Steroids aid in human decomposition fluid identification in soils of temporary mass graves from World War II

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Highlights

- Steroids used for the first time to identify temporary graves
- Tissue derived steroids (cholesterol, 5α-cholestanol and 5α-cholestanone) were ubiquitously present in soil pits and reference soil and could not be used as human decomposition fluid markers
- Faecal stanols (coprostanol, 5β-stigmastanol, epi-5β-stanols and 5β-cholestanone) gave an indication of a former input of human decomposition products in one of the putative mass graves
- Bile acids (lithocholic acid and isolithocholic acid) aided in the identification of human decomposition products in one of the putative mass graves

Abstract

Steroids are widely used for the detection of faecal matter and – in recent years – also for characterising human decomposition in the terrestrial environment. Until now it was not clear, whether all commonly used faecal (i.e. 5β-stanols, 5β-stanones and bile acids) and tissue derived steroids (i.e. cholesterol, 5α-cholestanol and 5α-cholestanone) could reveal the presence of human decomposition products in temporary graves. In this study, soil was sampled from three putative mass graves where concentration camp prisoners were temporarily buried for 10 months at the end of World War II (1944 - 45). We hypothesised that soil from the putative temporary mass graves exhibit elevated contents of faecal and tissue steroids compared to reference samples, thereby reflecting the former input of human decomposition products. Steroids (Δ^5-sterols, 5β-stanols, 5α-stanols, stanones and bile acids) from soil samples of three soil pits suspected to be putative mass graves as well as reference soil samples were extracted and analysed with gas chromatography-mass spectrometry (GC-MS). Cholesterol, 5α-cholestanol and 5α-cholestanone were ubiquitously present in all soil pits and reference samples and therefore not indicative for the former input of human decomposition products. Compared to the reference, increased contents of selected faecal steroids (coprostanol, 5β-stigmastanol, 5β-cholestanone, lithocholic acid, isolithocholic acid) gave a strong indication for the former input of human decomposition products for one soil pit (pit 2). Additionally, epicoprostanol and isolithocholic acid were unique components for soil pit 2 as they were neither found in the other pits nor in the reference soil. In combination with earlier findings, steroid and bile acid patterns indicate an input of faecal and tissue constituents from former buried bodies at least in one of the three soil pits.

Keywords

Biomarker, sterol, stanol, bile acid, grave soil, human decomposition

Introduction

In the 20th and 21st century advanced techniques of excavating mass graves were developed as a result of genocide, homicide and war crimes, particularly during World War I & II, in South America and in the former Yugoslavia (Hunter et al. 2013). Forensic and archaeological techniques facilitate the detection of clandestine mass graves (e.g. geophysical methods, remote sensing) and provide evidence about the interred bodies by analysing human decomposition products (e.g. carbon, phosphorus) and by identifying human remains (e.g. DNA) (Kalacska and Bell, 2006; Fiedler et al., 2009; Hunter et al. 2013). In cases of the temporary use of mass graves decomposition products of the former interred body remain preserved in the soil, while victims were removed to another site. Thereby additional evidence can be collected to identify the former use of a mass grave. In the past, mostly fatty acids, phosphorus and carbon were used to identify temporary graves and mass graves (Bull et al., 2009; Fiedler et al., 2009). However, compared to fatty acids, tissue and faecal derived steroids (Δ^5-sterols, stanols, stanones and bile acids) are more specific for their original source and relatively resistant against degradation in soils (Evershed and Connolly, 1994; Bull et al., 2002; Prost et al., 2017), thereby giving stronger indications for the presence of human decomposition fluids. Recent studies revealed the potential of Δ^5-sterols and stanols as human decomposition markers. Pickering et al. (2018) found tissue (cholesterol, 5α-cholestanol) and faecal derived (coprostanol) steroids in soil from an Anglo-Scandinavian grave (870-980 CE). In a forensic case study, von der Lühe et al. (2018) detected elevated concentrations of tissue (cholesterol, 5α-cholesterol) and faecal derived steroids (coprostanol, epicoprostanol, 5β-stigmastanol) in soil below a decomposing human body 11-16 days after deposition.

The common Δ^5-sterol of higher animal tissues is cholesterol, but it is also present as a minor constituent of plants, fungi and other eukaryotes and is thus ubiquitous in soils (Mouritsen and Zuckermann, 2004; Weete et al., 2010; Christie and Han, 2012). During digestion, cholesterol is

microbially reduced to coprostanol (5β-stanol) in the gut of higher animals (Björkhem and Gustafsson, 1971). In human faeces, coprostanol occurs in large amounts and is therefore utilised as human faecal marker in archaeological and environmental pollution studies (Bethell et al., 1994; Leeming et al., 1996). Additionally, the plant derived faecal 5β-stigmastanol is also found in large amounts in human faeces (Leeming et al., 1996; Prost et al., 2017).

In soils, the usual reduction product of cholesterol is 5α-cholestanol, but it can also be found in minor contents in animal tissues, faeces and plants (Murtaugh and Bunch, 1967; Daniellson and Tchen, 1968; Hatcher and McGillivary, 1979; Noda et al., 1988). When coprostanol and 5β-stigmastanol are excreted into the environment, they can be further transformed to epicoprostanol and epi-5β-stigmastanol, respectively (Wardroper et al., 1978; Quirk et al. 1980; McCalley et al., 1981). Stanones (5α-cholestanone and 5β-cholestanone) are intermediate steroidal ketones of the intestinal and environmental microbial Δ^5-sterol transformation to stanols and epi-5β-stanols (Björkhem and Gustafsson, 1971; Gaskell and Eglinton, 1975; Grimalt et al., 1990).

In contrast, bile acids are only produced by vertebrates and - compared to other steroids - are highly specific markers for vertebrate faeces (Haslewood, 1967; Hofmann and Hagey, 2008). Primary bile acids, e.g. cholic acid and chenodeoxycholic acid (CDCA) are synthesized from cholesterol in the liver of vertebrates. When excreted into the gut they get further microbially converted to secondary bile acids, e.g. lithocholic acid (LCA), deoxycholic acid (DCA) and isolithocholic acid (ILCA) (Hayakawa, 1973; Stellwag and Hylemon, 1979; Bull et al., 2002).

