more. It is very likely that pits would have opened over a considerable area and once emptied in the course of each year they would be unlikely to have been reworked, ie they would have been depleted. This means that although we assume today that only the area within these volcanic tuffs may have been worked, other areas outwith may also have been sampled as well and used for the preparation of the earths from a single pit or a number of pits.

Samples were taken at depth because the texts clearly suggest so. Depth is designated on the sample number; for example, LE6-1.60m signifies a sample taken at a depth of 1.6m. All samples are buff coloured like the grey Lemnian *sphragis* (Fig. 3b) with the exception of LE8-surf which was red and was collected from the surface. Samples were analysed with quantitative XRD to establish mineral structure and with ICP-MS for the purpose of assessing trace element content. The bioactivity of all was tested against two bacterial stands *staphelococcus aureus* and *pseudomonas aeruginosa*. To establish the nature of the molecule responsible for bioactivity liquid chromatography together with mass spectrometry (UPLC-MS) was carried out on a single sample of *sphragi*s (grey) and a single clay sample (LE5-3.3).

Materials and Methods

XRD**:** XRD analysis was carried out at the University of Crete on a Bruker D8 Advance Diffractometer, using Ni-filtered Cu Kα radiation (35 kV, 35mA) with a Lynx Eye strip silicon detector. Data were collected for 2θ values in the range of 3° to 70° with a step size of 0.02° and a count time of 1 second per strip step. The diffractograms were analyzed and interpreted with the Diffrac Plus software package from Bruker and the Powder Diffraction File (PDF). Samples with smectites were treated also with glycerol. The quantitative analysis was carried out by the Rietveld method using the TOPAS software package. The results are presented in Table 1.

ICP-MS: The samples were analysed with ICP-MS (7500CX coupled with Autosampler Series 3000, both by Agilent Technologies). About 200 mg of each sample were digested with 8mL aqua regia in a microwave digestion device (Multiwave 3000, Anton Paar), following the EPA3051 method. The method is not suitable for determination of Si due to partial dissolution of the silicates and S due to analytical constraints. Therefore the analysis of the major elements is not included. The precision of the analyses was tested using suitable standards. The results of the trace elements analyses are presented in Table 2.

Microbiological testing: Eight samples, five sedimentary clays and three *sphragides* were prepared for microbiological testing by grinding into fine powders and subsequent sterilisation by dry heat at 200ºC for 2 hours. Two fully susceptible bacterial strains, one Gram positive organism (*Staphylococcus aureus* ATCC 25923) and one Gram negative organism (*Pseudomonas aeruginosa* ATCC 27853), were chosen. A 0.5 McFarland dilution (a standard bacterial dilution utilising optical density to create a standard inoculum) of each bacterial strain was made using sterile saline and a densitometer. 400µl of this suspension was added to a sterile micro tube containing 200mg of each of the eight mineral samples. Controls were included consisting of a) bacterial suspension only (no mineral samples added) and b) with mineral samples only (400µl of sterile saline added). Each combination was set up in triplicate. The micro tubes were then incubated overnight on their side at 37ºC, on top of a rotating mixer to help prevent sedimentation. 10-fold serial dilutions of the original 0.5 McFarland dilution were made using sterile saline. 10µl of each dilution was then plated onto blood agar in duplicate and incubated overnight at 37ºC. Subsequent to the above, the number of colony forming units on each plate was counted in order to a) quantify the number of bacteria in the original 0.5 McFarland dilution of the bacterial strain prior to overnight incubation. b) following overnight incubation and c) following overnight incubation and mixing with sediments/*sphragides*. Results are displayed as graphs in Figs 3a,b and 4.

UPLC-PDA-MS: Only one sample of *Lemnian sphragis* ***(***LE2 (GREY) (bioactive)) was analysed and only one of the sedimentary clays ***(***LE5-3.3 (non-bioactive)). 20 mg of each sample was extracted in 1 ml 50% aqueous methanol. Each sample was sonicated, using a Soniprep 150 (MSE, UK), for 5 min in 1 min intervals (100 W, 17 kHz, max. amplitude). The samples were subsequently centrifuged at 13,000 g for 10 min and supernatants were transferred to HPLC vials.

