Stockholm University

Bachelor Thesis

Degree Project in Geochemistry 30 hp

Levoglucosan as a tracer for biomass burning

Modern and ancient fires in southwestern Peloponnese

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Abstract (English)

In this project, a method was set up for analysis of levoglucosan and the similar substances mannosan and galactosan, at the Department of Geological Sciences, University of Stockholm. These compounds are produced in fires through the breakup of cellulose, a process that especially produces levoglucosan. This makes it a valuable tracer for biomass burning. Following a method developed for high performance liquid chromatography – tandem mass spectrometry (HPLC-MS^2) by Hopmans *et al* (2013), the method was applied for analysis of modern soil samples from fire-raged areas in Peloponnese, Greece, as well as for samples from a dated core from the same region. Results showed a large instrument error and analysis did not go effortlessly. Still, a correlation could be seen between the occurrence of fire and of the tracers in the soil samples. There was also a good correlation seen in the core between the biomass burning tracers and hydrogen isotope values, another climate proxy. It was shown that levoglucosan, mannosan and galactosan could be separated for analysis with the help of a common lipid extraction fractionation scheme. The method is therefore promising for future development and could become a cost- and time-effective way to reconstruct the fire history of soil and sediment samples analyzed at the department, coupled with other analytical methods.

Sammanfattning (svenska)

En metod för att mäta halterna av levoglucosan och de liknande ämnena mannosan och galactosan sattes upp på Institutionen för Geologiska Vetenskaper, Stockholms Universitet. Dessa ämnen, av vilka levoglucosan är vanligast förekommande, bildas då cellulosa bryts upp under förbränning. Detta gör levoglucosan till en värdefull spårmolekyl för förbränning av biomassa. Projektet utgick från en metod för högpresterande vätskekromatografi kopplad till tandem-masspektrometri (HPLC-MS^2) utvecklad av Hopmans *et al* (2013). Metoden användes för analys av moderna jordprover från branddrabbade områden på Peloponnessos, samt för prover från en daterad borrkärna från samma region. Resultaten visade på dålig precision hos instrumentet och analysen gick inte problemfritt. Trots detta kunde en korrelation hittas mellan förekomsten av brand och halterna av levoglucosan, mannosan och galactosan. Ett samband sågs även i borrkärnan mellan dessa halter och förändringar i väteisotopsammansättning, en annan proxy för klimatförändringar. Det påvisades att levoglucosan, mannosan och galactosan kan separeras med hjälp av en vanlig metod för fraktionering inför analys i organisk geokemi. Den analytiska metoden är därför lovande för framtida utveckling, och kan bli ett kostnads- och tidseffektivt sätt att rekonstruera förekomsten av bränder i jord- och sedimentprover som analyseras på institutionen, även i kombination med andra analytiska metoder.

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Introduction

The understanding of climate systems is a broad and complex field that requires much research. This field may even be of larger interest than ever before, as we are living through times of global warming and rapidly changing ecosystems and environments (e.g. Barros *et al*, 2014). In order to understand the current climate shifts, one cannot put too much effort into decoding the climate shifts of older times. To do so, a very large number of proxies have been established, to understand climate parameters such as temperature and moisture content of older times (e.g. Zachos *et al*, 2001). As the climate is a complex system, the parameters cannot be few and narrowly localized, for a larger understanding to be achieved.

Given that forest fires break out and grow under drier and warmer conditions, knowledge of biomass burning in paleorecords gives a good indication of the climate of the time, in respect to these two parameters. In turn, large biomass burning events has the potential to affect climate. This can happen due to multiple causes, related to the release of chemicals such as greenhouse gases to the atmosphere, and the production of aerosols, which can affect the amount of incoming sun radiation (Crutzen and Andrea, 1990). An estimate of old fire frequency has traditionally been achieved by counting charcoal particles (e.g. Simoneit *et al*, 1999). For this work, which has mainly been performed on lake sediments, two different methods can be distinguished. Either the charcoal particles are studied from slides that are being prepared for pollen analysis, or by sieving and isolating larger charcoal particles. It is believed that the smaller particle size can be transported further from the source and therefore represent a regional area, while the larger particles would be representative for the local fire history. Thin-section analysis has shown to be both expensive and time-consuming, and is therefore hard to quantify (Carcaillet *et al*, 2001). Sieving for macroscopic charcoal particles still involves careful treatment, and chemical methods are used for removing or bleaching other sources of organic material to be able to perform the analysis (Schlachter and Horn, 2010).

Due to these difficulties, the use of geochemical tracers for biomass burning enables for faster and more quantitative analytic methods, and could possibly also contribute with information about the type of vegetation that burned (e.g. Hopmans *et al,* 2013, Simoneit *et al,* 2002) The use of new proxies for understanding past fire frequency is therefore a field of present research.

LVG as a chemical tracer for cellulose burning

Different molecules have been suggested as chemical tracers for wood burning. One example is Retene, which is formed by thermal alteration of diterpenoids in conifer wood. While this molecule rarely occurs without combustion taking place, it is not always found in detectible amounts (Simoneit *et al*, 1999). This is however not the case for Levoglucosan (1,6-anhydro-β-D- glucopyranose, LVG), LVG is an anhydrosaccaride that forms from cellulose and hemicellulose at temperatures exceeding 300°C, as these molecules split in smaller pieces through processes of fission, transglycosylation and disproportionation (e.g. Simoneit *et al,* 1999, 2002). During wood burning, two isomers of LVG also form, known as mannosan (1,6-Anhydro-β-D-mannopyranose, MAN) and Galactosan (1,6-Anhydro-β-D-galactopyranose, GAL)(figure 1). Still, while especially

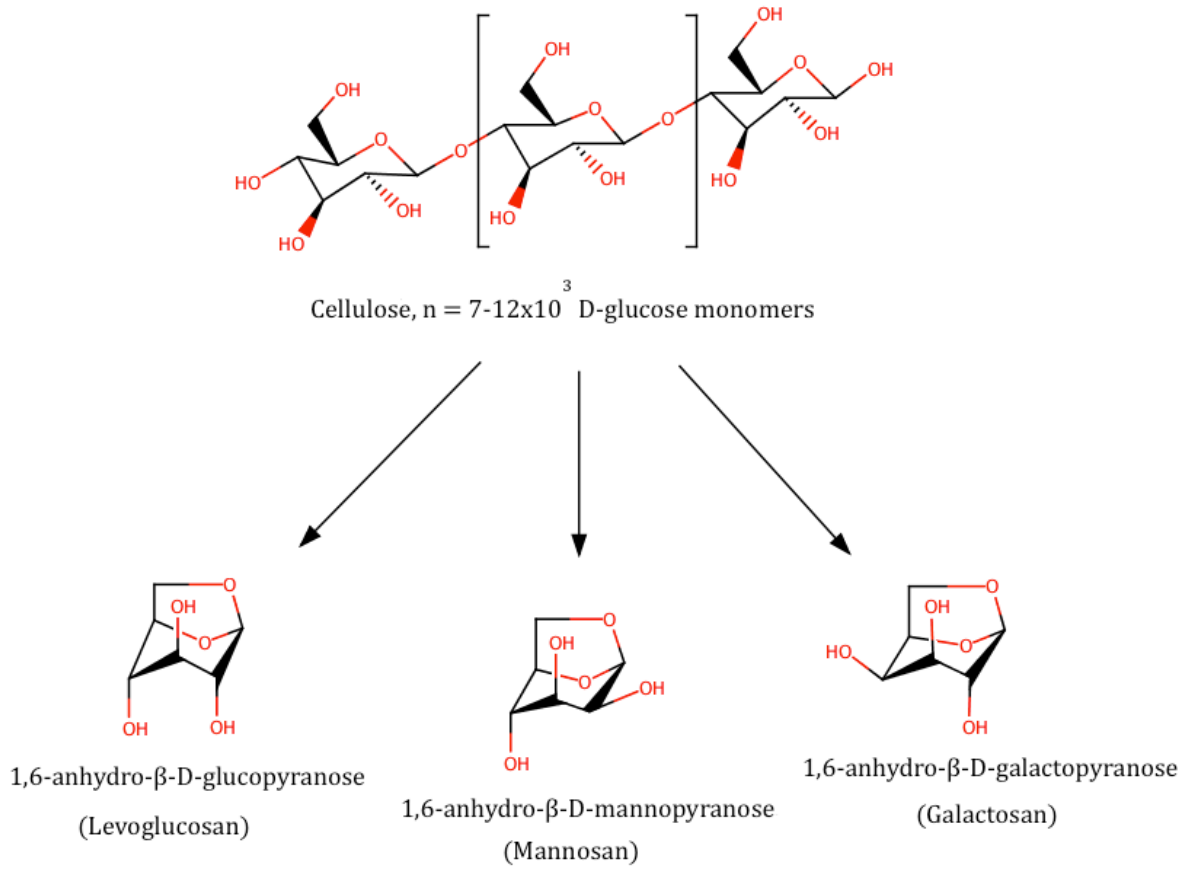


Figure 1: Schematics over anhydrosaccharides formed through combustion of cellulose, splitting the large molecule into smaller components. Chemical reactions and intermediate steps are left out for clarity. Reconstructed after Shafizadeh, 1984, and Jarosz and Nowogródzki, 2008.

MAN can be distinguished in wood smoke, the rates of these compounds seem to be one or two magnitudes lower than the rate of LVG (Simoneit *et al* 1999, Fine *et al* 2001, 2002). In favor of LVG as a molecular tracer, the amount of LVG emitted for different wood types have also shown to be largely constant, with an average of 100 +/- 40 mg LVG emitted per gram of fine particulate carbon. Softwood seems to emit somewhat lower amounts (Fine et al., 2001, 2002).

The unique formation conditions of LVG along with its high emission rates make it a very good indication of natural hardwood combustion. It is important to remember, however, that the occurrence of anthropogenic processes releases organic molecules to the atmosphere, thereby limiting the modern use of biomarkers for specific sources. In the case of biomass burning, heating by wood combustion is responsible for large emissions worldwide (e. g. Schauer et al, 2007). As the anthropogenic contribution for wood combustion is so significant, LVG has also been used as a tracer for anthropogenic wood smoke (e.g. Jordan *et al* 2006).

The benefits of using LVG as a chemical tracer for biomass burning also includes its stability in the atmosphere, as tested by Fraser and Lakshmanan (2000) as well as Jordan *et al* (2006). Matching of LVG rates to charcoal counts have also been attempted, with fairly good results (Elias *et al*, 2001).

Some disadvantages with LVG as a chemical tracer have also been found. Knicker *et al* (2013) measured the rates of LVG in organic matter after longer and shorter times of combustion at 350° and found that the amount decreased substantially after continuous combustion. They also submitted their samples to microbial incubation and found that microbes efficiently broke down LVG. The conclusion of the authors was that the use of LVG as a tracing molecule must be done with caution. LVGs susceptibility to be broken down and taken into the metabolism of yeast and fungi has also been studied and confirmed by earlier authors (e.g. Kitamura *et* al, 1991). Fabbri *et al* (2009) reported that a substantial amount of LVG was formed through burning of immature lignites, which in turn is used as a fossil fuel in some regions. The advantage of LVG as a source-specific biomarker could therefore be questioned in some cases. As he authors did not find GAL in these lignites, they suggested that a normalized value for LVG over GAL and/or MAN could be used to solve this problem.

Most commonly for geological materials, LVG has been quantitatively analyzed by gas chromatography - mass spectrometry (GC-MS). To gain results through this analytical equipment, derivatization of the alcohol groups on the compound with a protecting group like trimethylsilyl have been performed (e.g. Schkolnik and Rudich, 2006, Elias *et al* 2001, Simoneit *et al* 2001, Fabbri *et al* 2008, 2009)**.** Recently, a few methods for high-performance liquid chromatograpy (HPLC) have also been presented, in which no derivatization is necessary. Hopmans *et al* (2013) published a method for rapid separation with HPLC, and analysis with triple quadrupole mass spectrometry^2 (MS^2) analysis. This was based on a method used in a study by Gambaro *et al* (2008), who analyzed melted ice cores, but modified in order to be able to perform analysis on more complex matrixes, such as soil or sediment.