Because animal (including human) faeces comprise different steroid patterns, these patterns have been used to distinguish between faecal sources in environmental and archaeological studies (Leeming et al., 1996; Elhmmali et al., 1997; Bull et al., 2002; Prost et al., 2017). In archaeological studies, cholesterol (dating to 400 - 700 AD; Davies and Pollard, 1988) as well as LCA and DCA (dating to 7400 - 6000 BC; Shillito et al. 2011) were detected in graves containing human skeletal remains. Only recently, Pickering et al. (2018) demonstrated elevated coprostanol contents in soil

below a skeleton of an Anglo-Scandinavian grave (c. 870 - 980 AD). In a forensic case study, von der Lühe et al. (2018) found human tissue and faecal derived steroids in soil below a decomposing body 11 - 18 days post-mortem and after body-removal the preservation of those steroids after a period of one year (von der Lühe et al., 2018).

Due to their diagnostic potential and their long lasting presence in soils steroids are interesting markers for forensic and archaeological investigations, e.g. to identify human decomposition products. For this purpose, in recent studies steroid analyses were performed on soil samples, where the origin of the body buried in the grave (pig, human) was known (Davies and Pollard, 1988; Shillito et al., 2011; von der Lühe et al., 2013; von der Lühe et al., 2018). However, due to the fact that a temporary grave is characterised by a removal of the body before it completed decomposition and by a disturbance of the soil in the course of the removal, it is not clear whether a short term exposure of soil to human decomposition products followed by a mixture of this soil with the unexposed soil can give sufficient information to identify human decomposition products by steroid analysis.

This study investigated the use of steroid analysis as a specific tool to identify human decomposition products in temporary graves dating 67 years back. Soil samples were taken from putative mass graves in Germany that had been in use for 10 months during World War II (1944 - 45) (Fiedler et al., 2009), exhumed and refilled with soil thereafter. Soil samples were analysed for their steroid (Δ^5-sterols, stanols, stanones and bile acids) patterns and compared to reference soil samples taken in close proximity. We hypothesised that following decomposition of human bodies, tissue and faecal steroids are enhanced in the soils of the putative mass graves and show human-related particular patterns.

Material and Methods

Location of the mass graves: historical evidence, soil mapping and soil chemical analyses

During late 1944 to October 1945, 66 concentration camp prisoners were buried for a period of 10 months in a municipal forest close to Stuttgart, Germany. After World War II occupying forces exhumed the bodies and buried them in a cemetery nearby. 67 years later the knowledge about the exact location of those former mass graves was inconsistent (Faltin, 2008). Fiedler et al. (2009) located the position of the putative mass graves by using a combination of historical evidence, soil mapping and chemical analyses.

The results of Fiedler et al. 2009 are described here in brief: Historical evidence (eyewitnesses, aerial photograph) revealed that the location of the mass graves was a vegetation free remote area within the communal district Ramsklinge (close to Plattenhardt, no privately owned area). The mean annual precipitation in this area is 686 mm and the mean annual temperature 8.8 °C. The aerial photograph from the 10th of April 1945 showed that the area containing the mass graves was of 5,000 m^2 in extend. Eyewitnesses reported, that the bodies were transported in a truck and buried in 2 – 3 shallow rectangular pits (Back 2005). After exhumation, the bodies were washed in a spring 80 – 120 m away from the mass graves. In spring 2006, core samples were taken in a 5 x 5 m grid with 234 georeferenced sampling points (Berger 2006). For each sampling point, soil parameters (horizons, texture, colour, pH, $CaCO_3$ content, structure, moisture content and redoximorphic properties) and the soil type was recorded. For the identification of the mass graves specific features must be fulfilled: A low penetration depth was considered (≥ 6 cm per hammer stroke), because graves were opened twice which leads to a decrease in bulk density. The characteristic soils in the area were Luvisols, Planosols, Vertisols and Cambisols with more than 4 horizons in a depth of 0 – 1 m (IUSS Working Group WRB, 2006). The construction of the mass graves lead to a homogenisation of soil horizons and a reduction of the number of horizons to ≤ 3. After grave construction, homogenized horizons with a thickness of ≥ 40 cm were produced.

Soils with developed depths of ≥ 50 cm were considered to be sufficient to count as shallow graves. The highest degree of soil disturbance was confirmed at 11 out of 234 sampling points. Seven out of the 11 sampling points were within privately owned areas and excluded. Point 234, 242 and 241 were in close proximity and after a second field visit in May 2006, three shallow soil pits (pit 1 – 3) were discovered at these sampling points (Fig. 1a, Berger 2006). The alignment of the pit edges was still clearly visible and the maximum height difference of these shallow pits (in the following “pits”) to the surrounding area was - 20 cm. These soil pits were 50 m apart from a spring layer that is referred by local people as “Jew spring”. This coincides with the report from the eyewitness that bodies were washed at a close by spring. A woodland path is located 1 – 2 m from the pits, which could have been used to transport the bodies to the pits by trucks.

For soil stratigraphy and soil chemical parameter characterisation, a test trench (90 – 100 cm depth) was cut through pit 2 and pit 3 (pit 1 was not approved for investigation, Fig. 1b). Several corroded objects were found in pit 2 clearly pointing to an anthropogenic influence. Those objects were probably disposed in pit 2 after exhumation of the bodies. Pit 2 and pit 3 had no distinct horizons with a uniform soil colour (10YR 4/3). The undisturbed intermediate soil between the pits had soil horizons typical for Gleysol and Planosol soils and showed a colour differentiation from the top (10YR 6/3) to the bottom (10YR 3/1). In pit 2, the position of the grave was further confirmed by the discovery of a pivot crown (“Richmond” crown). The Richmond crown is a dental prosthesis used in Germany before the 1950s (Fiedler et al., 2009). Total phosphorus and organic carbon are often used to identify human influence and decomposition fluids in soil material (Dietz 1957, Holliday and Gartner 2007). Chemical analysis of soil revealed elevated total phosphorus (pit 2 & pit 3: ~ 500 - 900 mg P kg^{-1} at 50 cm, ~ 700 – 1000 mg P kg^{-1} at 75 cm) and organic carbon (pit 2 & pit 3: ~ 6 – 8 g kg^{-1} in 50 cm, 7 – 10 g kg^{-1} in 75 cm) in the subsoil compared to the undisturbed soil taken between the two pits (total phosphorus: ~500 – 600 mg P kg^{-1} in 50 cm, ~600 mg P kg^{-1} in 75 cm; organic carbon: ~ 3 – 4 g kg^{-1} in 50 cm, ~ 3 g kg^{-1} in 75 cm) (Fiedler et

al., 2009). The topsoil in soil pit 2 and pit 3 had lower phosphorus and carbon contents compared to undisturbed areas.