The combined system of chromatograph with mass spectrometry was a Waters Acquity Ultra performance LC coupled to a photodiode array and a Xevo quadrupole time of flight mass spectrometer (*UPLC-PDA-MS)*. A liquid chromatograph is used to separate the various components within a compound, as a function of retention time and as they travel through a column. However, the technique, while separating the various components, provides little information as to the molecules that make them up. In order to identify pure molecules it is essential to subject each separate component of the original compound that emerges out of the LC column to a mass spectrometer; on entering the mass spectrometer the solvent is removed and the constituent parts are ionised. The detector in the mass spectrometer generates a high-resolution spectrum, separating all ions according to their mass. Direct infusion- electron spray ionisation-is the method by which the sample is introduced to the mass spectrometer while positive and negative ionisation refers to the sign of the charge detected. In reverse chromatography a hydrophobic packing material, as opposed to the hydrophyllic one is used.

In our case, samples were separated on a BEH C18 column (100 x 2.1 mm; 1.7 μm particle size) which was maintained at 40°C. The mobile phase was a mixture of (A) Milli-Q Water plus 0.1% formic acid and (B) acetonitrile plus 0.1 % formic acid. Separation was achieved using a gradient increasing from 20% B to 70% B over 10 min, followed by a 100% B wash step and re-equilibration. Data was acquired in positive ion electrospray scanning from *m/z* 50 to 2000 with a scan time of 2 s and inter-scan delay of 0.1 s. Low voltage scans were acquired a 6 v and high voltage using a ramp from 25-40 V, providing parent ion and characteristic fragment data respectively. Instrument control, data acquisition (centroid) and processing were achieved using MassLynx v4.1. The results for direct infusion (positive and negative ionization) for both the sphragis and the local clay is shown in Figs. 5a and 5b and the reverse phase chromatograms for the same samples are shown in Fig. 6.

Results

Lemnos is dominated by young Cenozoic (Upper Eocene to Lower Oligocene) sedimentary rocks which consist of both marine and terrestrial sediments (Fig. 2) (Photos-Jones and Hall 2011). Such sedimentary rocks underlie the topographically gentle areas in the northern-central, north-eastern and southern parts of the island. Younger Lower Miocene volcanic rocks, both lavas and pyroclastics, outcrop in a general east-west zone through the central area of the island. Holocene alluvial sediments occupy extensive low ground in the centre and the east of the island. A significant concentration of intrusive rocks of calc-alkali mafic to felsic composition occur in sediments in the extreme southern and north-western parts of the island, but from their distribution on the geological map these do not appear to relate to well-defined magmatic centres. However, the notable development of andesitic to felsic lavas in the western part of the island attests to the presence of a volcanic centre. In the area of the extraction of the Lemnian Earth, there are light-yellow to yellow slightly altered pyroclastic volcanic rocks of biotite-hornblende dacitic-andesite composition. Within light-coloured surface material are rare relicts of fresh volcanic rock and isolated feldspar phenocrysts.

The results of the quantitative XRD analyses (Table 1) suggest that the three *sphragides* are not mineralogically identical: the white *sphragis* consists largely of dolomite and kaolinite, with some illite and quartz; the grey *sphragis* consists of montmorrilonite and the red *sphragis* consists mainly of illite, kaolinite and quartz. On the other hand four out of the five sedimentary clays are similar. LE8-surf, the fifth sample, a surface sample, is quite different consisting primarily of kaolinite, alunite and hematite. The main difference between clay sediments and *sphragides* is in the presence of calcite and chlorite which are both absent from the *sphragides*. If calcite was originally present it could have been removed by some sort of treatment, for example dissolution in some organic acid. However, the removal of chlorite by beneficiation is less likely.

Dolomite in LE1(WHITE) is absent from any of the sediments analysed here, while LE2(GREY) is particularly rich in montmorillonite suggesting a basic volcanic environment; on the other hand LE8-surf, with kaolinite and alunite, suggests an acidic volcanic environment. It is not clear to what extent the greater Kotsinas area was thought to have had, in the past, the same boundaries as today. The geological map of Lemnos in Fig 2 shows that the area consists largely of Miocene tuffs which border to the west by Holocene alluvium and to the east and south by shallow marine calcareous mudstones and sandstones. The calcareous mudstones may have given origin to the dolomite in LE1(WHITE) but it is not clear where the excess montmorillonite of sample LE2(GREY) was derived from other than beneficiation.

