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## EDITED BY

Aron L. Crowell,  
Smithsonian National Museum of Natural  
History (SI), United States

## REVIEWED BY

Anna Irto,  
University of Messina, Italy

## \*CORRESPONDENCE

Sabine Fiedler  
✉ fiedlers@uni-mainz.de

RECEIVED 12 December 2024

ACCEPTED 17 January 2025

PUBLISHED 06 February 2025

## CITATION

Fiedler S, Scherer S and Krause-Kyora B (2025)  
Fecal lipid markers in tandem with ancient  
sedimentary DNA as a tool for tracing past  
livestock farming from soils and sediments.  
*Front. Environ. Archaeol.* 4:1544307.  
doi: 10.3389/fearc.2025.1544307

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# Fecal lipid markers in tandem with ancient sedimentary DNA as a tool for tracing past livestock farming from soils and sediments

Sabine Fiedler<sup>1\*</sup>, Sascha Scherer<sup>1</sup> and Ben Krause-Kyora<sup>2</sup>

<sup>1</sup>Institute of Geography, University of Mainz, Mainz, Germany, <sup>2</sup>Institute of Clinical Molecular Biology, Kiel University, Kiel, Germany

Reconstructing past environments can be challenging when archaeological materials are missing. The study of organic molecules, which remain as traces in the environment over millennia, represents one way to overcome this drawback. Fecal lipid markers (steroids and bile acids) and ancient sedimentary DNA offer a complementary and cross-validating analytical tool to broaden the range of methods used in environmental archaeology. However, little is known about the benefits of combining these two approaches. We present a brief overview of the current state of knowledge on fecal lipid markers and ancient sedimentary DNA. We identify scientific and methodological gaps and suggest their potential relevance for a better understanding of dynamic, human-animal relationships of the past. With this review, we aim to facilitate new research avenues, both in established disciplines and in conjunction with analytical approaches that have rarely been combined to date.

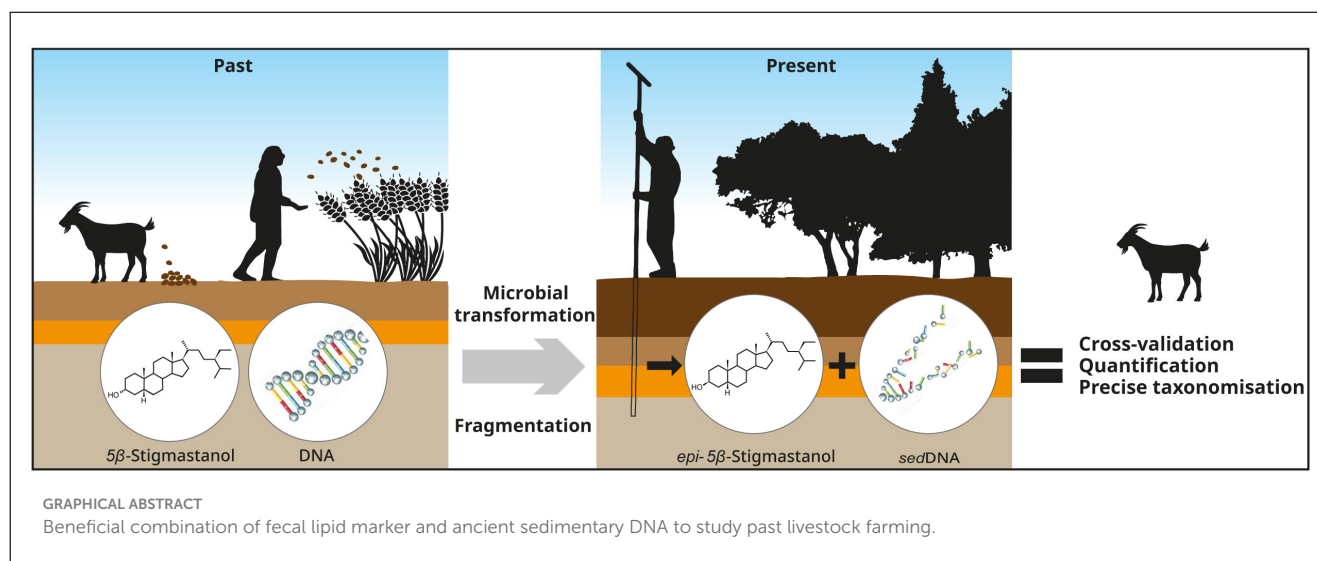
## KEYWORDS

past livestock farming, archaeology, steroids, bile acids, sedDNA

## 1 Introduction

Advances in analytical approaches for studying past environments have strengthened interdisciplinary collaboration between archaeology and geosciences. However, this can be challenging when archaeological materials are degraded or absent. Analyzing specific organic molecules that are recalcitrant against (e.g., aromatics) and protected from (encapsulated in aggregates) microbial turnover and therefore remain in the environment as traces over millennia, offers one approach to overcome this limitation (Curtin et al., 2021; Kjær et al., 2022).