Project Description and Hypothesis

In this project, the method developed by Hopmans *et al* (2013) was set up at the Geology Department of Stockholm University (IGV). The goal of the project was to implement and test the analytical method, as an expansion of organic geochemical methods currently in place at IGV. For testing the method, two main sets of samples was extracted and analyzed: A set of modern soil samples were taken on Peloponnese, Greece, from areas recently raged by wildfires. Extraction was also performed after the method described by Hopmans *et al* (2013). Samples from a sediment core taken in the same region, dated and analyzed for various environmental and paleoclimatic proxies during another project, was also analyzed for levels of LVG, MAN and GAL. The method was also used on a few samples from a core from a maar lake in Myanmar to evaluate its potential application for future research.

For the modern soil samples, a correlation between the chemical tracer levels in soil samples and the age and extent of the fire was expected, but the rates were also expected to change a lot depending on the various sample locations. By analyzing modern fire areas for LVG rates, a deeper understanding for the behavior of the tracer during the first years after the fire event would hopefully be achieved, as this aspect of the tracer does not seem to have been well covered in previous studies. As for the sediment core from the same region, a correlation was expected between the biomass burning tracer levels and other climate proxies analyzed for the same core. Such a

correlation could increase the confidence for the climate trends as indicated by the proxies.

Time spent on different aspects of the project is declared in **table 1**.

Moment	Description	Approximate time spent
Initial planning	Reading, planning for sampling trip	3 weeks
Sampling	Field trip to Peloponnese	1 week
Sample Treatment	Preparing samples for analysis: initial treatment, extraction	5 weeks
Method development	Setting up the method for analysis	2 weeks
Troubleshooting	Occupied with problems that occurred during analysis	4 weeks
Report writing	Finishing final report	3 weeks
		Total 18 weeks, 30 HP

Table 1: The different parts of the project, and the time spent for the different moments.

Study Area

Soil Samples

The Peloponnese peninsula in southwestern Greece is to a large part a rural district, but also contains high mountains and an overall dramatic landscape. With a drier climate in the Mediterranean region, the fire occurrence is also increasing, and this also affects Peloponnese. Here, devastating fires raged in 2007, affecting as much as a few percent of the area and also leading to a number of causalities. Since then, the fire frequency has also been quite high. Information about areas where major fires occurred in modern times was found through the NASA Firms Web Fire Mapper (2015).

Sample locations were as follows:

- Ancient Messini: On this inland location on about 400 m height above the sea lays the ancient remnants of the old Greek city of Messene. The city ruins on this location are still well preserved and dates as far back as to the antique Greek era. These ruins of large cultural importance were threatened as a fire raged in the area, bursting out on the 25th of august 2014. Village inhabitants tell of strong northeastern winds on that day, making the fire propagate eastwards and forcing the evacuation of four villages in the area (Daily Mail, 2015). The path of the fire can be clearly seen in the landscape, reaching the mountainside of Ithome and rounding the mountain, before continuing beyond the mountain in two small and separate tracks. Vegetation was burned to different extent in this area, in many cases burning crowns and charring the trunks of the trees and brushes affected. The vegetation burned mainly consists of large shrubs and small trees,

with the landscape being spotted by sole trees or groves of larger trees. 28 samples were recovered from this location, from different slopes and types of soil as well as from different parts of the burned area.

- Dorio: A few kilometers from the village of Dorio, a fire was reported in an uphill olive field in the summer of 2012**.** The vegetation in the area consisted of grasses, brushes and olive trees. No clear traces of this event could be seen in the landscape, and no information about the wind direction at the time of fire could be confirmed. The actual fire could however be confirmed by village inhabitants, and a few samples were recovered from the reported area.

- Kaiafas Lagoon: As part of the large fire event of august 2007, fires raged in the area of Kaiafas Lagoon, found on the west coast of Peloponnese. Today, only a few burned trees and bushes on the beach were found that could possibly be tied to the event. 23 samples were recovered from the area, of which 13 were taken along the beach line, a few were from wet areas, and another few from uphill locations and elevated olive groves.

- The Navarino area: Close to the Navarino Science center, some background samples of supposedly unburned samples were recovered. A few kilometers away on a cliff to the west of Gialova Lagoon, a small fire was known to take place in 2014, burning some bushes and trees. Some samples were taken in this area, as well as from another older burned trees found in the castle ruins on top of the hill. One sample was also recovered from the bank of the lagoon below.

Sample sites are marked out on the map in figure 2.

Agios Floros Core Sections

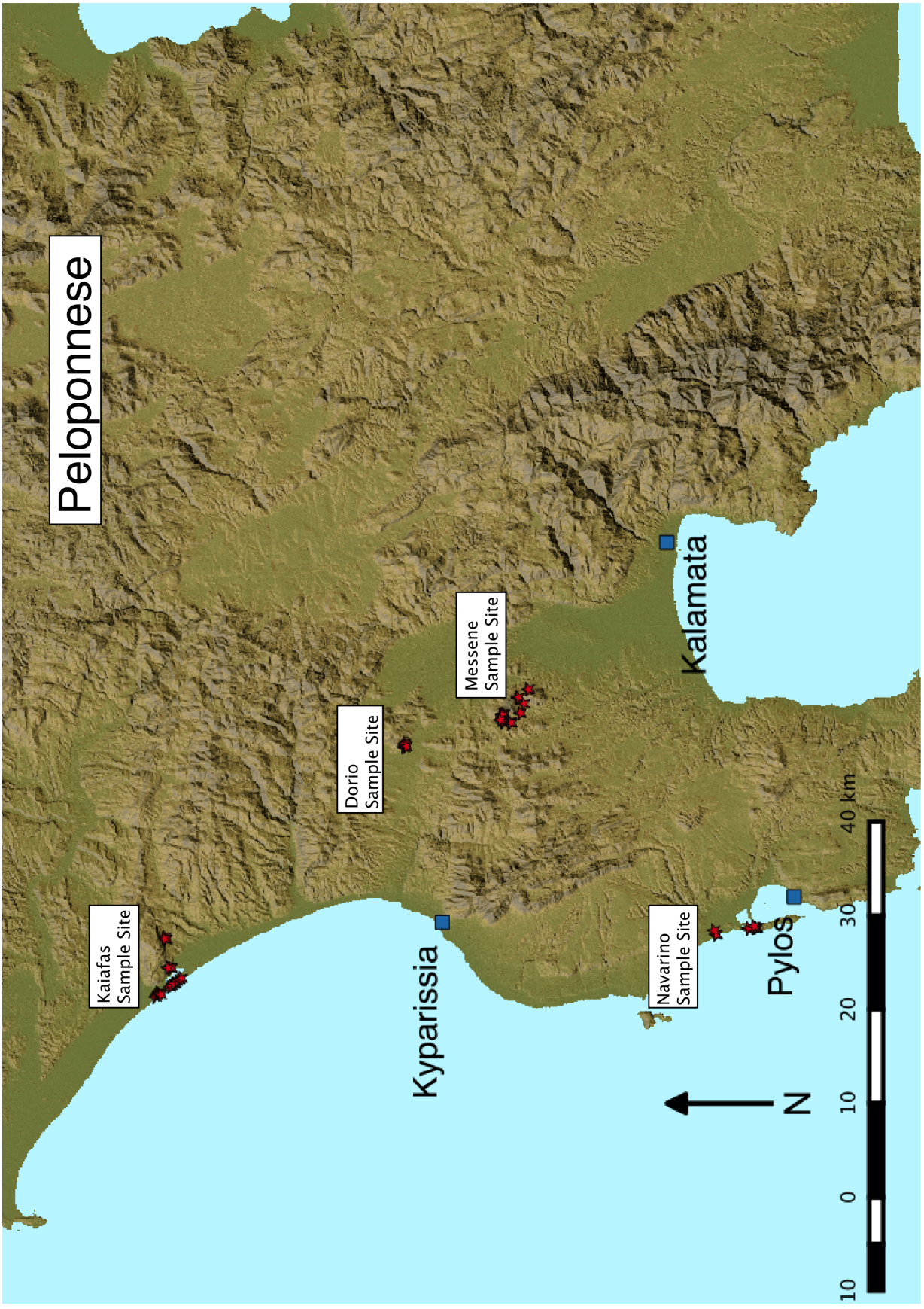
A sediment core, recovered from Agios Floros north of Kalamata, Peloponnese, was already studied in order to gain understanding about the Holocene history of the area (Katrantsiotis *et al, under revision)*. Studies have been carried out with diatoms as an environmental proxy (Katrantsiotis *et al, under revision)*, as well as lipid biomarker analysis, in particular compound-specific stable isotope analysis on leaf waxes (Norström, unpublished data). The core recovered is about 7 m long and was dated by ^{14}C back almost 6000 cal yrs BP. Sediment types change throughout the core. The uppermost two meters are dominated by fen peat, followed by alternating sections of gyttja and clay, sometimes also containing sand and silt (Katrantsiotis *et al, under revision)*.

One of the proxies analyzed in this core is hydrogen isotopic composition (δD) of *n*-alkanes. This composition is though to be connected to the composition of surrounding water, and could be a suitable proxy for recording the changes in isotopic composition in the hydrological cycle (Estep and Hoerig, 1980). This cycle changes in turn, as kinetic fractionation effects favor lighter isotopes for evaporation and precipitation, leaving excess of heavier isotopes in the oceans in times of large glaciations. The δD value is calculated with the help of a standard according to equation 1:

$$\delta D = \frac{R_{sample} - R_{standard}}{R_{standard}} \qquad (1)$$

Where R is the ratio between deuterium (D) and ordinary hydrogen (Sachse *et al*, 2012).

Figure 2 (next page)**:** Map over south-western Peloponnese, with the four sample sites marked out. More detailed maps over the sample sites can be found in figure 12**,** appendix.


Peloponnese
Kaiafas Sample Site
Dorio Sample Site
Messene Sample Site
Navarino Sample Site
Kyparissia
Pylos
Kalamata
N
10 0 10 20 30 40 km

Myanmar Maar Lake Core

Myanmar has for a long time been closed for foreign visitors and lacks well-established paleoclimate records. During a reconnaissance mission in 2013, a group of Maar lakes in central Myanmar were visited and short sediment cores were recovered. The area has a Monsoonal savannah climate where the Rahkine Mountains to the southwest shades the region and makes it relatively dry compared to the rest of SE Asia. The lakes themselves are so-called soda lakes with a pH of about 10-11, which receive only runoff from their relatively small catchment areas (Rienk Smittenberg, Pers. Comm.). Lipid extraction was already performed before the start of this project. A few extracts from one core were tested for approximate LVG concentrations.

Instruments

Principles of the HPLC-MS^2 Analytical Method

The HPLC analytical system follows the same principle as any chromatography system: compounds are separated on a column after their individual properties, causing them to interact at different rates between the mobile phase and stationary phase in a column. The compounds are thereby delaying to different extent, providing a possibility to identify them on a chromatogram based on the retention time (RT) of the peaks for the different compounds (figure 3). In a HPLC system, the mobile phase is a liquid, and selected for the compound of interest so that the compound travels through the system evenly, ideally giving a short but distinct peaks on the chromatogram.

The analytical method of MS depends on the separation of ions after their charge and mass. For the present HPLC-MS system, ionization is achieved through electrospray ionization (ESI). This way, ionization is achieved by the addition of a high voltage to the mobile phase. The energy also creates a fine spray of suspended droplets, enabling for evaporation and analysis in the gas phase in the MS system (Ho *et al*, 2003).

In a quadrupole mass analyzer system, consisting of four metal rods of an even distance, polarity changes between the poles at different rates. The frequency of these changes governs when ions of different mass and charge ratio (m/z) are allowed to come through. In a triple quadrupole MS^2-system, the analyte ion mass is detected by this method in the first quadrupole. When this ion is confronted with a collision gas in the second quadrupole, fragmentation occurs and the first ion fragmentizes into smaller ions, so-called daughter ions. The mass of these daughters can thereafter be detected by the third and final quadrupole, functioning after the same principle as the first in the row (Ho *et al*, 2003). This way, knowledge of the compound being analyzed can be more reliable, provided that different compounds give specific daughters when exposed to this fractionation (e.g. Hopmans *et al* (2013). Analysis of fractions formed from a selected analyte ion is known as Selected Reaction Monitoring, or SRM.