Table 2 presents the ICP-MS results for trace elements in an attempt to highlight potential differences in metallic element content which might have adverse effect on antimicrobial properties. Pb content is relatively elevated in the *sphragides* compared to some of the sediments but not all (ie LE6-1.60). Cu content is considerably higher in LE1 (WHITE) while As content is elevated in LE3(RED). Ti, Co, Cr, Ni, Mn are overall relatively lower in concentration in the *sphragides* compared to the sedimentary clays.

Table 1: results of quantitative XRD analysis for the three sphragides and a set of five sediments. Mineral composition in %.

	SAMPLE	DOLOMITE	ILLITE	KAOLINITE	QUARTZ	ALBITE	MONTMORILLONITE	HEMATITE	CHLORITE	GYPSUM	ALUNITE	CRISTOBALITE	TRYMITE	CALCITE
LIMN-1	LE1WHITE	65.2	9.9	17.3	7.6	-	-	-	-	-	-	-	-	-
LIMN-2	LE2GREY	-	18.1	-	6.9	9	66	-	-	-	-	-		-
LIMN-3	LE3RED	-	41	37.4	17.7	-	-	3.8	-	-	-	-	-	-
LIMN-4	LE6-1.6	--	22	1.3	23.8	10.1	16.9		9.9					16.1
LIMN-6	LE1-3.2	-	14.9	2	33.2	15.1	18.1		5.7	1	-	-	-	9.9
LIMN-7	LE2-2.7		12.2	1.2	23	6.9	30.5		10.4	-	-	-	-	15.8
LIMN-5	LE5-3.3	-	13.3	1	21	12.7	35.1	-	8.9	-	-	-	-	8.1
LIMN-8	LE8-surf	-	-	69.3	-	-	-	1.8-	-	-	22.5	4.5	1.9	-

Table 2. ICP-MS analysis: traces only, composition in ppm. (<DL= below limit of detection).(RPT=repeat)

	Li	B	Ti	V	Cr	Pb	Cu	Co
	Ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm
LE1(WHITE)	33	7	115	26	13	17	176	<DL
LE2(GREY)	2	12	827	71	7	40	31	4
LE3(RED)	24	16	216	51	38	10	17	1
LE 1-3.20	49	9	375	43	49	3	12	7
LE 5-3.30	76	12	485	49	62	8	14	9
LE 6-1.60	6	5	1478	95	32	33	28	14
LE 2-2.70	51	13	498	65	65	3	17	10
LE 2-2.70 (RPT)	46	13	468	61	61	2	16	9

	Zn	As	Rb	Sr	Mn	Cs	Ba	Ni
	Ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm
LE1(WHITE)	3	3	16	323	43	2	53	<DL
LE2(GREY)	29	5	25	307	258	4	629	9
LE3(RED)	14	77	52	175	65	12	136	4
LE 1-3.2	28	0	33	108	411	1	53	54
LE 5-3.3	35	0	38	92	291	2	78	82
LE 6-1.60	34	5	29	301	979	1	865	13
LE 2-2.20	38	<DL	46	120	729	<DL	79	74
LE 2-2.20 (RPT)	36	<DL	44	112	692	<DL	75	70

Having analysed the three Lemnian *sphragides* chemically and mineralogically, the three *sphragides* were subsequently tested microbiologically against *S aureus* and *P aeruginosa*. The results are shown in Fig. 3a. A logarithmic scale on the y axis of the graph displays the bacterial counts for both *S. aureus* and *P. aeruginosa*, expressed as the mean number of colony forming units per 10 microlitres. The graphs illustrate the changes in bacterial counts, both after an overnight's (18 h) incubation and with or without addition of mineral samples. Error bars demonstrate the standard error of the mean (SEM), calculated on Microsoft Excel by first working out the Standard Deviation of sample results and then dividing this number by the square root of the number of samples. Significance (p value) is relative to the positive control (bacterial suspension, at 18 h), and was determined on Microsoft Excel using a two-tailed t-test. Only p values < or = 0.05 were deemed significant.