The analysis of fecal lipid markers (FLM) such as steroids and bile acids has attracted considerable interest as complement to traditional approaches (e.g., coprophilous fungal spores, coprolites) to investigate the implications of past fecal inputs (Davies et al., 2022; Sistiaga et al., 2014). FLM in sediments have been used to reconstruct the onset of human occupation (Argiriadis et al., 2018; Ossendorf et al., 2019) and population fluctuations (Birkett et al., 2023; Keenan et al., 2021). They can provide in-depth insights into past land use, particularly livestock farming and manuring (Bull et al., 2001; Lavrieux et al., 2012), but also inform on functional areas of past settlements (Hjulström and Isaksson, 2009; Scherer et al., 2021).



Ancient sedimentary DNA (*sedDNA*) analysis is revolutionizing archaeology (Özdoğan et al., 2024). It enables deciphering ancient ecosystems, biodiversity, and even human activity through the study of genetic traces preserved in soils and sediments. By analyzing *sedDNA*, precise information on the presence, taxonomy and diet of animals can be collected, allowing for unraveling past livestock farming strategies (Giguët-Covex et al., 2014; Zampirolo et al., 2024). Also, *sedDNA* can provide new insights into the composition and functions of human and animal gut microbiota (Borri et al., 2020).

In this mini review, we address the analysis of FLM and *sedDNA* from soils and sediments used to study past livestock farming. A brief summary of the current state of knowledge is followed by the identification of future research avenues for a joint application of FLM and *sedDNA* analysis.

## 2 Fecal lipid marker (FLM)

The study of FLM has a long tradition, beginning in the 1980s (Knights et al., 1983; Pepe et al., 1989) with pioneering analytical work by Evershed's group (Evershed, 1993; Evershed et al., 1997; Evershed and Bethell, 1996). Many excellent reviews followed, summarizing the transformation processes of fecal lipids in the vertebrate gut and the environment, documenting their relevance in addressing research gaps in archaeology (Cramp et al., 2023; Evershed, 2008; Harrauld et al., 2019).

Each type of diet has an individual steroidal profile with different proportions of steroidal precursors entering the digestive tract (Tyagi et al., 2009a). While cholesterol is omnipresent in eukaryotic cells (Gerber et al., 2015),  $\beta$ -sitosterol is predominant in plant biomass (Christie and Han, 2010). In the vertebrate gastrointestinal tract,  $\Delta^5$ -sterols are biosynthesised to  $5\beta$ -stanols (coprostanol,  $5\beta$ -stigmastanol) (Cuevas-Tena et al., 2018).  $5\beta$ -stanols are useful not only for detecting fecal input in general, but also for distinguishing between omnivorous and herbivorous lifestyles (Leeming et al., 1996; Zocatelli et al., 2017). In a prominent example, Vachula et al. (2019) analyzed coprostanol from lake sediments in northern Alaska and provided evidence that eastern

Beringia was inhabited before the formation of the land bridge across the Bering Strait during the last glaciation.

Through microbial reduction in soils, sterols are often converted to  $5\alpha$ -stanols ( $5\alpha$ -cholestanol,  $5\alpha$ -stigmastanol) and *epi*- $5\beta$ -stanols (*epi*-coprostanol; *epi*- $5\beta$ -stigmastanol), mainly when fecal deposition has occurred long ago (Bull et al., 1999; Isaksson, 1998). Stanones are intermediates formed microbially during the conversion of  $\Delta^5$ -sterols to  $5\beta/5\alpha$ -stanols, and their epimers in the intestines of higher animals and the environment (Grimalt et al., 1990). They are thought to have potential as biomarkers comparable to  $5\beta$ -stanols but have rarely been used to study past lifeworlds (Knights et al., 1983). Since not all steroidal compounds are exclusively attributed to feces, numerous ratios of distinct fecal lipids are calculated to interpret soil-specific degradation effects and to differentiate between omnivorous, herbivorous and plant-derived steroidal inputs (Bull et al., 2002; Lerch et al., 2022; Vázquez et al., 2021).

Bile acids (BAs) are the end products of cholesterol catabolism and are found exclusively in vertebrate feces. Individual patterns of BAs are the result of evolutionary variation and, to a lesser degree, diet (Hagey et al., 1993). Their analysis permits the differentiation between pig and human feces, which is not applicable with  $5\beta$ -stanols (Prost et al., 2017; Zocatelli et al., 2017). The dominant secondary BA in pig feces is hyodeoxycholic acid (HDCA), which is not present in human or ruminant feces (Elhmmali et al., 1997; Tyagi et al., 2009b). Lithocholic acid (LCA) and deoxycholic acid (DCA), among others, are typical of human biochemistry (Perwaiz et al., 2002). A reliable source identification of fecal inputs combines both the analysis of (*epi*-) $5\beta$ -stanols and bile acids (Prost et al., 2017).