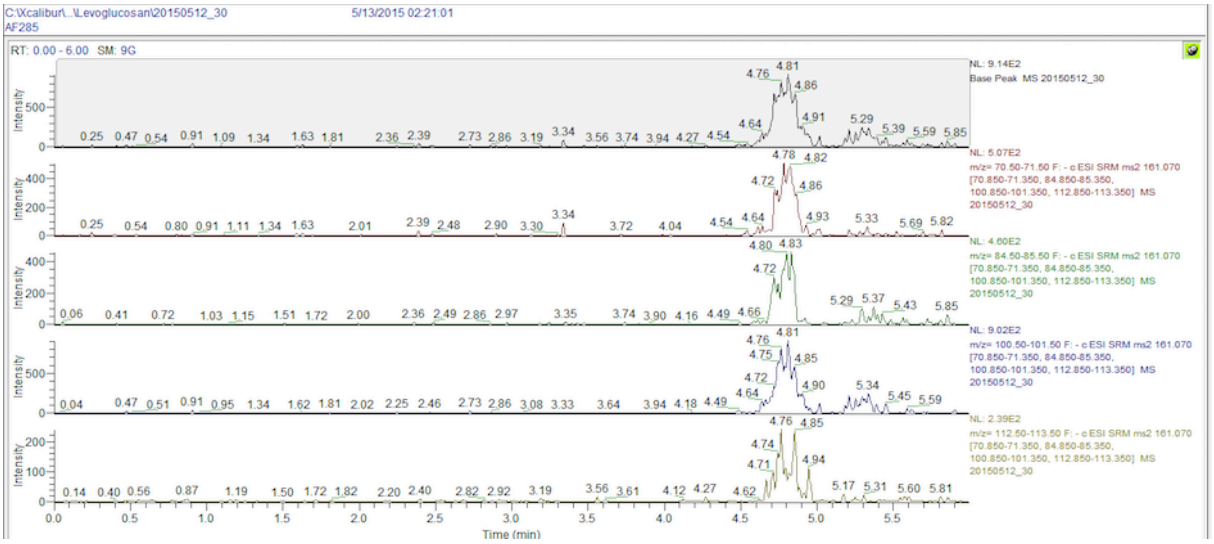


Figure 3: an example of a SRM chromatogram from the HPLC-MS^2 system. This chromatogram shows the analysis for Agios Floros core section depth of 285 cm.

Principles of the SIL Organic Carbon Analytical Method

The instruments setup in the stable isotope lab (SIL) at Stockholms University enables for analysis of the isotopic composition for a range of different sample types. The equipment for this analysis consists of continuous flow mass spectrometer, connected to different elemental analyzers. For this project, no isotope ratio information would be necessary, but instead a value for the total percentage of organic carbon in the sediments (% ORG C) would be required for normalization of the HPLC-MS^2- results. In the elemental analyzer, an adjustable magnet separates molecules after their elemental masses, providing the possibility to target different masses and thereby achieve isotope composition ratios. Upon analysis for elements such as carbon, samples are combusted. The masses of interest are therefore not the pure elemental mass, but the gas phase in which the element is included (Heike Siegmund, pers. Comm).

Methods

Sampling and initial treatment

Sample areas were chosen to be within daily driving distance of the Navarino Environmental Observatory in Navarino, southwestern Peloponnese, where accommodation was arranged. Sampling took place 8-13 February 2015. The sampling locations were selected with diversity mind, aiming to cover different biotopes and environments. Samples were also taken to cover the different ages of the fires, ranging from the big wildfire event in august of 2007 to a fire within the past six months. A complete sample list can be seen in table 6, appendix.
Samples were collected with a small shovel, taken from clean soil surfaces with vegetation and other matter being removed. Quantities of about 30-40 ml were taken at a soil depth of one to a couple of centimeters. Samples were placed in small sample bags and the location was marked with a Garmin Etrex 10 GPS. Notes about the locations were made in a notebook. A photo of each sample location was also taken.

The larger particle fraction of the soil samples was removed by the use of a 2mm sieve. Samples were collected in 40 ml jars, and freeze-dried for three days. Samples with high clay- and moisture content were not put through the sieve, but larger particles were removed by hand.

Early HPLC-MS^2 Method development

The instrument method was setup on the Dionex UltiMate 3000 HPLC system, connected to a TSQ Quantum Access Max MS analyzer. The outset for the method development was the method used by Hopmans *et al* (2013). A big difference between this method and the one developed was the choice of column; the column of choice for Hopmans *et al* (2013) was a Phenomenex Luna NH2 column. When ordering a column from manufacturer Phenomenex, recommendations were given to instead use a Kinetex 5u EVO c18-column, which reportedly would be a good or even better alternative compared to the older Luna NH2 column. During method testing with standards of LVG in solvent mixtures, it was early discovered that the RT had greatly diminished with the use of this column, now pushed back from a RT of about 5 minutes (Hopmans *et al*, 2013) to only about 1,6 minutes. The instrument settings went through a few changes during the course of the method testing, but the final settings used for the analyses are presented in table 2. SRM was performed on mass fractions of m/z 161 to m/z 71, 85 101 and 113, after the method of Gambaro *et al* (2008). No column backflush was set up in the early method development. Solvent composition and ratios were tested and the final settings used for analysis can be seen in table 2. Ammonium hydroxide was added to the solvents in order to change the pH, as triethylamine did not seem to change this sufficiently in the way described by Hopmans *et al* (2013).

ESI settings	Value
Spray Voltage	4000
Vaporizer Temperature	0
Sheath Gas Pressure	20
Ion Sweep Gas Pressure	0
Aux Gas Pressure	35
Capillary Temperature	270
Tube Lens Offset	130
Skimmer Offset	0
Collision Pressure	0
Collision energy	-10
Scan for ion type	Negative
Chromatography settings	**Value**
Flow Rate	0,2 ml/min
Oven Temperature	25 +/- 3

Table 2: Final instrument settings utilized for the sample analysis.

Solvent A, 90%	Solvent B, 10%
Acetonitrile (ACN) 99,9 %	Water (H2O) 99,9 %
Ammonium Hydroxide (NH4OH) 0,1 %	Ammonium Hydroxide (NH4OH) 0,1 %
Triethylamine (Et3N) 0,01%	Triethylamine (Et3N) 0,01%

Table 3. The composition of solvents used as mobile phase for analysis and for dilution of samples.

Extraction

A test sample of fireplace soot was first extracted in order to test the method. 1 g of soot was ground and put into a 40 ml vial. 3 ml of Methanol (MeOH) was added to the sample, which was put in ultrasonic bath for ten minutes and centrifuged at 2500rpm for 10 minutes in a Jouan MR 22 centrifuge. The supernatant was then collected into a 15 ml test tube. This was repeated three times. The sample was then filtered trough pipettes with a fine powder of Na_2SO_4 to remove remaining suspended ash particles, and put into pre-weighed 3ml vials. These were blown down with N_2 gas, and weighed again before kept in storage until further analysis.

Soil samples were weighed up into amounts of approximately 10 g into 40 ml vials. Samples were subsequently split into two vials. 10 ml of MeOH were added to each vial. Sample vials were then shaken, and placed in an ultrasonic bath for ten minutes. They were then centrifuged in a Jouan MR 22 centrifuge at 2500 RPM for 10 minutes, alternatively at 1000 rpm for 12 minutes in a Scanvac Scan Speed 40 centrifuge. Another 10 ml of MeOH were added to each vial, and this procedure was repeated three times. Samples were left overnight, allowing particles to settle. The solutions were then pipetted over to new vials, and the solvents were removed by either gentle blowing with N2 gas, or alternatively by vacuum centrifugation in a Scanvac Scan Speed 40 system. The two vials for every sample were rinsed with Acetonitrile (ACN) containing 0,01% of triethylamine (Et_3N). Each vial was rinsed three times each. The extract was then filtered through a syringe filter with the diameter of 0,45 μm into 4 ml vials. A total amount of about 3-4 ml extract was collected for each sample. The content of the vials were blown down with N2-gas again.
The vials were rinsed with 2x50 μl of the HPLC solvent mixture (table 2) and the extract were added to inserts placed in 1,5ml autosampler vials, for analysis in the HPLC system.

Core samples had been extracted previous to the start of this project. The extraction had been performed with a mixture of Dichloromethane (DCM) and methanol (MeOH) of ratios 9:1, which is a standard way for lipid biomarker analysis. This extraction method was tested and compared to pure MeOH extraction, showing indifferent results but with slightly higher matrix interference for the DCM:MeOH extraction method (Hopmans *et al*, 2013). For the purpose of varying biomarker analysis, the total lipid extracts (TLE) of this core was separated into four fractions of different polarity. This was achieved by applying the TLE to silica gel (5% deactivated) columns and eluting with hexane, DCM, DCM:MeOH 1:1, and pure MeOH. The sediment samples from the Myanmar core was vaporized, and then the lipids were dissolved with ACN + 0,01% Et_3N. The standard

method was continued from there. For each extract, two fractions were analyzed: Fraction 3, following a 50:50 DCM:MeOH rinse, and Fraction 4, following a pure MeOH rinse. This double analysis was performed since it remained unclear in which fraction LVG would show up. For the Agios Floros samples, fraction 4 was not available for processing, but fraction 3 was was reportedly eluted with a large amount of solvent. Samples from this core had been evaporated before this project. Out of convenience and due to problems when dealing with too small amounts of solvent, these samples were dissolved in 100+100 ul of solvent mixture instead of 50+50.

Because the project involved also method development, a few exceptions were made from the main method described above. Exceptions of the final method are listed below:

Sample M01 to M06 along with sample M15 were split into three parts, and about 3 ml of solvent were added to these samples at a turn in the extraction process. This amount were decreased to 20 ml of solvent added per turn to approximately 10 grams of sediment split into two 40ml vials. This change was made, as the amount of solvent and vials used was too large to be easily handled.
Some samples were centrifuged at 1000 rpm for 12 minutes in a Scanvac Scan Speed 40 centrifuge, instead as in the Jouan system.

Method testing

Analysis of the ash sample showed large amounts of LVG. the first soil samples (samples M03-13, M15) did also give results, however corresponding to very low amounts of LVG. These samples had been dissolved into 1000 μl of solvent mixture, of which 20 ml were injected. Therefore, the concentration was increased tenfold by adding 100 μl of solvent mixture to samples instead of 1000 ul. This method was applied for all remaining soil samples. At the time of the first analysis, a tendency for polymerization of the samples were recognized, after they had been in the sampler cabinet with a temperature set to 4°C. The cooling was suspected as a cause for the polymerization and the cooling was switched off for the sampler cabinet.
A few samples from the Myanmar core were analyzed next. The extracts from different depths were treated as the main Greek soil samples, now with increased concentrations. Upon analysis, it stood clear that while most samples seemed to contain LVG, none of the samples contained significant amounts but were close to the detection limit. The total amount of LVG per sample ranged between 0,25-1 ng. Also, the F4 fraction, resulting from a pure MeOH rinse, contained most if not all of the LVG. This varied slightly between samples.

Standards of MAN and GAL were also prepared, diluted in the usual solvent mix to concentrations ranging from 5ng/ul to 0,005 ng/ul. These were also run through the HPLC. It stood clear that the response in the instruments gave peaks with a similar shape and RT as the peak for LVG. Also, all three compounds broke into the same daughter ions. Therefore, the differentiation between these isomers could only be made through differences in RT. In the results of Hopmans *et al* (2013) the authors managed to separate the peaks from each other due to the different RTs of the different molecules, although the peaks for MAN and GAL sometimes occurred together. Most likely the possibility to separate the compounds disappeared with the decreased RT, following the

use of different columns. However, as GAL, MAN and LVG are products of cellulose burning, the bulk concentration of these compounds together would not significantly alter the importance of the concentrations seen in the samples. Still, when compared together, LVG, GAL and MAN making the same daughter ions would be equally indicative for the process of interest. (e.g., Fine *et al*, 2001, 2002).

Troubleshooting

When preparations for final sample analysis were coming to a close, all of the Greek soil samples showed strange sections in the total ion chromatogram (TIC), where practically no signal at all came through for the RT interval of about 1,3 to 1,6 minutes. As Ion suppression is a reoccurring problem in MS analyses, this was suspected to be the cause for this. Ion suppression is usually caused when less volatile compounds changes the efficiency for droplet formation or evaporation in the MS, and this way can influence the amount of charged ions in that reaches the detector in gas phase (Annesley, 2003). Suspicion first arose that this was activated due to a too high concentration put on column, and therefore a series of lower injection volumes were analyzed. However, this did not show any improved results.
Speaking against ion suppression was the fact that samples that had previously showed results, such as sample M03 (figure 4a) now did not show any signal at all (figure 4b). The flat baseline gap was also very consistent, always seeming to show up in the same time interval. Troubleshooting was therefore performed on the instrument method next, and the MS was optimized and instrument equipment cleaned. No improvement came from this treatment. Also, it was noticed that the standards did not seem to be affected by in the same way as the samples, once again pointing towards that the cause for the cancellation was to be found in the sample matrix. To test for ion suppression, Sample M03 was tested again, this time mixed LVG standard of known concentration. This was compared with the pure standard, so that the same amount of known LVG was put on column, enabling for a direct comparison and evaluation of matrix effects. The analysis of the standard/sample mix showed that a peak appeared just after the suspected retention time, but when compared to the pure standard, it rather appeared like the peak was cut off by the disturbance. A full scan analysis showed that, indeed, a large amount of an unknown compound appeared just at the time of the gap in the chromatogram, first occurring around 1,3 minutes..