The combination of the evidence found by Fiedler et al. (2009) can be supported by steroid analytics for an improved identification of human decomposition fluids in the soil material from the pits.

Soil sampling for steroid analyses

In 2012, 67 years after exhumation, soil was sampled for steroid analysis with a Pürckhauer auger (1 m). Two augers (depth ~ 0 – 80 cm) were taken in each soil pit 1 - 3 (n = 2, in total n = 6; Fig. 1a). Three auger samples (n = 3) were taken as reference at ca. 10 m distance and slightly uphill south-east to pit 1 and pit 2 (Fig. 1a). We were not able to penetrate the augers to a depth of 1 m, because bedrock appeared at a depth of 60 – 80 cm. For a better comparison of steroid concentrations between pit and reference samples, soil samples were divided into 10 cm depth samples. To avoid contamination, the auger and sampling equipment (sampling knife) were cleaned with water and ethanol between each sample. Soil samples from the subsoil (40 - 50 cm, 50 - 60 cm) and from the topsoil (0 - 10 cm, only pit 2 and reference) were further investigated on their steroid proportions. Samples were stored at -20 °C until analysis.

Steroid extraction

For steroid extraction, purification and analysis a previously published method was used (von der Lühe et al., 2018). Bile acid extraction was adopted in a modified form from Birk et al. (2012). Investigated compounds including suppliers for analytical standard material are listed in Table S1. Soil samples were freeze-dried for 24 h, sieved ≤ 2 mm, and finely ground in a ball mill. Samples were weighed (10 g) into fibreglass thimbles and covered with quartz sand (thimbles and sand combusted at 300 °C for 12 h). Extraction was carried out with a Soxhlet apparatus, using a dichloromethane/methanol (2:1, v/v, 150 ml) solvent system for 36 h. Solvent was removed by rotary evaporation and the total lipid extract (TLE) was dried under nitrogen (N_2).

A Δ^5-sterol internal standard (IS) (cholesterol-d_7, 50 µl, 10 ng µl^{-1}), a stanone IS (5α-pregnan-3-one, 250 µl, 2 ng µl^{-1}) and a bile acid IS (isodeoxycholic acid, 250 µl, 2 ng µl^{-1}; Tab. S1) was added to the TLE. Saponification was carried out with 1.5 ml 0.7 M methanolic KOH at room temperature (21 °C) for 12 h. Extracts were heated to 50 - 60 °C, 0.5 ml H_2O were added, and neutral lipids (containing Δ^5-sterols, stanols and stanones) were separated from the aqueous phase by liquid-liquid extraction with 4 x 1.5 ml *n*-heptane. To obtain bile acids, the aqueous solution was acidified to pH ≤ 2 with 6 M HCl and bile acids were extracted with 4 x 1.5 ml chloroform. Both fractions were evaporated under N_2.

The neutral lipid extract was transferred with 500 µl *n*-heptane to solid phase extraction (SPE) glass columns (ø 11 mm) containing 1 cm silica gel (60 Å, 0.063 - 0.200 mm (Merck, Darmstadt, Germany)), combusted at 380 °C for 4 h and activated at 200 °C for 6 h, preconditioned with 2 x 1.5 ml *n*-heptane). Columns were washed with 2 x 1.5 ml *n*-heptane to elute hydrocarbons. The fraction containing steroids was collected by adding 4 x 1.5 ml *n*-heptane/ethyl acetate (80:20, v/v). Steroids were silylated with 12.5 µl of *N,O*-Bis(trimethylsilyl)-trifluoroacetamide + trimethylchlorosilane (BSTFA+TMCS; 99:1, v/v) and 37.5 µl pyridine at 90 °C for 1 h. Eluates were dried with N_2 and 100 µl of 5α-cholestane (5 ng µl^{-1} in toluene, IS II; Tab. S1) was added.

The fraction containing bile acids was methylated by adding 1 ml dry 1.25 M HCl in methanol (Fluka, Sigma-Aldrich, St-Louis, MO, USA) and heating at 80 °C for 2 h. Methyl esters were extracted by adding 1 ml H_2O and 3 x 1.5 ml *n*-hexane. SPE was carried out with glass columns (ø 11 mm) containing 1 cm silica gel (Polygoprep 100-130 (Macherey-Nagel, Düren, Germany), activated at 120 °C for 12 h, preconditioning 2 x 1.5 ml *n*-hexane). Extracts were transferred with 500 µl *n*-hexane and fatty acid methyl esters were eluted to waste with 4 x 1 ml dichloromethane/*n*-hexane (2:1, v/v). Bile acids were collected by adding 4 x 1 ml of dichloromethane/methanol (2:1, v/v). Bile acid methyl esters were silylated with 100 µl of BSTFA/1-(Trimethylsilyl)imidazole (98:2,

v/v; Supelco, Bellefonte, PA, USA) and 50 µl toluene at 90 °C for 1 h. The derivatisation agent was removed with N_2 and 100 µl of 5α-cholestane (5 ng μl^{1} in toluene, IS II; Tab. S1) was added.

GC-MS analysis and quantification

Analysis was performed on an Agilent 6890 gas chromatograph coupled to a 5975B mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). A DB-5ms Ultra Inert fused silica capillary column (30 m x 250 µm x 0.25 µm) was used with He (99.9995 %) as a carrier gas at 1.1 ml min^{-1} constant flow.

For Δ^5-sterols, stanols and stanones, the injection port was maintained at 250 °C and 1 µl was injected in splitless mode. The initial oven temperature was held at 80 °C for 1.5 min, then programmed at 12 °C min^{-1} to 265 °C, at 0.8 °C min^{-1} to 280 °C and at 10 °C min^{-1} to 300 °C, and held for 12 min. For the analysis of bile acids 1 µl was injected in splitless mode, the injector port was set at 290 °C and column flow was 1.0 ml min^{-1}. The initial temperature was held at 80 °C for 1.5 min, then programmed at 20 °C min^{-1} to 250 °C, at 1.2 °C min^{-1} to 280 °C and at 10 °C min^{-1} to 300 °C.