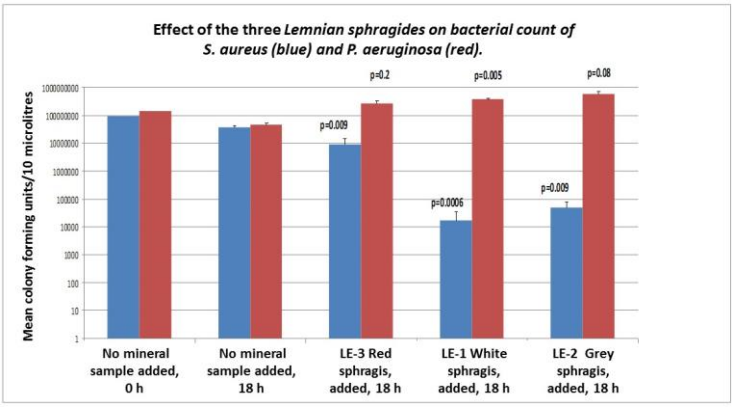


Fig. 4a Effect of the three Lemnian *sphragides* on bacterial count of *S aureus* and *P aeruginosa*. All three *sphragides* showed an antibacterial effect against *S aureus* but not against *P aeruginosa*. The white *sphragis* being the most significant and the red being the least.

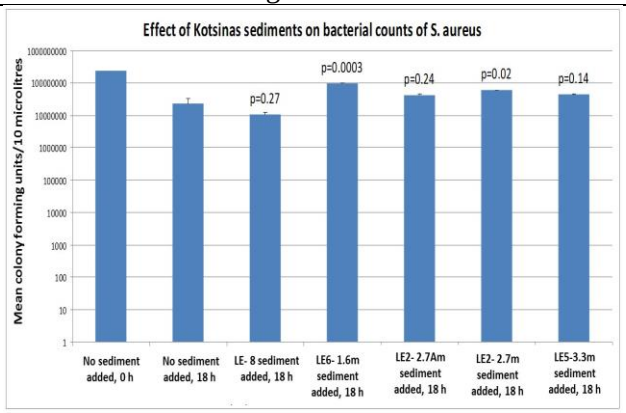


Fig. 4b Effect of the Kotsinas sediments on bacterial count of *S aureus*. None of the five sediments showed an antibacterial effect against this pathogen.

All three varieties of *sphragides*, were associated with a statistically significant reduction in bacterial counts of *S. aureus*, but not *P. aeruginosa* (Fig. 4a). The most significant antibacterial effect was elicited by the white and grey Lemnian *sphragides.* Bacterial counts of *S. aureus* were reduced by 3 log (p=0.0006 and 0=0.009, respectively). Although the red Lemnian *sphragis* reduced bacterial counts of *S. aureus* by less than 1 log, it reached statistical significance (p=0.009); it also affected the morphology of the bacterial colonies (Fig. 5).

The five Kotsinas sedimentary clays were subsequently tested against *S. aureus* but not against *P. aeruginosa.* As can be seen in Fig. 4b none of them reduced significantly the bacterial counts of *S. aureus*. Sample LE8-sulf was associated with a slight reduction in the bacterial count of *S. aureus*, but this was a reduction of less than 1 log and did not reach statistical significance (p=0.27). The bacterial colonies of *S. aureus* which grew following exposure to the Lemnian *sphragides* were noticeably smaller and paler than the positive controls (Fig. 5) suggesting stunting of bacterial growth. The appearance of *S. aureus* colonies returned to normal when they were subjected to a fresh agar plate, suggesting that the damage was reversible.