With a likely cause for the problem identified, different problem solutions were tested. pH of samples and oven temperature was tested and experiments was made changing these values, but to no avail. In fact, lower pH even seemed to make the cancellation gap larger. The sampler cabinet cooling system, which had previously been shut off, was turned on again. Still, repeated tests with standards and sample/standard mixtures revealed that the cancellation gap still existed.

In attempts to filter away the compounds causing the ion suppression, a standard and a test sample was put on an amino solid phase extraction column. The column was rinsed three times, and each time a fraction was recovered. The first fraction was rinsed with a mixture of 90% ACN and 10% H2O. The second fraction consisted of equal parts H2O and ACN, while the last part consisted entirely of ACN. Fractions were evaporated and dissolved in solvent mixture once again, and analyzed in the HPLC. While the fractions showed different results, the cancellation gap did not go away. Rather interestingly, it also showed up in the standards after this treatment, where it had never been observed

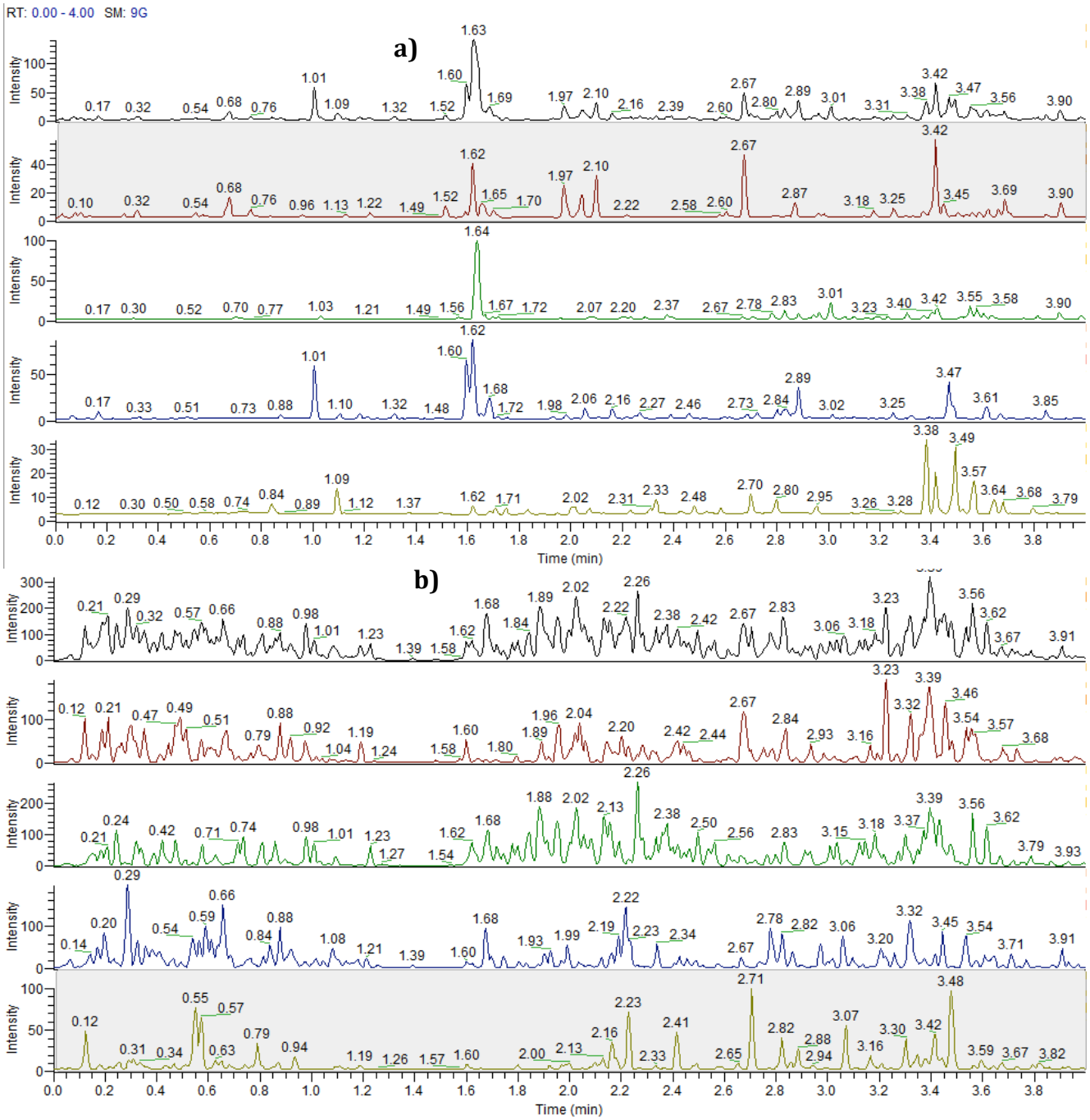


Figure 4. Chromatogram of the same sample tested a) before and b) after the appearance of the flat baseline gap between RT 1,3-1,6. Notice how the small peak at RT 1,62 is completely absent in the later image.

before. In short, the SPE method did not bring any solution to our problem.

Filtering over anhydrous Na_2SO_4 and silica powders over glass pipettes was also tried for both standards and samples. Na2SO4 filtering was performed by Hopmans *et al* (2013), but had been omitted in favor of syringe filtering. The silica columns were rinsed three times with different ratios of dichloromethane (DCM) to MeOH, going from pure DCM in the first fraction to pure Methanol in the last one, with an intermediate step of DCM to MeOH relations 1:1. Rinsing was performed with one ml of solution in each step. This was similar to the different fractions, through which the cores samples from Myanmar and Agios Floros had been prepared, with the only difference that the first

fraction, consisting of pure hexane, was left out. Analysis of these filtering methods showed that NaSO4 filtering did not help with the samples, as the gap was still present and a very high noise level occurred in the sample. The analysis of the different silica column fractions for the samples showed an occurrence of LVG already in the first fraction. Here, the flat baseline gap was also absent. The second fraction showed a peak at a retention time of 1,75 minutes, but only the fraction ion with mass 84 was present, questioning the credibility of the source for this ion. Also, this fraction contained a very high noise level as well as the flat baseline gap at a similar RT. The third fraction showed the highest peak, at a RT of about 1,67, but here the gap also occurred again. The fact that LVG was present already in the pure DCM rinse fraction was surprising, as hardly any LVG was found in the DCM/MeOH 1:1 rinse fraction for the Myanmar samples. This pattern was however not seen again in the standards going through the same treatment. Instead, the silica gel fractions for the standards indicated that LVG ended up in equal parts in the second and the third fractions. In the second fraction, the noise level intensity decreased from about 100 to only 3 between RT 1,3-1,4. Although it is hard to know for sure, perhaps this could be a trace of the ion suppression gap seen in the second and the standard runs. This did not return in the third fraction.

As a part of the LVG content seemed to end up in the DCM:MeOH 1:1 fraction, a hypothesis was put up that with a well-rinsed second fraction, all of the LVG would be rinsed down already in the second step. This was tested by repeating the silica-gel step, with the same standard as before. For the DCM/MeOH 1:1 intermediate fraction, the amount of rinsing solution was increased from 1 ml to 4 ml. Analysis of this fraction showed that indeed, all LVG of the standard seemed to end up in the second fraction. The dated core samples from Agios Floros on Peloponnese, collected and already extracted by Elin Nordström, was reportedly well-rinsed with solvent in the third fraction, corresponding to the second in this experiment. It was therefore likely that we would see a signal in this fraction corresponding to the whole or most of the LVG in the samples. This would be even though the F3 fraction of the samples already tried from the Myanmar core showed no or very small levels of LVG in the F3 fraction, and that most of the LVG were found in fraction F4.

However, at this time, the analysis was put on hold until a new column, a Luna NH2 column – the exact same column as the one used by Hopmans *et al* (2013) arrived, hoping that the new column would solve the problem with the signal cancellation gap.

Total Carbon Analysis

A total of 45 soil samples were not in any way spoiled during the process or being discarded due to time issues, and was therefore analyzed for % ORG C. Samples were carefully weighed up in small silver containers in amounts of 5-10 mg. The amount of sample shifted due to the expected amount of carbon for each sample, where carbon-rich samples would result in a smaller amount of sample. The rough estimate of the carbon content for each sample was made from the color of the sample, with a darker shade most likely representing a more carbon-rich soil. For a majority of the samples, the scales would keep increasing the values on the microgram scale, possibly since the freeze-dried soils, now in contact with the surrounding air, tended to accumulate moist continuously. To get a reproducible value for each sample, the scales were allowed to

stabilize, and about ten seconds were counted before the value on the scales were noted down.
All the samples were treated with a 2M HCL solution in amounts about 100 – 200 μ l per sample. The exact amount varied between samples, as the goal was to cover the samples completely with the solution. Through this treatment, the samples were expected to be completely free from carbonates at the time of analysis, and indeed some samples reacted quite heavily with the acid. Samples were left to dry for about 24 hours at 60° in an oven. The containers were then closed with the use of two tweezers, and curled up and compressed into small, compacted packages. Unfortunately, during this step a few of the packages ripped open due to inexperience. In most cases the loss was considered minor, but in at least two or three cases the loss was significant. The losses were noted in the data sheet. Samples were analyzed in a Carlo Erba NC2500 system, coupled with a Thermo Scientific Delta V advantage MS. Standards used was Acetanilid (%C = 71,09) and an internal sediment standard (%C = 3,32). Analysis was supervised by personnel in the SIL Laboratory, Stockholm University, and administered by dr. Heike Siegmund.

Final analysis

After the Luna NH2 column was installed, the ion suppression problem also seized. The retention time for LVG also increased to values of around 4-4,5 minutes, more similar to those reported by Hopmans *et al* (2013). With the longer retention times, standard mixtures between LVG, MAN and GAL showed that it was possible to separate the two latter from the first. A notable retention time difference of about 6-7 seconds could also be observed between MAN and GAL. However, this retention time difference was too short to be quantifiably used for the different samples.
Calibration sets were analyzed of concentrations between 0,25-7 ng/ul, confirming that the response signal for all three substances could be interpreted as linear. The detection limit for samples appeared around 0,25 ng/ul, as it was not always possible to separate this amount from the background noise. Test samples were run confirming that a more complex matrix also showed results with the new column.
A new analysis method was put up, now following the method of Hopmans *et al* (2013) more accurately. The authors went with a runtime of 10 minutes, following a backflush of 20 min during which solvent B was increased to 30% and re-equilibrating for another 20 minutes. In this experiment, the retention time was cut down to 6 minutes, and an isocratic backflush was utilized for 10 minutes per sample. This was due to the theoretical aspect that whatever made it through the column in six minutes would also have sufficient time to go back again. Reoccuringly after every 16-20 samples run, a backflush method more similar to the one of Hopmans *et al* (2013) was performed to remove any possibly remaining contaminating compounds on the column. Here, solvents with the concentrations of 70%A and 30%B were run for 20 minutes, after which the system was allowed to re-equilibrate to starting conditions. This was in order to thoroughly clean the column every once in a while, and was always performed between the analysis of a standard and a blank. While the total protocol of samples and calibration standards to be analyzed in the final run exceeded 100, following the protocol of Hopmans *et al*, the sequence would have taken almost three days straight. By changing the method slightly the time could be decreased to less than half.
When the final analysis was initiated, samples were run approximately 16 at a time, followed by a standard mixture, a blank, and calibration standards for LVG, MAN and

GAL of concentrations ranging between 0,25-7 ng/ μ l.