## 3 Ancient sedimentary DNA (*sedDNA*)

The ability to decode species presence and biodiversity dynamics has been revolutionized by *sedDNA* analysis. Organic-rich sediments, such as those found in permafrost soils and caves, have proven to be excellent sources for recovering animal DNA (Boessenkool et al., 2014; Ficetola et al., 2008). Early

studies demonstrated that *sedDNA* could identify extinct species in soils and sediments, offering new insights into past ecosystems (Pedersen et al., 2016; Rawlence et al., 2016; Willerslev et al., 2014). The long-term preservation of *sedDNA* in stratified archaeological layers allows scientists to track changes in animal populations in response to climate shifts, farming activities, and human migration (Pedersen et al., 2016; Willerslev et al., 2003). *sedDNA* has been used to trace animal domestication, revealing early evidence of domesticated species such as cattle, pigs, and sheep at prehistoric sites (Giguet-Covex et al., 2014). Haile et al. (2007) found that *sedDNA* could identify species, like moa and sheep, even without macrofossils, but they also revealed challenges in non-frozen environments where DNA leaching between layers can compromise chronological accuracy. This issue was illustrated by the presence of sheep DNA in older layers, likely due to downward migration. While *sedDNA* remains a valuable tool for reconstructing past ecosystems, non-frozen conditions pose significant risks for contamination.

Zampirolo et al. (2024) demonstrated the utility of *sedDNA* in tracing the introduction of pastoralism in Central Europe. By analyzing sediment samples from the Velký Mamuták rock shelter, modern-day Czech Republic, they reconstructed animal and plant DNA profiles across stratigraphic layers, revealing a gradual shift in herding practices. Their findings suggest herding began in the Late Neolithic with sheep, while pigs began to appear in the Late Bronze Age. This gradual transition, challenging to capture from fossil records alone, highlights *sedDNA*'s potential to reveal human impacts on ecosystems.

## 4 Fecal lipid markers in tandem with *sedDNA*

By combining the analysis of FLM and *sedDNA*, researchers continue to broaden their understanding of human-animal relationships in archaeological contexts, helping to answer long-standing questions about past biodiversity and animal domestication (Slon et al., 2017; Zeder, 2015). To our knowledge, there are only a few studies that combine the analysis of FLM and *sedDNA*, but they are pioneering.

There is still much debate about the route taken by the Carthaginian commander Hannibal in the 2<sup>nd</sup> Punic War. To address this debate, Mahaney et al. (2017) examined a significant accumulation of mammalian dung in the upper Guil Valley near Mt. Viso dating to 2,168 cal <sup>14</sup>C year. They were able to extract DCA, 5 $\beta$ -stigmastanol and genes of Clostridia, which were interpreted as evidence of the passage of the Hannibalian army.

Another remarkable study addresses the hitherto mystery as to whether the Vikings first colonized the Faroe Islands in the mid-ninth century. Apart from a few charred grains of barley dating to 351–543 BCE, there is no archaeological evidence of human activity before the Viking Age. Curtin et al. (2021) investigated lake sediments by combining FLM and *sedDNA* analyses and demonstrated that the Faroe Islands were colonized by humans and their livestock 300 years earlier than the Vikings' arrival.

In another study, geochemical and palynological indicators were used in combination with 5 $\beta$ -stanols, BAs and *sedDNA* from lake sediments to reconstruct human activity at medieval Celtic

crannog settlements in Ireland (Brown et al., 2021) and Scotland (Brown et al., 2022). The results show a direct link between human presence, the processing of specific crops, livestock farming and local slaughters.

Chen et al. (2024) showed that the presence of coprostanol in Antarctic lacustrine sediments does not necessarily indicate human waste. In conjunction with high-throughput sequencing of the 16S rRNA gene, the conversion of seal feces-based cholesterol to coprostanol by anaerobic bacteria (*Eubacterium coprostanoligenes*) was illustrated. This example reveals that analyzing FLM and *sedDNA* in tandem enables for a better understanding of natural processes and helps avoiding misinterpretation.

Individually, FLM and *sedDNA* analyses already provide valuable insights in different cultural-historical contexts, but both methods have interpretive limitations. FLMs provide information on past fecal input and its quantification, whereas FLM cannot differentiate between non-/domesticated animal species. While *sedDNA* cannot quantify animal stocking, it gives precise data on animal phenotypes, physical traits and adaptations of past populations. The combination of both groups of molecular biomarkers as a complementary and a cross-validating analytical tool opens new windows into the past for reconstructing human-animal interactions.