Electron ionisation was set at 70 eV. Samples were run in total ion monitoring mode (TIC, *m/z* 50-500) and selected ion monitoring mode (SIM) for quantification (Tab. S1). Concentrations in ng $g_{freezed\ dried\ soil}^{-1}$ (in all following sections ng g^{-1}) were calculated with an external calibration ($R^2 \geq 0.98$) of each individual analyte in respect of the injection IS II. Recovery surrogates of the sterol and stanol IS I was 127 ± 7 %, the stanone IS I was 109 ± 19 % and the bile acid IS I was 100 ± 19 %. Example GC-MS chromatograms of silylated Δ^5-sterol, stanol, stanone and bile acid methyl ester SIM runs are shown in Fig. S1 and Fig. S2.

Results

Steroids were found in the top- and subsoil of all soil pits and in the reference samples. Highest concentrations of cholesterol (pit 2: 800 ng g^{-1}; reference: 2,820 ng g^{-1}), β-sitosterol (pit 2: 10,108 ng g^{-1}; reference: 29,835 ng g^{-1}) and stigmasterol (pit 2: 1,287 ng g^{-1}; reference: 3,770 ng g^{-1}) were found in the topsoil (0 – 10 cm) of pit 2 and in the reference (Fig. 2). In the subsoil there was no clear difference of Δ^5-sterols between the pits and the reference (Fig. 2). Cholesterol contents ranged between 122 and 153 ng g^{-1} in pit 2 and between 29 and 295 ng g^{-1} in the reference subsoil (Fig. 2).

Derivatives of cholesterol reduction were slightly (5α-cholestanol: 87 - 114 ng g^{-1}) up to moderately elevated (5α-cholestanone: 39 - 49 ng g^{-1}) in the subsoil of pit 2 compared to the reference (5α-cholestanol: 6 - 86 ng g^{-1}; 5α-cholestanone: 1 - 6 ng g^{-1}; Fig. 2). In the topsoil of pit 2, 5α-cholestanol exceeded the concentrations found in the subsoil (pit 2: 148 - 208 ng g^{-1}; reference: 232 - 340 ng g^{-1}) while 5α-cholestanone was not detectable in the reference topsoil (Fig. 2). The sum of tissue-derived cholesterol and its derivatives (5α-cholestanol, 5α-cholestanone) was 21 - 212 ng g^{-1} in the reference subsoil and between 244 - 462 ng g^{-1} in pit 2 (Table S1).

In general, soil from pit 1 and pit 3 had comparable low contents of steroids in the subsoil likewise to the reference samples (Fig. 2). Whereas in the subsoil of pit 2 elevated contents of steroids typical for human faecal material namely 5β-stanols and 5β-stanones (Fig. 2). Thus, steroids found in the subsoil of pit 2 were additionally separated from the results of the other pits in Fig. 2 to emphasize the exceptional findings.

In the subsoil of pit 2, elevated concentrations of the faecal 5β-stanols coprostanol (59 - 81 ng g^{-1}), 5β-stigmastanol (28 - 32 ng g^{-1}) and the stanone 5β-cholestanone (50 - 59 ng g^{-1}) were found, whereas only relatively low amounts could be detected in the reference subsoil (coprostanol: 0.9 - 2 ng g^{-1}; 5β-stigmastanol: not detected; 5β-cholestanone: 2.2 ng g^{-1}) (Fig. 1). Coprostanol was increased in the reference topsoil (23 - 75 ng g^{-1}), while there was no 5β-stigmastanol detected

(Fig. 2). Epicoprostanol (12 - 20 ng g^{-1}) was exceptionally found in pit 2, neither pit 1 and pit 3 nor the reference contained epicoprostanol (Fig. 2). Epi-5β-stigmastanol was detected in selected samples of pit 1 - 3 (pit 1: 4.1 – 4.5 ng g^{-1}; pit 2: 14 ng g^{-1}; pit 3: 3.3 – 3.9 ng g^{-1}) and was absent in the reference (Fig. 2). Proportions of coprostanol were compared to 5α-cholestanol in order to detect an enhanced input of faecal material into the soil pits (coprostanol / coprostanol + 5α-cholestanol; with a ratio of > 0.7 clearly indicating an input of faecal material; Grimalt et al., 1990; Fig. 3). In pit 1 and pit 3 as well as in the topsoil of pit 2 and in the reference this ratio was ≤ 0.2 (Fig. 3). In the subsoil of pit 2 the ratio was increased to 0.39 - 0.44 (Fig. 3). By adding epicoprostanol to this ratio (coprostanol + epicoprostanol / coprostanol + epicoprostanol + 5α-cholestanol; with a ratio of > 0.7 clearly indicating an input of faecal material; Bull et al., 1999), values were increased to 0.44 - 0.48 in the subsoil of pit 2, while reference values were ≤ 0.16 (Fig. 3). The 5α-cholestanol / 5α-stigmastanol ratio was elevated in two pit 2 subsoil samples (0.95 - 1.06; Fig. 3) and did not differ in all of the other measured samples ≤ 0.71 (Fig. 3).

The bile acids lithocholic acid (LCA) and deoxycholic acid (DCA) were elevated in all soil pits but were also found in lower abundances in the reference soil (Fig. 4). Highest concentrations of LCA (pit 2: 22 ng g^{-1}) and DCA (pit 1: 322 ng g^{-1}) were found in the subsoil of the pits, while the reference samples revealed LCA contents ≤ 13 ng g^{-1} and DCA contents ≤ 246 ng g^{-1} in both soil depths (Fig. 4). Isolithocholic acid (ILCA) was exceptionally found in all pit 2 subsoil samples (2 - 6 ng g^{-1}) and in one subsoil sample of pit 3 (2 ng g^{-1}, Fig. 4). Chenodeoxycholic acid (CDCA) was relatively low in all subsoil pit samples (4 - 21 ng g^{-1}) and reference samples (5 - 19 ng g^{-1}, Fig. 4) and no clear difference was detected. In the topsoil of pit 2 and the reference CDCA levels were increased up to 123 ng g^{-1} (Fig. 4).

Regarding summed concentrations of faecal steroids and their epimers significant for human faecal matter identification (coprostanol, 5β-stigmastanol, epicoprostanol, 5β-cholestanone and

bile acids) only pit 2 revealed elevated concentrations in the subsoil (244 - 490 ng g^{-1}) compared to the reference (21 - 212 ng g^{-1}).