Unfortunately, during the final run an error occurred after about 40 samples, as the sample needle hit the glass wall of one of the inserts in the sample vials. The analysis was allowed to continue, but after a while the concentration of samples and standards alike decimated into much lower signals than seen before. Unfortunately, this problem was not recognized for a few days. Worried that an unknown amount of liquid could have disappeared through evaporation during the time since analysis, All soil samples after K17 (see table 6, appendix) and all sediment samples needed to be blown down, refilled, and vortexed before re-analysis. The amount of solvent mixture added corresponded to the calculated theoretical remnant of the samples after the prior injection, in order to regain the original concentration of the samples. Unfortunately, this made an earlier method problem worse by mistake, as explained in the possible error section (page 29). Another attempt was made, picking up where the error occurred**.** Due to the error, only the lowest calibration standard concentrations were trustfully analyzed. However, with the restart, added calibration standards seemed to go well together with the old ones, leading to the conclusion that only insignificant amounts of liquid had disappeared through evaporation..

The second attempt finished the analysis of the Greek soil samples, but did also have to be cancelled, since the response from the standards started to decrease once more. Most notably, the standard mix between LVG, GAL and MAN disappeared completely during analysis, even though the amount on column would equal 2,5 ng for each substance. The signal went from being quite strong to completely disappear. A signal decrease was also observed in the samples.

Before the third attempt was made, the ESI needle was cleaned and sonicated. This time, a complete calibration series between 0,25-2,5 ng OC was run both in the beginning and end of the series. The standard mixture never showed any results in the third attempt either, but the calibrations in the end and the beginning of the run showed to be fairly constant. The conclusions drawn were therefore that the samples analyzed in this run were trustworthy, and that whatever happened to the standard mixture was an isolated phenomenon unlikely to also affect the samples. Also, the cleaning of the ESI needle showed to increase the clarity of the signal, now giving a less amount of background noise and lowering the limit of detection. Now, the 0,25 ng OC calibration standard was also clearly distinguishable for all three isomers, significantly increasing the low-level detection certainty.

After all samples were run, sample M05 was run ten times in a row, to test the instrument error for the analysis.

Processing and calibration

As the different runs ended up with different noise levels, a number of processing methods had to be constructed. The settings for these are presented in table 4**.** Two separate calibrations also had to be performed, where the first one represented the first two analysis attempts and was made by composite calibration runs from both the two earlier runs, and the second one represented the clearer, final run. Due to the problems associated with analysis, there was never a chance to make duplicates of the calibration standards for the soil samples. For the sediment samples, two sets of calibration

standards were weighed in for consideration.

During the processing, another problem appeared when dealing with the data. While the RT for MAN and GAL varied somewhat, MAN appearing slightly before GAL, it was impossible to do this separation when they appeared in the sample together. This was also the case for runs performed by Hopmans *et al* (2013). However, GAL gave a smaller signal per concentration unit compared to MAN. The practical implication for this is therefore that for any given concentration observed at the expected RT for MAN and GAL, the composition between these defines the amounts observed and no practical method were observed to distinguish the two from each other**.** Also, on multiple occasions, peaks in sample grew together, making it hard to accurately attribute the right area to the right isomer, also in the case for MAN and GAL appearing together. Assumptions were therefore made, assuming a relation between MAN and GAL of 5:1, calculated after table values from Fine *et al* (2001, 2002) and assuming a linear calibration between the samples. The linear fit was forced through origo to more accurately calculate for the low values of MAN and GAL found in the samples.

Processing Method Name:	Expected RT (min)	Window (sec)	Smoothing Points	S/N Treshold	Expected Width (sec)	Min. Peak Height (S/N)
LVG Soil samples 1	4,31	31	11	3	17	3
MAN+GAL Soil samples 1	3,8	40	11	50	55	4
LVG Soil samples 2	4,5	31	11	3	17	5
MAN+GAL Soil samples 2	4,05	40	11	50	50	4
LVG Sediment samples	4,75	19	11	3	15	20
MAN+GAL Sediment samples	4,25	19	11	3	15	20

Table 4: Settings for the different processing methods used. Soil samples were run in two runs for which two different processing methods were used. Sediment samples were run all in a sequence. Different settings were also used for the different components.

Results

An example of the calibration curves used is shown in figure 5. The rest of the calibration diagrams can be found in the appendix. Table 5 presents the instrument error for the different components, judged by the run of the same sample multiple times in a row. This row was however not calibrated and the area compares poorly to the earlier soil sediment runs. Error bars for standard deviation are therefore left out of the main sample graph seen in figure 6.

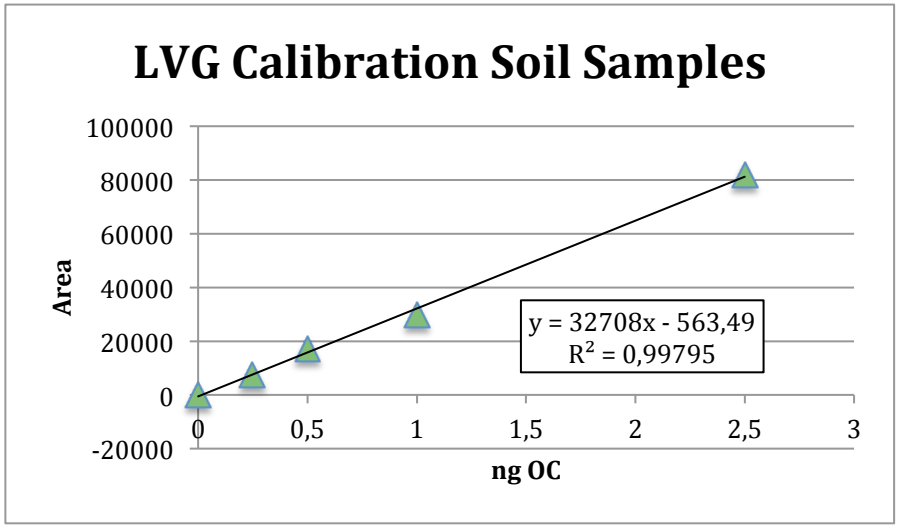


Figure 5: Example of calibration curve. This one was applied for LVG in the soil samples.

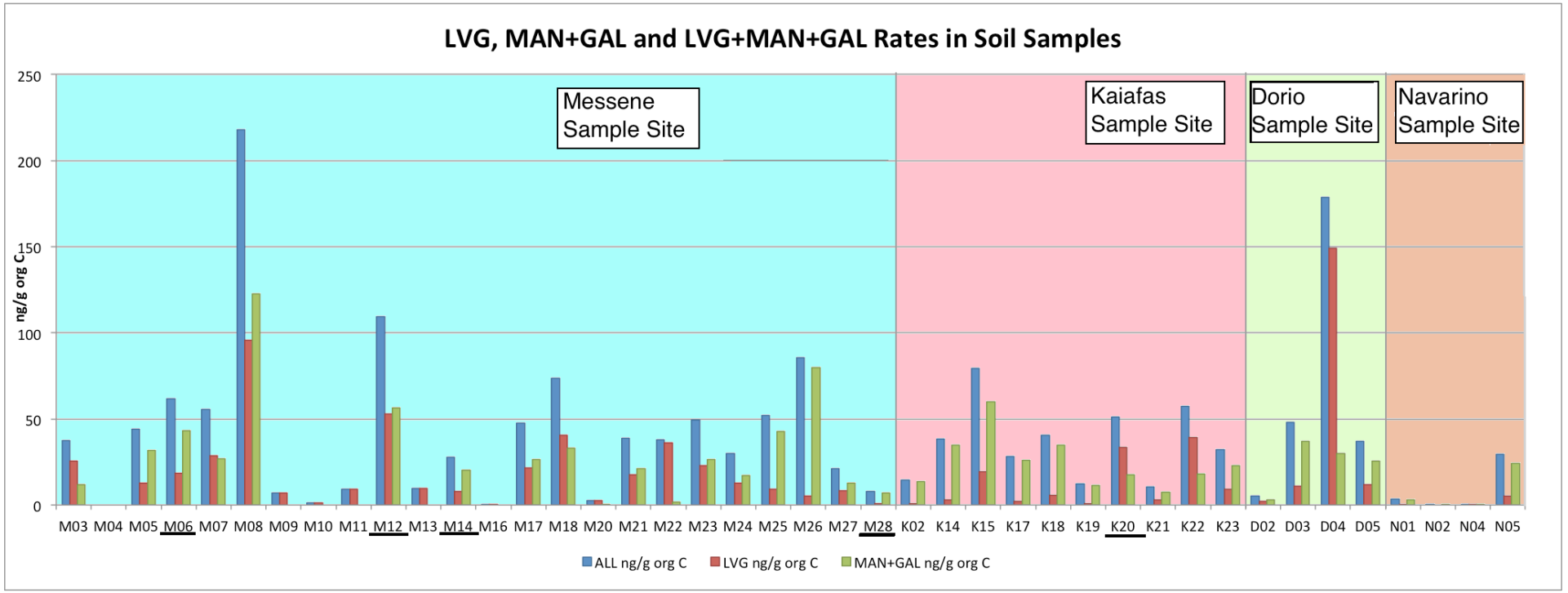
Levels for LVG, MAN+GAL and LVG+MAN+GAL for all analyzed soil samples are displayed in figure 6. In the final results, samples with known or unknown errors have been removed. This regards samples that were accidentally consumed or destroyed during method testing and preparation, for example through broken bottles, mix-up between bottles, unknown amounts of solvents added etc. A few samples were also discarded out of time limitation or due to being judged as inappropriate due to course particle sizes, leading to only 42 of the total 69 samples recovered being analyzed and processed. In the chart, the five samples for which a known or potential volume loss occurred during the SIL analysis are still included. However, these are marked out in the figure, as they may report higher concentrations of the anhydrosugars than may actually be the case. These are however not incorporated in the mean value graph (figure 7), in order to remove the risk for adding data uncertainties. In this graph, sample N05 was also discarded since it was taken close to burned vegetation. This way, the Navarino area samples displayed in the figure represent areas of no known fires in modern days.

Components:	Average area	SD +/-	SD %
LVG	49304,84	25499,91	51,72
MAN+GAL	103543,42	52174,60	50,39
TOT	152848,26	63322,77	41,43

Table 5: Standard deviation calculated for the different components for sample M05, analyzed ten times after one another after the final sample analysis.

In **figure XXX,** the biomass burning tracers are also plotted against δ D-data for the same core, provided by Elin Norström (Norström, unpublished data). In this figure, the mean value for δ D was calculated from the δ D of *n*-alkanes of chain lengths of 21, 23, 25, 27, 29, 31, 33 and 35 carbon atoms. These were in turn normalized after the relative abundance of each chain length. Generally, the δ D of *n*-alkanes can vary largely within a single sample, due to different water sources being used for biosynthesis (Sachse *et al*, 2004). The mean value was used in order to see the main trend of the δ D value of this site over time.

Figure 6 (next page): The rates of LVG, MAN+GAL and LVG+MAN+GAL displayed together from the different sample sites. Underlined samples suffered a more or less significant weight loss during TOC analysis, and do therefore likely show slighly higher values than the actual case.