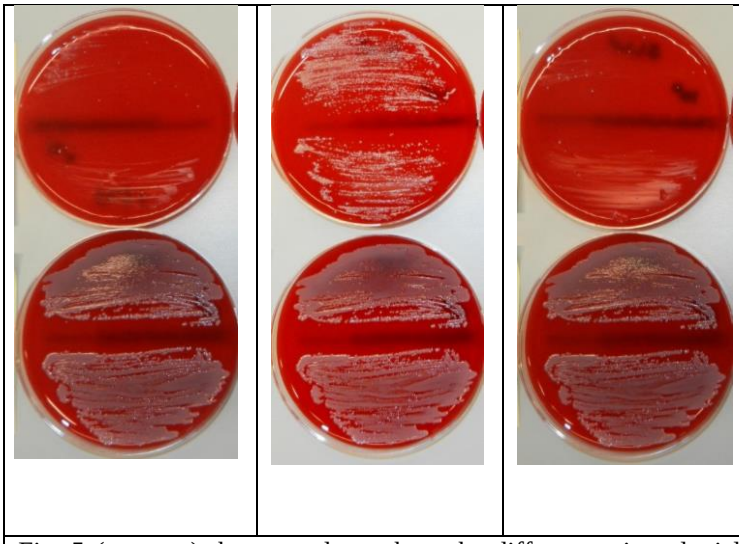


Fig. 5 (top row) the agar plates show the differences in colonial appearance when *S. aureus* was mixed with a Lemnian *sphragides* sample; from left to right, white, red and grey Lemnian *sphragides*: (bottom row) normal colonies of *S. aureus* which grew from our positive control. Both the number and size of colonies has diminished. Such an effect was not seen with *P. aeruginosa*. The appearance of *S. aureus* colonies returned to normal when they were subjected to a fresh agar plate.

Having assessed the bioactivity of the three Lemnian *sphragides* it is now essential to direct attention to the molecules that might be responsible for it. Direct infusion with positive ionisation of the extract from Lemnian *sphragis* gave a spectrum with an intense ion at m/z 579.2228 whereas no prominent ions were detected in the extract from LE5-3.3 (Fig. 6). Matching the main peak to a natural compound database (Antibase 2013) revealed that it has a close similarity to a family of compounds of fungal origin known as bioxanthracenes. These are a group of fungal metabolites which have antimalarial and cytotoxic properties (Laatsch 2013).

Further to the above, the extracts were separated by reversed-phase chromatography using a method routinely used for analysis of natural metabolites from cyanobacteria (REF?). The ion chromatograms revealed that the Lemnian *sphragis* contained many peaks with mass between 300-750 amu in addition to that at m/z 579 and also 705 which were not present in the extracts (Fig.7).

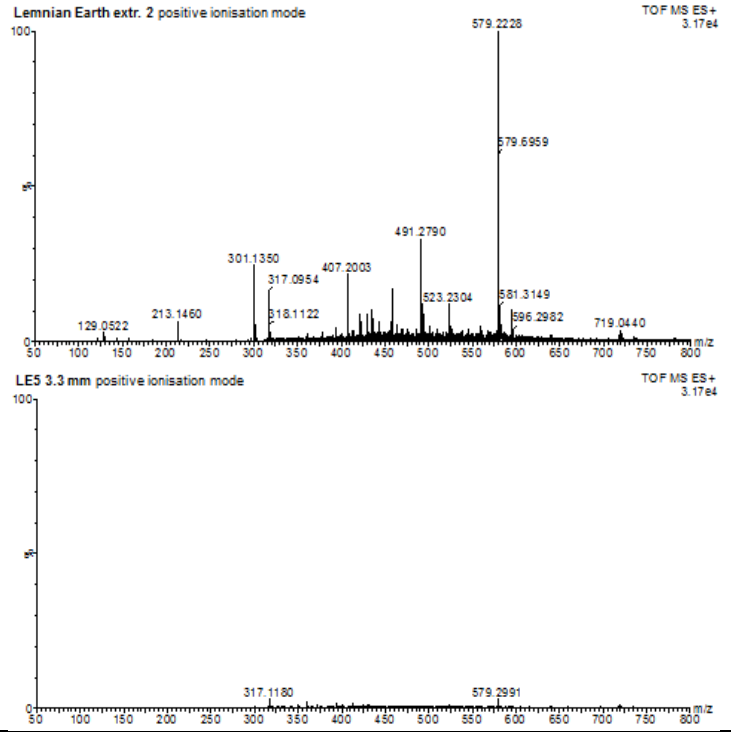


Fig. 6a positive ionisation spectrum of (top) LE 2(GREY) bioactive sphragis and (bottom) LE5-3.3 sedimentary clay. There clay displays far fewer peaks than the sphragis.