Discussion

By combining the evidence found by Fiedler et al. (2009), the soil pits were the only location in the area that were most likely used as former mass graves. The soils of the potential former mass graves had undergone a high level of disturbance during grave construction and reopening after a period of 10 months and refilling the empty graves with soil material. This process was confirmed by the homogenisation of the soil horizons in pit 2 and pit 3 (Fiedler et al., 2009). As a consequence, the natural distribution of steroids along the soil profile - as detected in the reference soil - was disturbed by the human impact. The *in situ*- and decomposition fluid-derived steroids were most likely homogenised in the mass graves. In the topsoil the decomposition-derived steroid concentrations were most likely altered by organic matter input and decomposition during the period of 67 years. In the subsoil relevant information about the former input of human derived decomposition products were most likely reflected by the preserved steroid patterns. In pit 2 several evidence (disturbed horizons, corroded objects, pivot crown, elevated total phosphorus and organic carbon; (Fiedler et al., 2009)) points to the former use as mass grave. Additionally, the largest steroid concentrations were detected in pit 2 with a predominance of faecal steroids and a lack of tissue derived steroids which is further discussed in detail.

High contents of the Δ^5-sterols cholesterol, β-sitosterol and stigmasterol in the topsoil were considered as being the natural steroid background in the study area. In the soil pits and the reference, the ubiquitous presence of cholesterol, β-sitosterol and stigmasterol was attributed to other input sources than human bodies, like root exudates, plant material, fungi and other eukaryotes (Thompson and Hale, 1983; Mouritsen and Zuckermann, 2004; Weete et al., 2010; Christie and Han, 2012). Cholesterol concentration in the reference topsoil were within the range of agricultural topsoils (200 – 2100 ng g^{-1}; Puglisi et al., 2003, Meli et al., 2006) and from shrub woodland (500 – 600 ng g^{-1}; von der Lühe et al., 2018). In the subsoil of the soil pits, cholesterol was not indicative for human decomposition products as levels did not differ from the reference

(Fig. 2). In archaeological and forensic investigations, high contents of cholesterol were found in close proximity (few centimetres below the soil surface) of human remains, demonstrating its origin from human body decomposition (Davies and Pollard, 1988; Shillito et al., 2011; Luong et al., 2018; Pickering et al., 2018; von der Lühe et al., 2018). It is likely that higher levels of cholesterol from the body decomposition were formerly present in the soil pits. But it can be assumed that a following reduction of cholesterol to 5α-cholestanone and 5α-cholestanol and decomposition led to cholesterol levels comparable to those of the reference (Bull et al., 2002; Pickering et al., 2018; von der Lühe et al., 2018).

Cholesterol from human remains can be rapidly converted to 5α-cholestanol in soil (Evershed and Connolly, 1994; Pickering et al., 2018; von der Lühe et al., 2018) while 5α-cholestanone is an intermediate in this reduction process (Evershed and Connolly, 1994; Bull et al., 2002). In the soil pits and the reference sub- and topsoil samples, the natural microbial reduction is reflected by similar 5α-cholestanol / 5α-stigmastanol ratios (Fig. 3), although slightly enhanced ratios in pit 2 could indicate a former input of decomposition products of human tissues. Elevated concentrations of 5α-cholestanol and 5α-cholestanone in pit 2 might derive from the reduction of cholesterol from the human tissue as well as from the presence of human faeces (Daniellson and Tchen, 1968; Prost et al., 2017). However, it cannot be excluded that 5α-cholestanol and 5α-cholestanone could originate from topsoil organic material incorporated into the subsoil during mass grave construction. Concentrations did not clearly differ from the reference, thus the human tissue associated steroids cholesterol, 5α-cholestanol and 5α-cholestanone were not indicative for human decomposition products in this study.

Pit 2 contained substantial larger concentrations of faecal stanols and stanones in the subsoil (coprostanol, epicoprostanol, 5β-cholestanone and 5β-stigmastanol; Fig. 2) compared to the reference. Coprostanol and 5β-stigmastanol are the major stanols in human faeces, epicoprostanol and 5β-cholestanone are minor constituents (Bull et al., 2002; Gérard, 2013; Prost

et al., 2017). Only recently, faecal stanols have been indicated as constituents of decomposition fluids in soil below a human body (von der Lühe et al., 2018) and below pig carcasses amongst tissue derived steroids (cholesterol, 5α-cholestanol; von der Lühe et al., 2013). Because of the nearly absence of faecal stanols and stanones in the subsoil of the reference samples, the presence of faecal stanols and 5β-stigmastanol in the soil pits was attributed to originate from human decomposition products, presumably from the concentration camp prisoners. As 5β-stigmastanol was a minor constituent in topsoil of the reference samples, the input of herbivore faeces from wildlife was precluded in this study (Bull et al., 2002; Prost et al., 2017). Epicoprostanol was found as singular steroid in the subsoil of pit 2. It can be found in higher concentrations in faecal material from horse, aged sewage sludge, and compost (Wardroper et al., 1978; Simpson et al., 1998; Prost et al., 2018) and is known to be microbially epimerised from coprostanol under anaerobic conditions (McCalley et al., 1981; Bull et al., 2002). In pit 2, the waterlogged soils (Planosols) likely favoured anaerobic conditions and a microbial epimerisation of – the for human faeces typical steroid - coprostanol to epicoprostanol. Likewise to 5α-cholestanol, coprostanol could have been also incorporated into the subsoil during grave construction, however the epicoprostanol level suggest that higher coprostanol contents might have been previously present by the input of decomposition products. The narrow ratio of coprostanol to epicoprostanol of 4 - 6 (Table S1) differs from ratios in fresh human faeces at 66 - 80 (Leeming et al., 1996; Prost et al., 2017) assuming that intensive coprostanol epimerisation took place in the subsoil of pit 2. A comparable process was neither found in the reference soil nor in the other soil pits suggesting that an input of faeces in pit 2 most likely occurred.