LVG, MAN+GAL and LVG+MAN+GAL Rates in Soil Samples
ng/g org C
250
200
150
100
50
0
Messene Sample Site
Kaiafas Sample Site
Dorio Sample Site
Navarino Sample Site
M03 M04 M05 M06 M07 M08 M09 M10 M11 M12 M13 M14 M16 M17 M18 M20 M21 M22 M23 M24 M25 M26 M27 M28 K02 K14 K15 K17 K18 K19 K20 K21 K22 K23 D02 D03 D04 D05 N01 N02 N04 N05
ALL ng/g org C
LVG ng/g org C
MAN+GAL ng/g org C

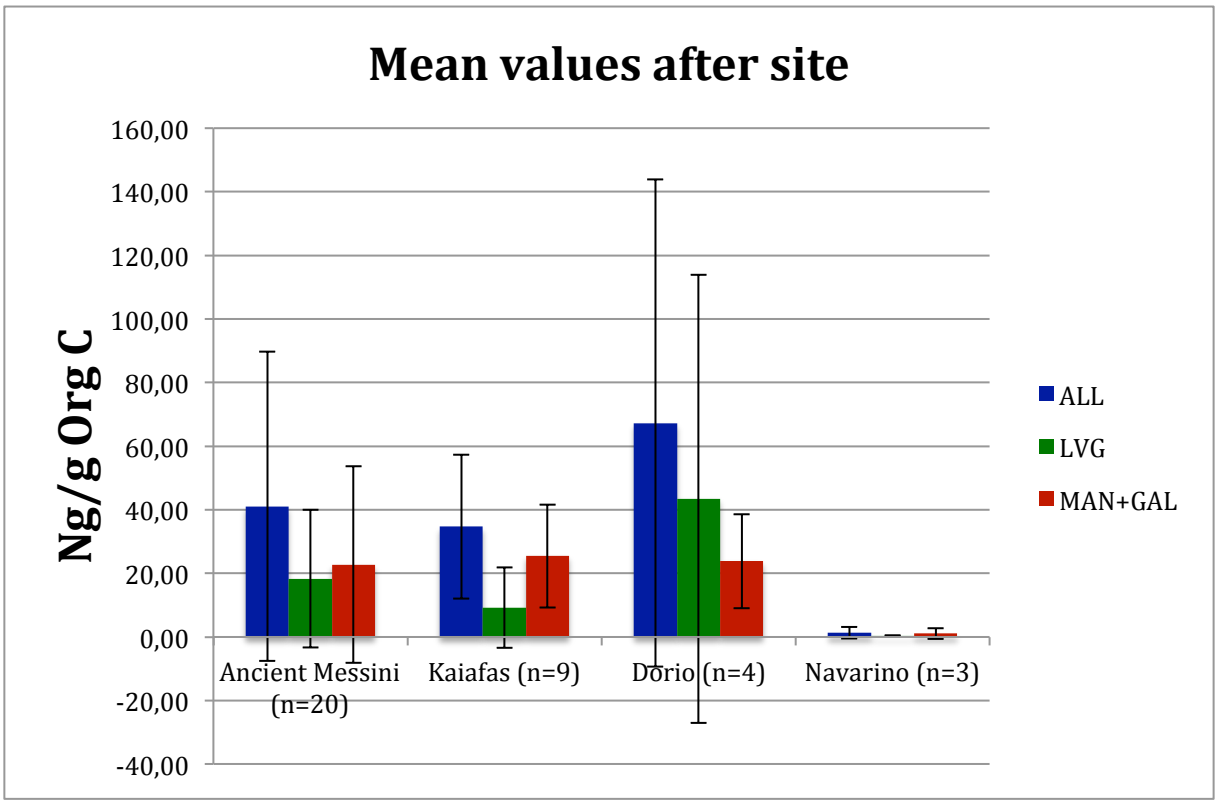


Figure 7: rates of LVG, MAN+GAL and LVG+MAN+GAL for the soil samples, grouped after sample sites. The error bars show the standard deviation for the values.

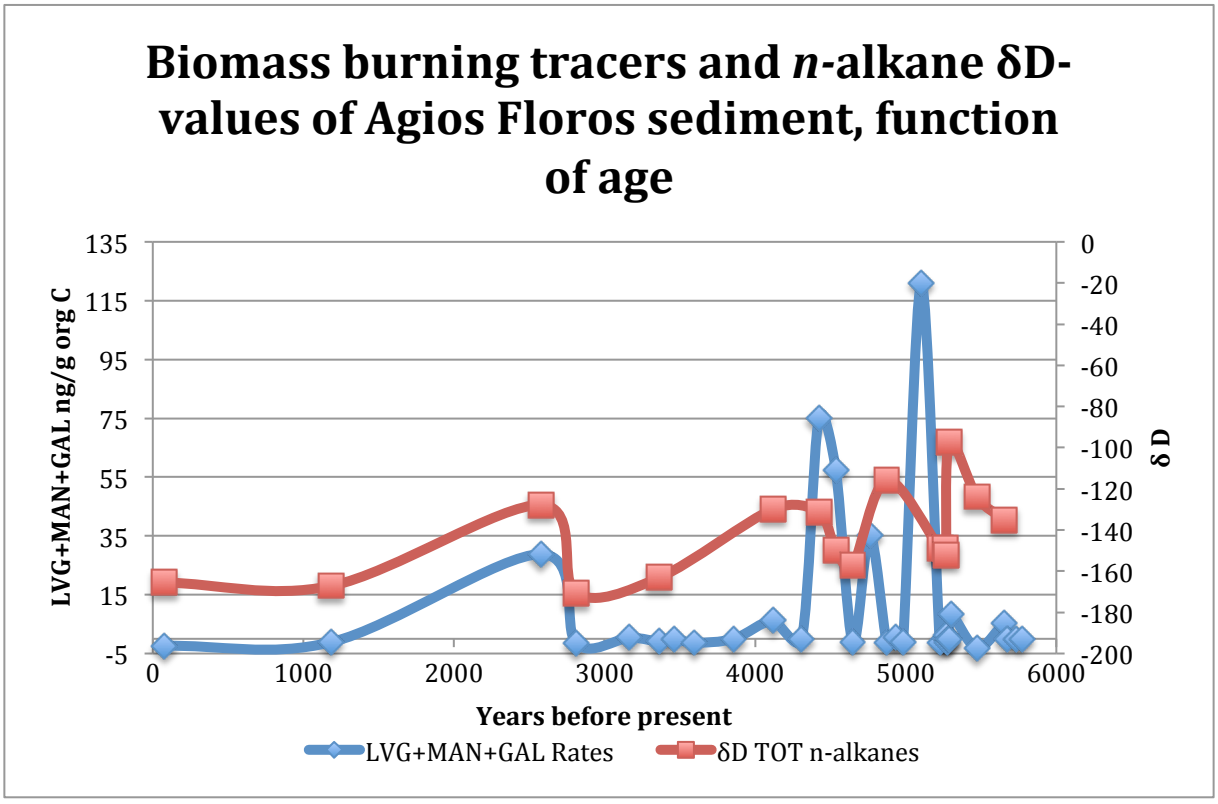


Figure 8: comparison between LVG+MAN+GAL rates to δD-values of all measured lengths of *n*-alkanes. The δD-values of the individual chain lengths for *n*-alkanes are displayed in **figure xxx**, appendix.

Discussion

Greek soil samples

The soil sampling was performed with the different aspects of the environment in mind: The slope, vegetation and soil type was considered as different variables that may affect the rates of LVG and its isomers in the environment. The characteristics of the fire was also expected to play part, with aspects such as age since fire, fire extent, wind direction and distance from burned trees serving as important variables. In this experiment, the results are not precise enough to be interpreted on this level of detail. The reason for this lack of precision is partly due to different sources of error, but mainly due to a large instrument error, as displayed by table 5. Additional instrument error was introduced for the soil samples, since the analysis was interrupted due to instrument errors, and must be run in two separate sessions. This introduced both a method error (see error section) and potential instrument errors due to time passed between analyses. Still, when samples are compared together after site, some general indications can be seen in figure 7.
Given the recent and extensive fire at the Messene sample area, this area was expected to show high rates of LVG and its isomers. The 20 samples included in the mean calculation come from varying locations in the large fire area, including wet areas and areas outside of the main sampling area. The also quite well covered area of Kaiafas Lagoon shows similar levels, indicating that most of the tracers stayed stable in the ground during the eight years since the major fire event.

In all sample areas, MAN and GAL are emitted in high quantities when compared with LVG. This is not consistent with earlier studies, showing that MAN and GAL are emitted in values one or two magnitudes lower than LVG from plenty of hardwood and softwood species (Fine et al., 2001, 2002). This raises the question if the RT is sufficient to distinguish between the three isomers, or if, in samples with complex matrix, LVG actually shifts in RT and therefore has been mistaken for peaks of both MAN and GAL at multiple occasions. In any case, by adding all three isomers together, one could avoid this possible error and get a good average value of the total biomass burning-generated anhydrosugars, which, with an identical source and similar molecular structure, still would be valid as a biomass burning biomarker. As mentioned in the method section, this would introduce another error due to the lower response factor observed for GAL, but perhaps this could be viewed as an error of acceptable magnitude due to the response factor for LVG and MAN being largely similar, and since GAL has been reported to be emitted in the lowest quantities of the three (Fine et al., 2001, 2002).
The Navarino location samples do indeed show the smallest signal, effectively acting as a blank when compared to the other sites in figure 7. Still, the strongest mean signal comes from the sample location of Dorio, where a fire of a smaller scale occurred. While it may seem surprising that this fire should leave the strongest signal, it is also important to remember that this only four samples were tested from this location and that this result therefore is quite questionable. Sample D04, containing one of the highest rates of biomass burning tracers of this study, could for example accidentally have been sampled close to a modern fireplace or similar. As mentioned above, instrument precision error could also affect the result.

Agios Floros core samples

As mentioned, the analysis of the Agios Floros core extracts went more effortlessly as all were analyzed in one session, calibrations went well and the noise level during analysis was low. Also, no extraordinary problems or losses occurred during the preparations of these samples, see the possible errors – section for problem possibly affecting all samples.
Perhaps due to the increased clarity of the signal in these runs, the amount of LVG was significantly higher compared to MAN+GAL. This, therefore, bears a larger similarity the expected ratios of these isomers presented by Fine *et* al (2001, 2002). Where MAN and GAL do occur, however, the ratios between them and LVG are still much larger than previously visualized, perhaps meaning that also in this case there may be an interpretation error associated with distinguishing the isomers from another based on retention time. In figure 8, the total of these isomers are therefore presented together, to minimize the interpretation errors. By adding MAN and GAL to the visualization, the trends seen in the diagram are only amplified, as the significant features presented there are also present when only LVG data is modeled.

In figure 8, it can quite clearly be seen that a correlation exists between the δD record and the biomass burning tracers. Although the interpretation of the Agios Floros lipid-δD is under progress (Norström, pers. Comm.), one could hypothesize that a warmer and drier climate may be represented by a higher δD value (Sachse *et al*, 2012). Ideally higher δD values would therefore correlate with increasing values of LVG, MAN and GAL found in the core. For the beginning of the record, between approximately 5,6-4,3kyrs BP, the correlation is not very straightforward. However, this could be due to resolution issues, as the δD data is in lower resolution than is the biomass burning data for the same interval. It is therefore possible that important features seen in the biomass burning tracer record have been cancelled out in the δD record. Still, both these record shows a general, large variability during this period. Between approximately 4 – 3 kyrs BP, the δD record shows a slow decrease towards lower values, although the actual features may be hidden by the low resolution for this interval. For the same time period, the signal for biomass burning disappears, making correlation hard for this interval.

Although only a few data points exist for the last 3 kyrs BP, the correlation between the records is very significant from this point forward. A pretty sharp shift towards drier and hotter conditions are indicated to have taken place between approximately 2,8-2,6 kyrs BP. This seems to be followed by a 1,5 kyrs transition period into wetter and/or colder conditions, after which the biomass-burning signal completely disappears.

The results displayed in figure 8 do not correlate significantly with general climate reconstructions for the Mediterranean region (e.g. Finné *et al* (2011)). It is possible that the results seen in this record would represent climate variations on a more local scale. These results will be complemented as analysis of the core is still under progress, also adding charcoal counting to the proxies used for climate reconstruction. The results will be presented in a scientific article in the future, focusing on the past climate of the Agios Floros area (Norström, pers. Comm.).

It is important to remember that there are many different factors affecting the rates of LVG, MAN and GAL found in sediments or soils after a fire event. First off, the rates

depend heavily on the kind of vegetation burned (e.g. Simoneit, 1999, Fine, 2001, 2002) where grassland and other vegetation not containing cellulose or hemicellulose would not be expected to leave these molecules behind after fire. The conservation of LVG has also been indicated to change a lot depending on environment (Knicker *et al*, 2013). Therefore, the usefulness of this proxy is probably more limited to semi-quantitative works. LVG and its isomers can indicate that a fire has occured, but for the time being the image is too complicated for the proxy to be used for any more than a measure for relative abundances. Problems with the instrument precision and method errors associated with this project are therefore not devastating for the results achieved, as the method used has not changed significantly between samples, and sample preparation was made by one person only. As long as samples are compared between another and no absolute value is sought for, the method setup in this project is already useful and could possibly be improved by further development.