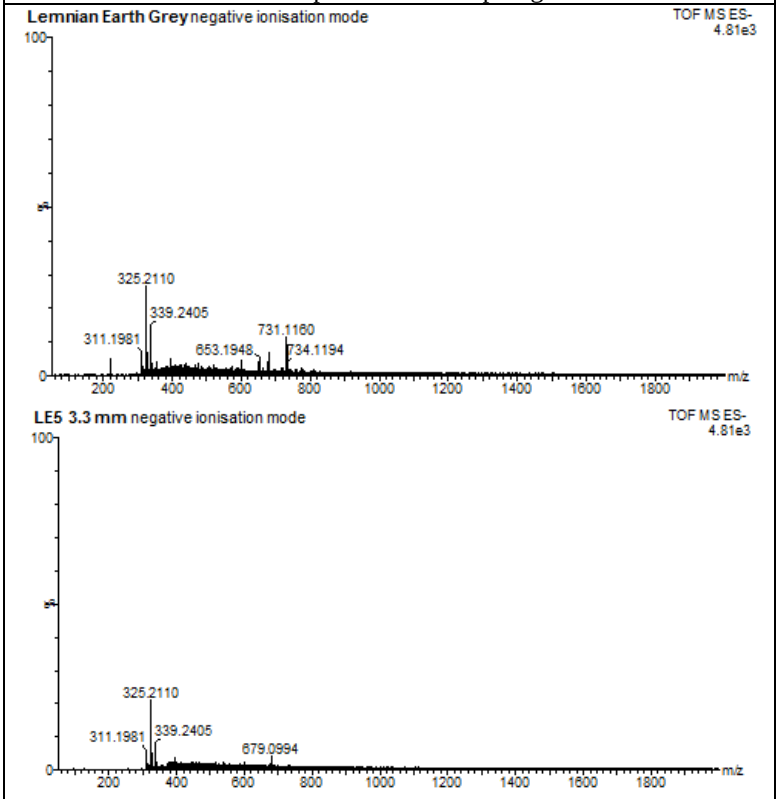


Fig. 6b negative ionisation spectrum of (top) LE 2(GREY) bioactive *sphragis* and (bottom) LE5-3.3 sedimentary clay. There is overlap between clay and *sphragis* for some peaks like 325.2110. However other peaks like 731.1160 is present only in the *sphragis.*