The input of human faecal material (potentially derived from the intestinal contents of the decaying bodies) in pit 2 is more clearly indicated when typical ratios of faecal matter detection are applied (Fig. 3). The coprostanol / coprostanol + 5α-cholestanol (Grimalt et al., 1990) and coprostanol + epicoprostanol / coprostanol + epicoprostanol + 5α-cholestanol ratios (Bull et al., 1999) in pit 2 ranged from 0.39 - 0.44 and 0.44 - 0.48 (Fig. 3). None of the reference and the other soil pit

samples reached ratios ≥ 0.2 (Fig. 3). Regarding the usually applied threshold value for faecal matter input of 0.7 introduced on marine sediments by Grimalt et al. (1990), all ratios in pit 2 failed to reach the threshold. Similar ratios to pit 2 were found in archaeological settings (Bull et al., 2001; Birk et al., 2011; Prost et al., 2017) presuming a generally decline due to gradually decomposition and dilution effects of faecal stanols in soils over a period of 67 years. Instead of considering the threshold value of 0.7, Bull et al. (1999) recommended the comparison of ratios to reference samples. By considering the local background contents of faecal steroids in the reference samples and the degradation effect in pit 2, an input of the intestinal contents of the decomposing bodies in pit 2 is likely. In order not only to show an input of faecal material, but to be able to point to a source, bile acids, as additional steroid biomarkers have to be included in the analysis, as human faecal material and those of pigs show similar patterns of stanols (Prost et al., 2017).

The input of bile acids in the topsoil of the reference was attributed to faecal matter input from wildlife. CDCA is a primary bile acid and produced from cholesterol in the liver (Bull et al., 2002). It was detected in all soil pits and the reference and did not show any particular difference, due to its ubiquitous and wide distribution in mammalian species (Matschiner, 1971; Prost et al., 2017). There is no published data on natural bile acid distributions in Middle European forest soils and it is thus difficult to evaluate and quantify natural bile acid distributions in the reference samples.

In terms of concentrations, the order of bile acids found in human faeces is DCA > LCA > ILCA > CDCA (Prost et al., 2017). High abundances of DCA (subsoil of all pits) and enhanced LCA (subsoil of pit 2 and pit 3) might be attributed to an input of bile acids via human decomposition products including faecal material. DCA and LCA are used as typical indicators of human and cattle faeces (Matschiner, 1971; Subbiah et al., 1976; Elhmmali et al., 1997), but cattle faeces have considerably lower contents of LCA (Tyagi et al., 2007a; Tyagi et al., 2007b). In this study, a contribution of cattle bile acids can be ignored, because cattle faeces have dominant

abundances of 5β-stigmastanol (Bull et al., 2002; Prost et al., 2017), which was absent in the reference soil. Although lower concentrations of DCA were detected in the reference samples, all soil pits showed also a wide range of DCA concentrations and it was not clear if DCA from topsoil material was incorporated in the subsoil of the soil pits. It was also not clear, why the reference subsoil samples contained higher DCA concentrations than in the reference topsoil. DCA is an ubiquitous bile acid that has also been found as most prominent bile acid in reference soil material in archaeological studies (Prost et al. 2017). DAC is also the most prominent bile acid in the soil pits and the reference. Its high abundances in the soil pits might be explained by its prominent abundance in natural soil material. The increased concentrations in the subsoil of the pits might be an indicator of the human impact, weather DCA was incorperated from human decomposition fluids (most likely pit 2) or originated from other sources is not understood yet. We were not able to detect hyodeoxycholic acid in any soil sample. This excludes a contribution of porcine faeces (e.g. from *Sus scrofa*) in the soil pits and the reference samples, which is in this study a strong indication towards human faecal sources. The secondary bile acid ILCA was found in pit 2 (in all samples) and pit 3 (in one sample). It is a major human faecal bile acid (Hofmann and Hagey, 2008) and strengthens the notion of an input of human derived bile acids to those pits. Compared to DCA, ILCA was only present in pit 2 and 3. Consequently, bile acids revealed that human decomposition products might have been introduced at least into pit 2 and pit 3, due to the higher abundances of LCA and ILCA and the presence of DCA. It is still not clear yet, why pit 3 did not contain any faecal stanols. Thus, pit 2 shows a stronger evidence of the former input of human decomposition products than pit 3. As far as known there is only one study that analysed bile acids in graves containing human skeletal remains. Shillito et al. (2011) found LCA in one and DCA in two of five burial samples at Neolithic Çatalhöyük (c. 7400 - 6000 BC). However, no reference sample was analysed in that study, it is thus uncertain if DCA and LCA were introduced into the soil from the bodies or were naturally present.

Conclusions

This study showed that steroids could be useful biomarkers to detect human decomposition fluids in soils. The presence of human decomposition fluids was only confirmed by the detection of human faecal steroids (coprostanol, epicoprostanol) rather than the presence of tissue-derived steroids (cholesterol, 5α-cholesterol, 5α-cholestanone). Thus, the hypothesis of this study was only partly confirmed. Nevertheless, results should be compared to reference soil material from the same study area, as only the comparison of steroid contents with the natural background can indicate an input of human decomposition products. It is also important to combine steroid analysis with other evidence (e.g. pivot crown, grave stratigraphy) to strengthen the evidence of a temporary grave. So far, this is the first study that proofed the presence of human derived steroids in temporary graves. Further work in forensic and archaeological sciences are necessary to understand soil profile depth distributions of steroids and the preservation of tissue and faecal derived steroids in the course of time.

Acknowledgements

Jochen Berger is thanked for support during soil sampling. We thank Tobias Fischer for supporting Soxhlet extraction of the soil samples.

Competing Interests Statement

The authors declare no competing interests.