Possible Errors

The largest possible error occurred consequently in all sample preparations. As samples were transferred to sample vials and being diluted into the solvent mix during the last step of the sample preparation, a misunderstanding occurred. A quantitative way of doing this sequence would have been to transfer the sample by diluting it in an excess of solvent mixture, rinse multiple times, evaporate the sample and thereafter add the wanted concentration of solvent mixture to achieve the wanted concentration. Instead, the samples were dissolved in the exact amount of solvent mixture directly, and while this was done in two turns and involved careful rinsing of the container to make sure that no extract was left behind, a small amount of the sample was always left on the walls of the container of the previous vial after transfer. By this mistake, the repeatability of the experiment was severely affected. As for the comparative results between samples in this report, all samples were prepared in the same manner and the individual changes in treatment between samples are therefore estimated to be very small. Still, as the final sample analysis had to be performed in multiple steps and sample evaporation and re-dilution was performed on a number of samples in order to guarantee an even concentration of the remaining samples, this would affect the concentration of the remaining samples when compared to the preceding ones. Roughly estimated, 20 μ l of the sample remained in the previous vial after transition. For samples only diluted in 100 μ l of solvent mixture, this would mean that the evaporation and refill process would dilute the samples by about 1/5. For Agios Floros core samples, being diluted in 200 μ l, the dilution of these samples would be about 1/10 compared to those analyzed in the first run. Samples M03 to K17 were analyzed during the first attempt, and would therefore be slightly stronger when compared to the preceding samples analyzed. By introducing an internal standard to the samples during future analysis, it should be possible to account for sample losses associated with the preparation.

Other significant errors include the difficulty in separating MAN from GAL in the chromatograms, as discussed above. The large instrument error and variability, as seen in table 5, also increases the possible error for all the samples, and also for the total area of the chromatogram. That means that using the total area for LVG+MAN+GAL to determine a relative value for biomass burning cannot completely solve this problem.

Conclusion

In this project, a method for determining fire history using chemical tracers was developed at IGV, and applied to modern soils and old sediments. A few problems with the resolution of the method cripples the actual result of the study performed. However, as so many factors play part in the spread and breakdown of the tracer in the environment, LVG is capable of telling whether there has been a fire event or not and is likely less useful for distinguishing the size of the fire. Therefore, relative abundances can still be used to compare areas with one another, as long as the method is consistent. From that aspect, the results for this study are still useful. The biomass burning tracers compare well with the δ D record of the Agios Floros sediment core, strengthening the image of the regional climatic and environmental situation of the site. The soil sample study also show credible and comparable values, when the areas are compared to one another. These results also show that a normal lipid extraction method could be sufficient for separating out LVG for analysis, if eluted by sufficient amounts of solvent in the DCM:MeOH 1:1, v/v step of a common fractionation method. To include this method together with other analyzes performed on soil and sediment samples at IGV would therefore be a simple, quick and cost-effective way to improve the diversity of independent proxies for climatic and environmental reconstruction.

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Appendix

Table 6: List of soil samples taken and short descriptions of the sample sites. Samples marked with stars were successfully analyzed during the run of the project.

Locality	Name	Short description	Collected on
Messini	M01	Pretty flat location, among burned shrubs	08-feb
	M02	Pretty flat location, among burned shrubs	08-feb
	M03	Pretty flat location, among burned shrubs	08-feb
	M04	Flat area, outskirts of burned area	08-feb
	M05	Relatively flat area in a slope. Surrounded by burned bushes and trees	08-feb
	M06	In the middle of burned trees, sloping	08-feb
	M07	Burned grove in a field. Wet and flat	09-feb
	M08	Relatively flat area in a slope. Surrounded by burned bushes and trees	09-feb
	M09	Flat, wet area. Surrounded by burned bushes and trees	09-feb
	M10	Sample taken from a shallow puddle	09-feb
	M11	Sloping area, sample taken from burned grove	09-feb
	M12	Sample taken from a shallow puddle	09-feb
	M13	Gentle slope, some burned trunks	09-feb
	M14	Steep slope, close to burned trees	09-feb
	M15	Flat location, close to burned trees	09-feb
	M16	Steep slope, close to burned trees	09-feb
	M17	Gentle slope, close to burned trees	09-feb
	M18	Close to heavily burned trees	09-feb
	M19	In grove with burned trees	09-feb
	M20	Gentle slope, meadow with charred trees	09-feb
	M21	flat location, no sign of burning	13-feb
	M22	flat location, no sign of burning	13-feb
	M23	Flat area, outskirts of burned area	13-feb
	M24	Pretty flat location, among burned vegetation	13-feb
	M25	Relatively flat area in a slope. Just outside of burned area	13-feb
	M26	Relatively flat area in a slope. In the middle of burned area	13-feb
	M27	Relatively flat area in a slope. Burned area, close to high trees	13-feb
	M28	Slope, outside of burned area	13-feb

Locality	Name	Short description	Collected on
Kaiafas	K01	Pretty flat location, dune environment	10-feb
	K02	Flat location, grass spot	10-feb
	K03	Flat location, lld fireplace	10-feb
	K04	Flat location, sand dune	10-feb
	K05	Flat location, grass spot	10-feb
	K06	Sand dune	10-feb
	K07	Sand dune	10-feb
	K08	Sand dune	10-feb
	K09	Sand dune	10-feb
	K10	Sand dune	10-feb
	K11	Sand dune	10-feb
	K12	Sand dune	10-feb
	K13	Sand dune	10-feb
	K14	Gentle slope, overall steep slope on a hill	10-feb
	K15	Steep slope	10-feb
	K16	Outskirts of swamp	10-feb
	K17	Sample taken from a deep puddle	10-feb
	K18	Sample taken from a flooded field	10-feb
	K19	Steep slope close to a garden	10-feb
	K20	Gentle slope, outskirts of an olive grove	10-feb
	K21	Gentle slope, outskirts of an olive grove	10-feb
	K22	In grove with dead trees	10-feb
	K23	Gentle slope, outskirts of an olive grove	10-feb
Dorio	D01	Sample taken in an olive grove	11-feb
	D02	Slope, outskirts of an olive grove	11-feb
	D03	Flat area, close to a dead tree	11-feb
	D04	Close to brushwood, outskirts of an olive grove	11-feb
	D05	Close to brushwood, outskirts of an olive grove	11-feb
Navarino	N01	Under bushes on the beach	12-feb
	N02	Under bushes on the beach	12-feb
	N03	Under bushes on the beach	12-feb
	N04	In olive grove	12-feb
	N05	By burned shrubs	13-feb
	N06	Some burned shrubs	13-feb
	N07	On a slope, 50 m from burned trees	13-feb
	N08	By burned trees	13-feb
	N09	Flat area in a meadow, above burned trees	13-feb
	N10	Flat area, some dead trees	13-feb
	N11	Flat area, thick grass. Surrounded by some burned trees	13-feb
	N12	Dense forest	13-feb
	N13	On a bank of clay	13-feb

Table 7: % C ORG analysis results.

Sample name	% Corg
M02	24,80
M03	7,38
M04	11,02
M05	30,59
M06	23,52
M07	22,66
M08	7,28
M09	18,04
M10	2,50
M11	14,68
M12	4,46
M13	14,54
M14	6,49
M15	3,06
M16	6,96
M17	16,98
M18	4,70
M20	4,55
M21	8,19
M22	1,02
M23	7,17
M24	8,73
M25	10,89

Sample name	% Corg
M26	4,96
M27	3,70
M28	5,94
K02	2,02
K14	9,10
K15	8,08
K16	6,31
K17	11,59
K18	5,24
K19	2,83
K20	7,38
K21	5,03
K22	2,04
K23	11,58
D02	6,89
D03	5,86
D04	2,09
D05	4,05
N01	7,96
N02	2,72
N04	3,45
N05	6,78

Figure 9: Soil sample calibrations. Only a single set of calibration standards were analyzed. a) MAN b) GAL.

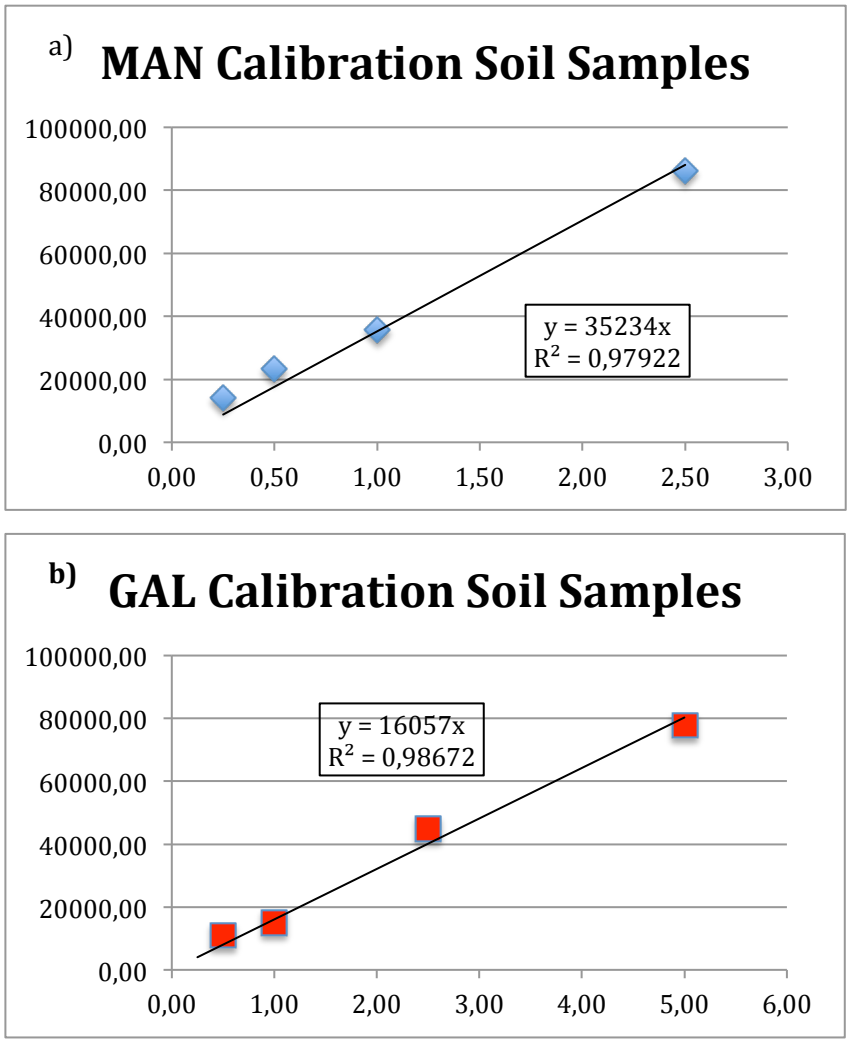
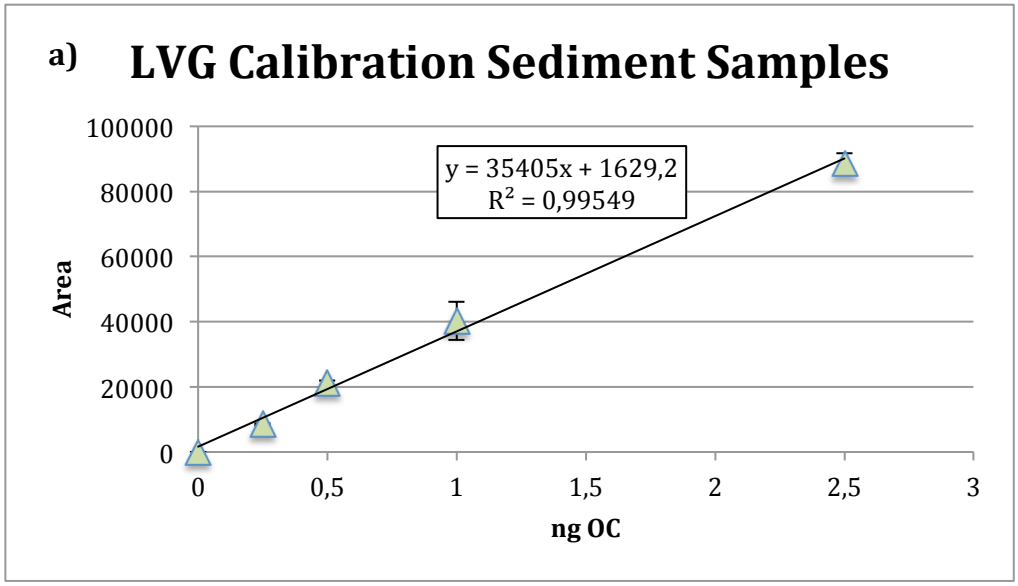
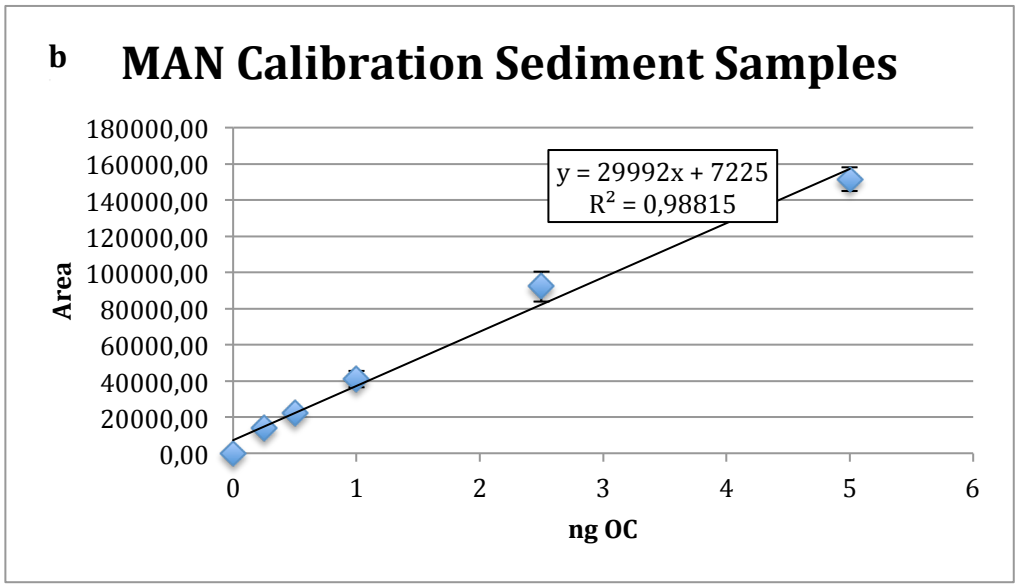
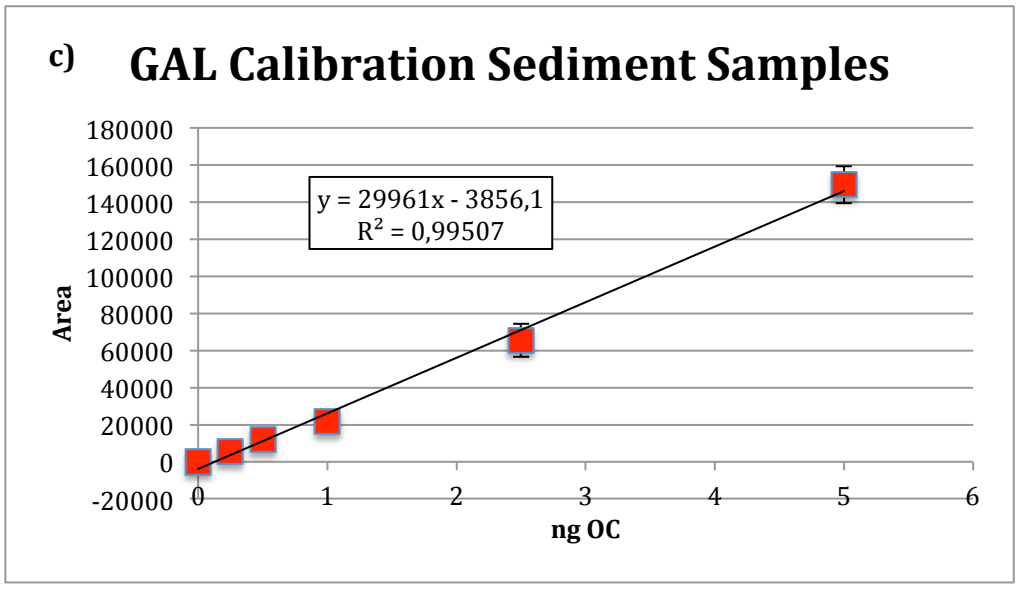