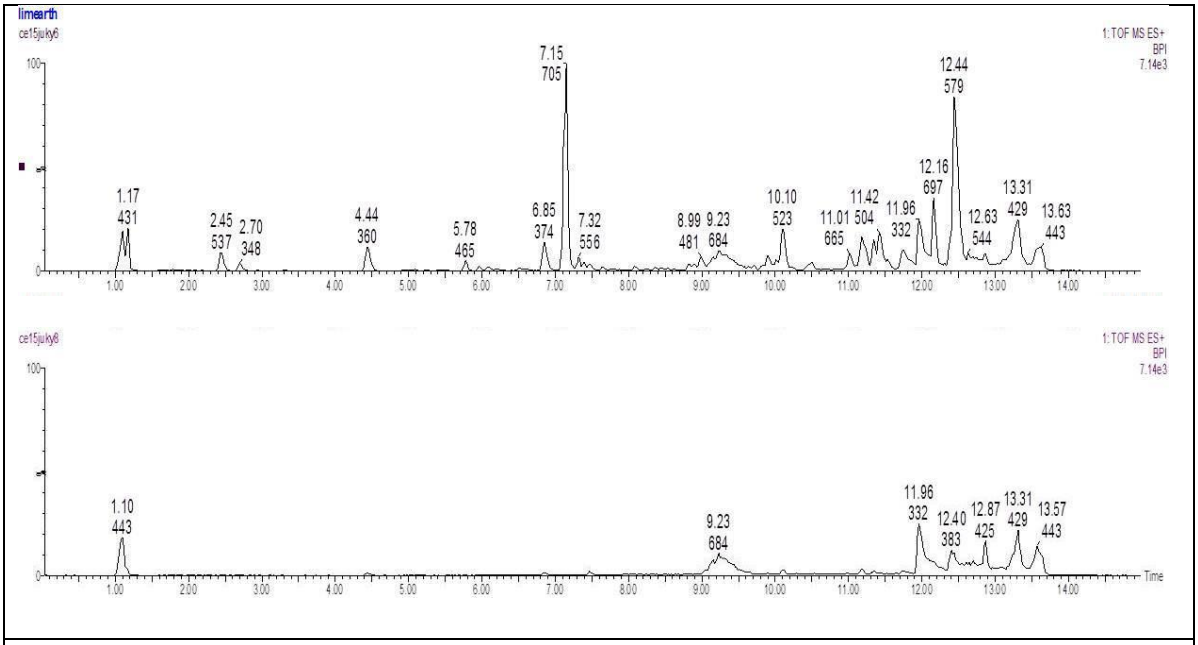


Fig. 7 Reverse phase chromatography of (top) LE 2(GREY) *sphragis* and (bottom) LE5-3.3 sedimentary clay. Two numbers are given per peak: the top number corresponds to the time of discharge from the column while the bottom corresponds to the mass of the molecule. Two major peaks are seen in LE 2(GREY) which are absent in the clay, ie m/z 579 and 705. Decimal points in Fig.6 have been rounded off compared to Fig. 6.

Discusssion-Conclusions

On the basis of mineralogy and geochemistry it is clear that the three Basel Lemnian *sphragides* could not have originated from any of the sedimentary clays discussed here. It is still possible that the three *sphragides* did originate from within Lemnos and the greater Kotsinas, the óriginal' boundaries of which cannot be known. Furthermore, it is not certain that all three may have undergone the same 'washing' stages. These beneficiation stages could have taken place either during the course of the year, ie via clay levigation within the pit or in the course of the day of the extraction or a combination of both. So a number of parameters are potentially at play here which derive a) from the sedimentary stratigraphy within each pit in the greater Kotsinas area and b) from the various recipes for production of the *sphragis* either by mixing of various 'different' raw materials or the beneficiation of the raw materials, or a combination of the two. It is simply not possible to expect the travellers' texts to provide us with the level of detail that it is required here, to reconstruct the process. One additional factor may be variation in the process over a period of four or five centuries, whereupon the process became more involved or simpler as per prevailing social or economic or political circumstances.

Over and above the differences in geochemistry and mineralogy and the interesting questions they raise are the fascinating results that arise from the microbiology tests and the search the critical molecule(s) responsible for bioactivity. All three of the Basel Lemnian *sphragides* displayed antibacterial properties. Of the three the dolomitic (white) one is the most antibacterial followed by the montmorillonitic (grey) and finally the kaolinitic+illitic (red). No antibacterial effect was demonstrated against *P. aeruginosa*. It is suggested that the role of the bioactive component may have been bacteriostatic rather than bacteriocidal. None of the Kotsinas sedimentary clays demonstrated a significant antibacterial effect against *S. aureus*. UPLC-MS spectra obtained from the analysis of the LE2(GREY) *sphragis* show the presence of organic component(s) which is/are absent from the sample of the sediment analysed. After consultation

with a natural products data base (Laatsch 2013), it is suggested that the peak with mass 579.2228 might belong to a family of fungal secondary metabolites, bioxanthracenes which are known to have beneficial rather than harmful effects and are used as antibiotics and against malaria (Moss 2011). There is also the possibility of a brominated metabolite but the identity of that is not presently known.

Having established the presence of a possible secondary metabolite it is important to be able to eliminate the possibility that fungal growth responsible for bioactivity may have been acquired not as a result of 'deposition', namely from long term storage, but rather as a result of beneficiation. It is concluded that until such time as the molecules responsible for the bioactivity of the three Basel Lemnian *sphragides* are fully identified it is advisable to keep an open mind. Nevertheless, there is sufficient ground at this stage and while work is still in progress, to argue that a 'trivial thing' like the Lemnian *sphragis* may have not been made special simply by means of a ceremony but rather by means of an active ingredient within.

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