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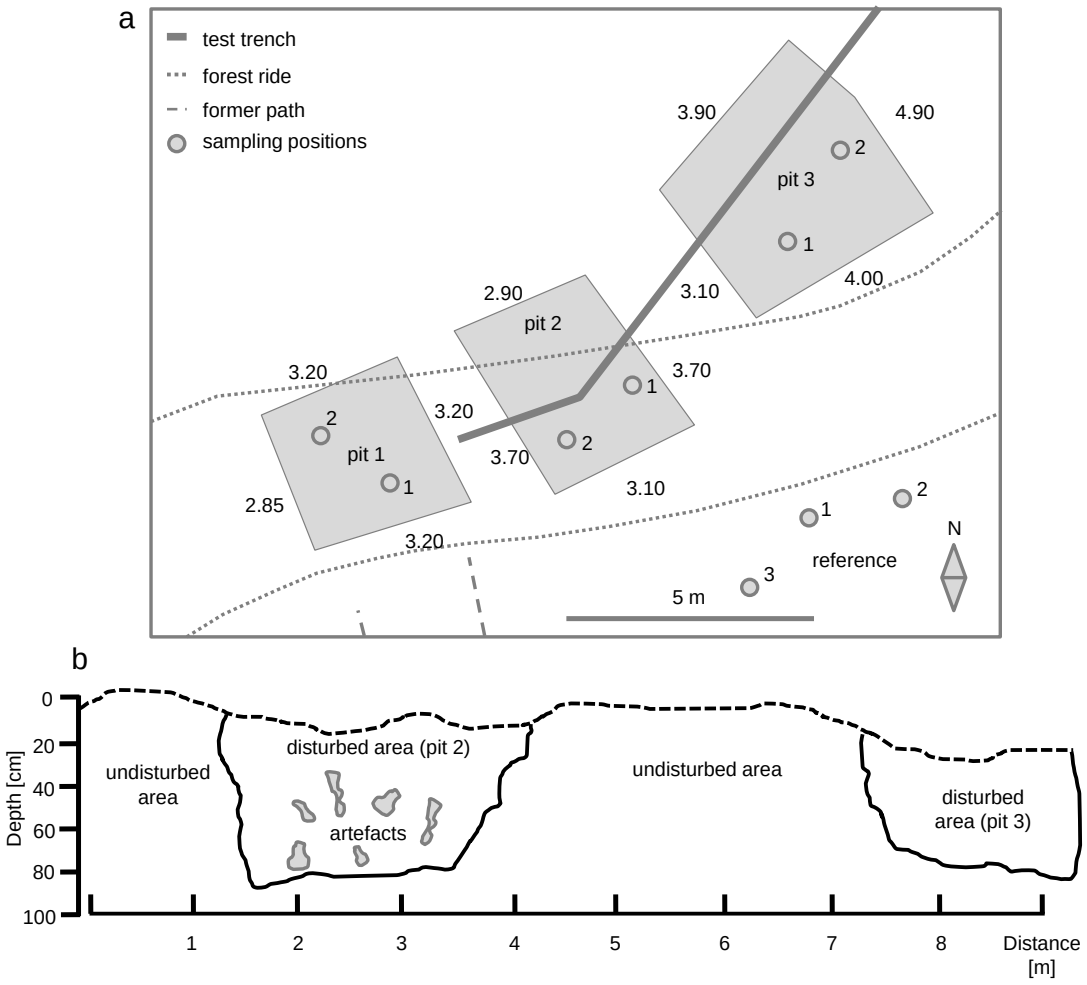


413

414 **Figure 1:** (a) Map (after Fiedler et al. 2009) showing the location of the temporary mass graves (burial of 66 bodies for
415 10 months) from late 1944 to early 1945 in a municipal forest close to Stuttgart, Germany. The test trench was
416 constructed during the first search of the former mass graves in 2009 (Fiedler et al. 2009). Position of the soil augers
417 are marked in the soil pits, augers from the reference samples were taken south-east and slightly uphill from pit 2 and
418 pit 3. (b) Section (test trench) of the disturbed areas of pit 2 and pit 3 with undisturbed intermediate areas.