Figure 10: Sediment sample calibrations. Double sets of calibration standards were analyzed. Error bars show standard deviation. a) LVG b) MAN c) GAL.







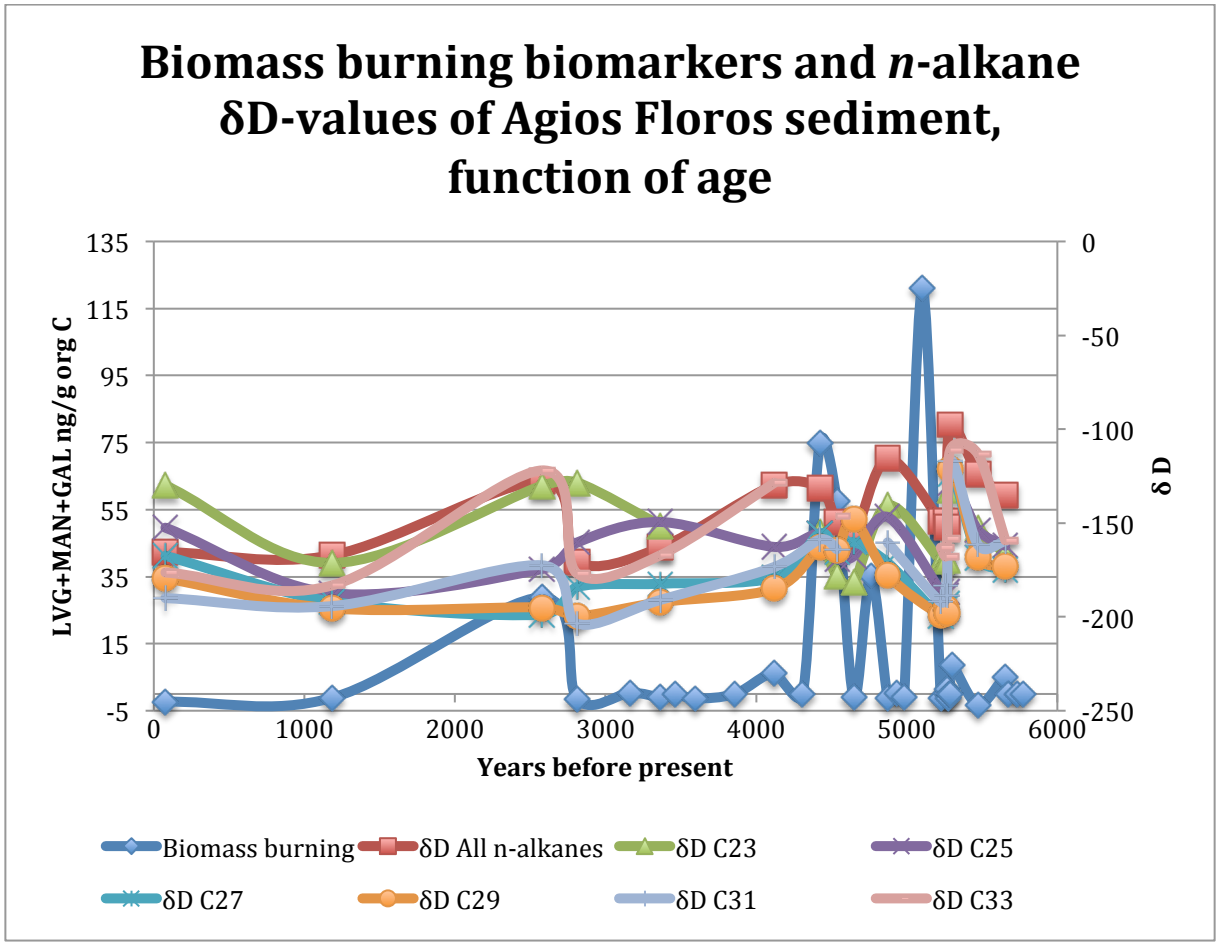
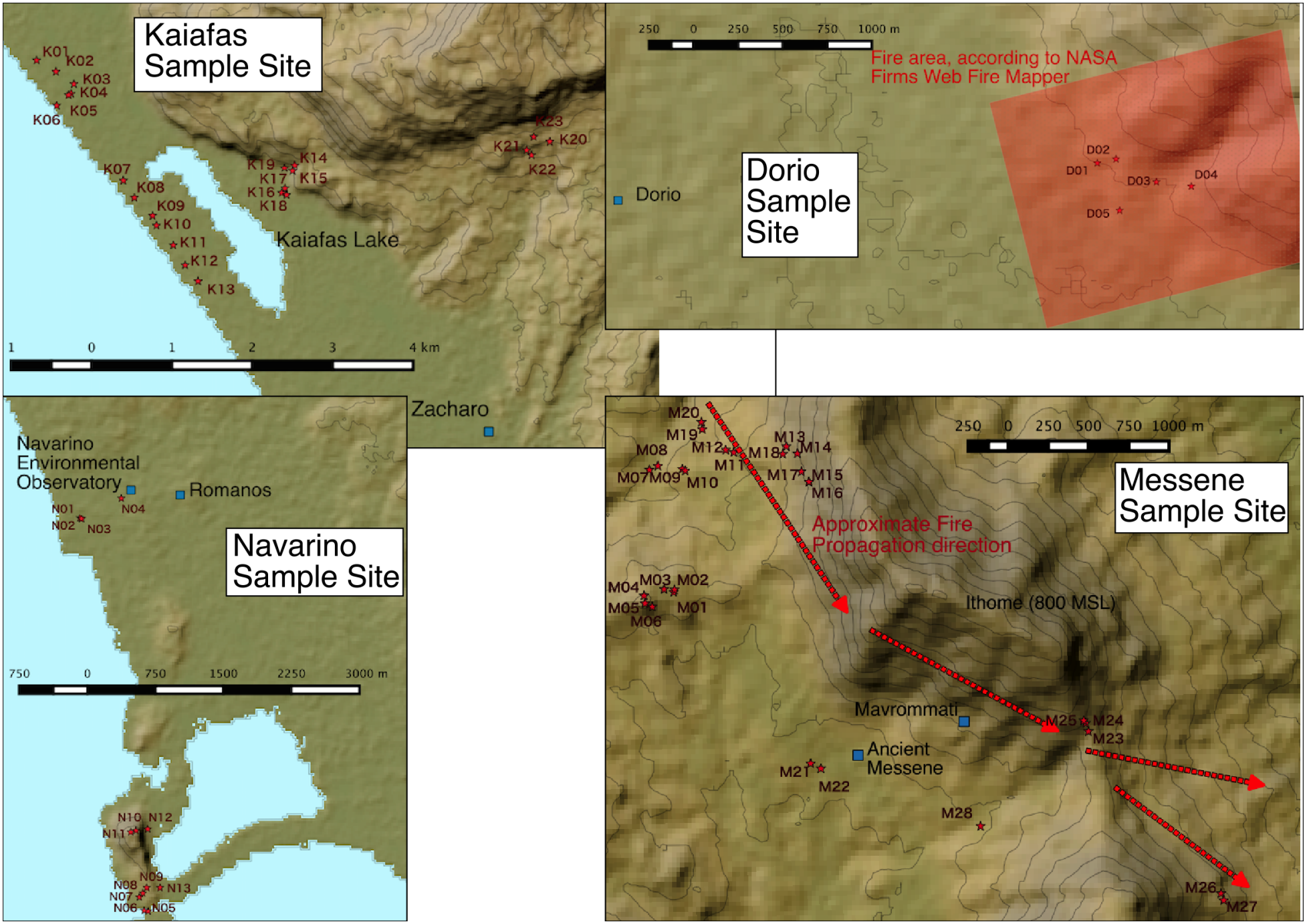


Figure 11. Comparison between biomass burning tracers and δ D values of different *n*-alkanes.

Figure 12 (next page): Soil sample sites in more enhanced maps. Topography lines equals 50 m of elevation. See scale bars for scale and notes on map.


Kaiafas Sample Site
K01
K02
K03
K04
K05
K06
K07
K08
K09
K10
K11
K12
K13
K14
K15
K16
K17
K18
K19
K20
K21
K22
K23
Kaiafas Lake
1 0 1 2 3 4 km
Zacharo
Dorio
Dorio Sample Site
250 0 250 500 750 1000 m
Fire area, according to NASA Firms Web Fire Mapper
D01
D02
D03
D04
D05
Navarino Environmental Observatory
Romanos
N01
N02
N03
N04
Navarino Sample Site
750 0 750 1500 2250 3000 m
N05
N06
N07
N08
N09
N10
N11
N12
N13
Messene Sample Site
250 0 250 500 750 1000 m
Approximate Fire Propagation direction
M01
M02
M03
M04
M05
M06
M07
M08
M09
M10
M11
M12
M13
M14
M15
M16
M17
M18
M19
M20
Ithome (800 MSL)
Mavrommati
Ancient Messene
M21
M22
M23
M24
M25
M26
M27
M28