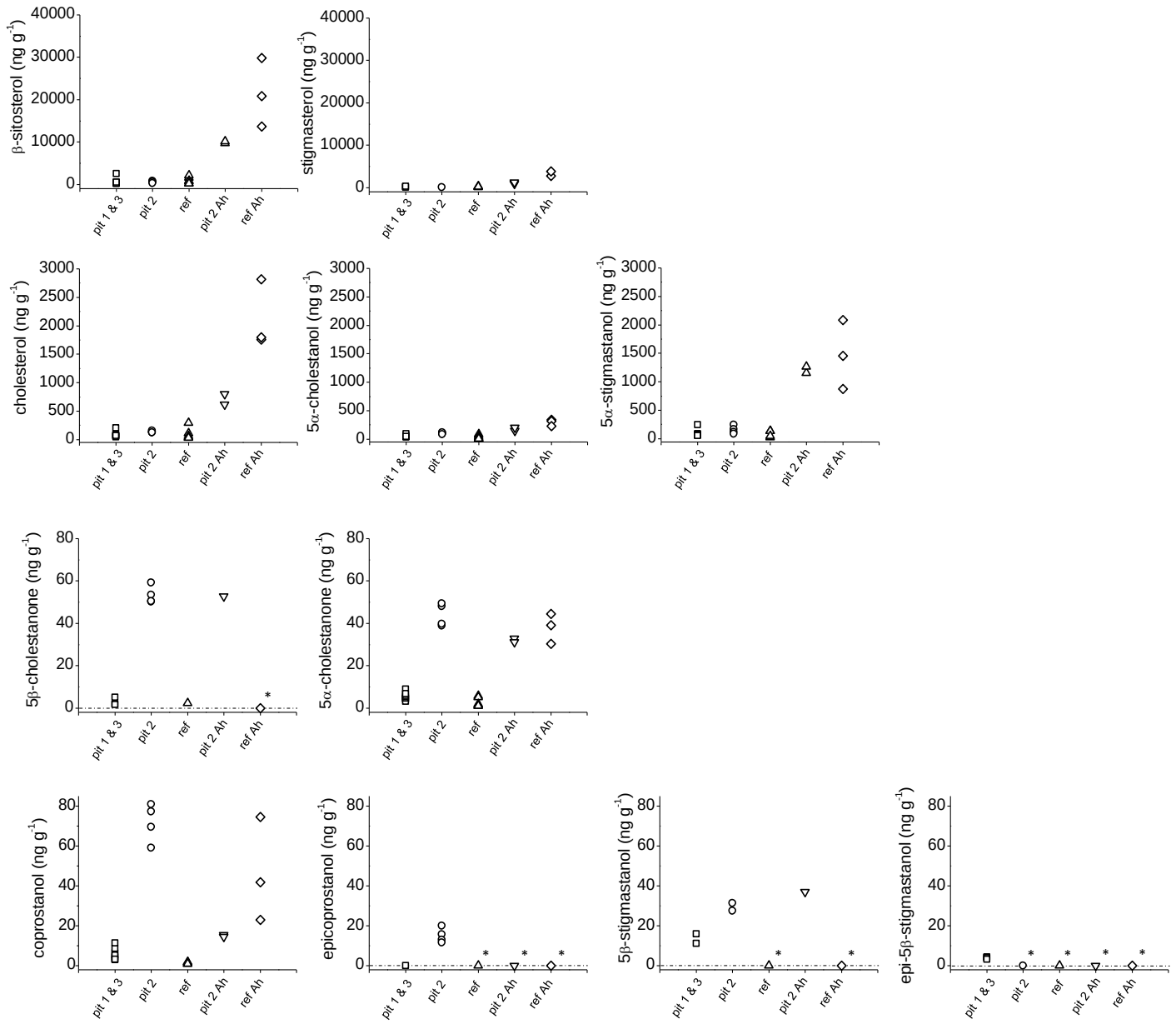
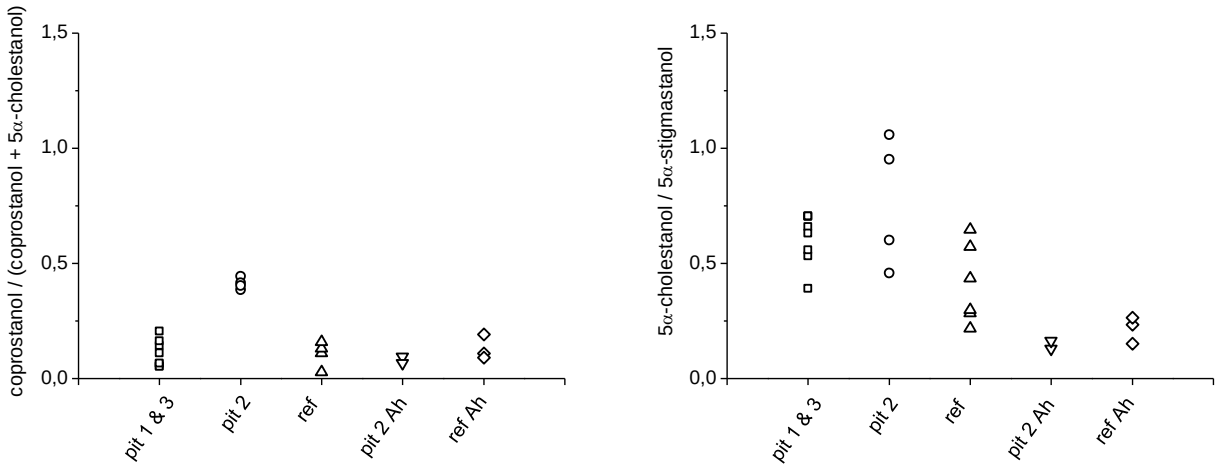
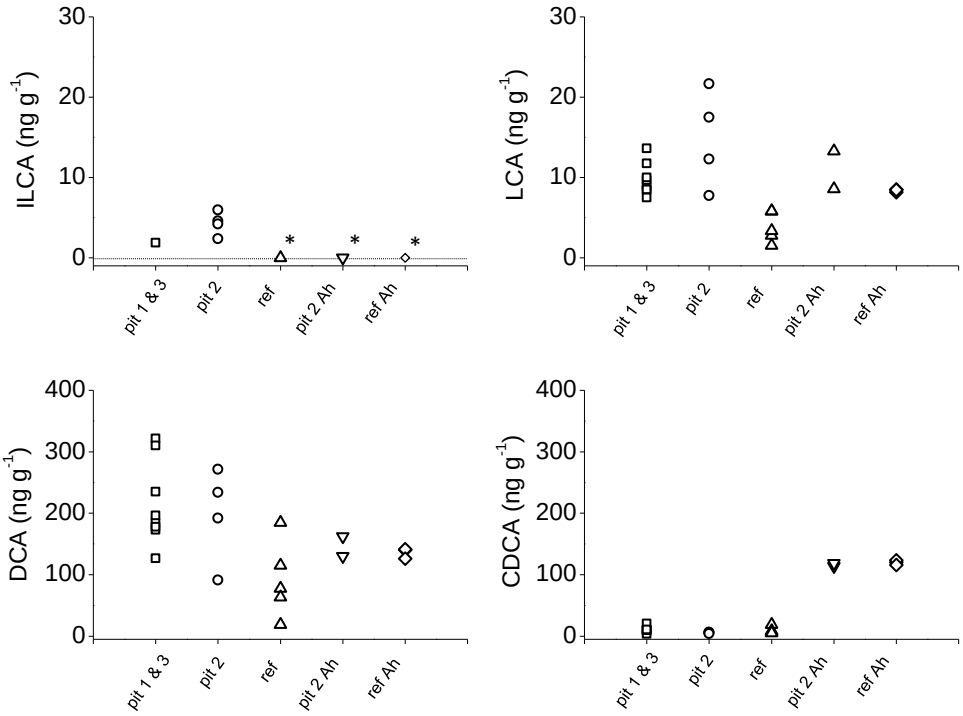


Figure 2: Silylated Δ^5-sterols, stanols and stanones (ng g^{-1}) extracted from soil from putative temporary mass graves (pit 1 - 3) and reference soil. Subsoil samples of pit 1 - 3 were taken in 40 – 50 cm (n = 2) and 50 - 60 cm (n = 2) of soil depth. Subsoil reference samples were taken in 40 - 50 cm (n = 3) and 50 - 60 cm (n = 3) of soil depth. Topsoil (Ah) samples of pit 2 (n = 2) and the reference (n = 3) were taken in 0 - 10 cm soil depth.



424

425 **Figure 3:** Ratios of 5β- and 5α-stanols for faecal source identification. Subsoil samples of pit 1 - 3 were taken in 40 -
426 50 (n = 2) and 50 - 60 cm (n = 2) of soil depth. Subsoil reference samples were taken in 40 - 50 cm (n = 3) and 50 - 60
427 cm (n = 3) of soil depth. Topsoil (Ah) samples of pit 2 (n = 2) and the reference (n = 3) were taken in 0 - 10 cm soil
428 depth.



429

430 **Figure 4:** Bile acids (as silylated methyl esters, ng g^{-1}) extracted from soil from putative temporary mass graves (pit 1 -
431 3) and reference soil. Subsoil samples of pit 1 - 3 were taken in 40 – 50 cm (n = 2) and 50 - 60 cm (n = 2) of soil depth.
432 Subsoil reference samples were taken in 40 - 50 cm (n = 3) and 50 - 60 cm (n = 3) of soil depth. Topsoil (Ah) samples
433 of pit 2 (n = 2) and the reference (n = 3) were taken in 0 - 10 cm soil depth. Asterisk = not detected.

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