Furthermore, FLMs are less frequently faced with cross-layer leakage, a common problem with *sedDNA*. Therefore, FLM analysis may serve as an effective pre-screening tool, allowing *sedDNA* analysis to focus on samples with a higher chance of DNA recovery. This reduces the risk of false positive signals and improves the detection of modern contaminants. Consequently, FLM and *sedDNA* analysis in tandem offer quantitatively interpretable, more precise and reliable datasets. This synergy permits the detection of subtle environmental changes and anthropogenic influences that may be less discernible when each approach is applied separately.

## 5 Research challenges and perspectives

Understanding the interaction between soils and organic marker molecules is crucial for assessing the explanatory power of the latter. Ideally, unambiguous compounds that entered the soil simultaneously and are sufficiently preserved over millennia are used (Salisbury et al., 2022; van Mourik and Jansen, 2013). The following substance-specific and analytical challenges highlight potential pitfalls and research avenues for a combined application of FLM and *sedDNA* in soils and sediments.

### 5.1 The fate of fecal lipid markers and *sedDNA* in soils

Both FLM and *sedDNA* behave differently under varying soil conditions (e.g., particle size distribution, mineral composition, organic matter, redox and pH state, hydrology), indicating a high mutual dependency (Barnes and Turner, 2016; Islam et al., 2023; Jia et al., 2022; Thomsen and Willerslev, 2015). Organic molecules in soils are stabilized by highly reactive surfaces (organic matter,

oxides, clay minerals) (Freeman et al., 2023; Gande et al., 2024; Islam et al., 2023; Lejzerowicz et al., 2013; Lloyd et al., 2012). Although our understanding of organic molecule behavior in soils still needs to be improved, some characteristics make such molecules promising candidates for advanced marker analysis.

FLMs are highly resistant against microbial turnover (Bull et al., 2002; Dubois and Jacob, 2016) primarily due to their aromatic ring structures and strong bonding to organic and mineral matter (Islam et al., 2023). Their hydrophobic nature inhibits large-scale translocation across stratigraphic layers, whereas a coarse soil texture, lower abundance of soil organic matter and co-transport by particulates are reported to have influence of FLM migration (von der Lühse et al., 2018; Lloyd et al., 2012).

*sedDNA* degradation occurs over time due to microbial enzymatic activity, mechanical damage, and spontaneous chemical reactions like hydrolysis and oxidation. Preservation is strongly influenced by environmental factors: Extreme pH (acidic or basic) and optimal conditions for microbial turnover (temperature, oxygen exposure and moisture) accelerate degradation. Cold and anoxic environments significantly enhance preservation, enabling *sedDNA* to persist for millennia (Kjær et al., 2022; Murchie et al., 2023; Parducci et al., 2017). In aquatic environments and through percolating water, *sedDNA* can be leached over considerable distances and across stratigraphic layers (Deiner et al., 2017; Haile et al., 2007). This transport is critical for interpreting genetic traces in chronological contexts. Giguët-Covex et al. (2019) emphasized the role of erosion in the preservation and transport of *sedDNA* within lacustrine environments. Their findings demonstrated how different soil horizons, vegetation cover and hydrographic connectivity influence *sedDNA* deposition and quality. Additionally, they highlighted the role of soil organic substance and soil milieu on *sedDNA* degradation, offering crucial insights into improving the reliability of datasets.

Future research is needed to refine our understanding of how environmental conditions affect the preservation, stabilization and transport of FLM and *sedDNA* in soils and sediments (Taberlet et al., 2018). In this context, column experiments with both FLM and *sedDNA* could help to identify differences in their transport sensibility to estimate their complementary and cross-validating character (Poté et al., 2007).

## 5.2 Optimizing methodology

FLMs are leached from soil samples using a mixture of organic solvents (commonly used: dichloromethane:methanol, 2:1, v/v) by various extraction methods (Birk et al., 2012; Manley et al., 2020). Scherer et al. (2024) demonstrated that Soxhlet (SOX) extraction achieves significantly higher yields and better reproducibility of FLM than microwave-assisted and accelerated-solvent extraction. However, traditional SOX extraction methods are time-consuming (>24 h extraction time) and consume high volumes of solvent. Therefore, optimisation and automatization of SOX extraction is crucial to achieve higher sample throughput, which enables larger data volumes with a higher reliability (by using replicates). Furthermore, methodological approaches from related disciplines could be promising to integrate into studies on past environments.

Can the fecal BAs assay developed for analyzing medical plasma also be used for soil samples? Is it possible to develop non-destructive and extraction-free methods to pre-screen samples for certain substances (e.g., coprostanol)? One approach could be the use of high-resolution mass spectrometry (e.g., Orbitrap) combined with direct ionization technology (ambient ionization mass spectrometry) from flowing atmospheric pressure afterglow ion sources, allowing organic molecules to be desorbed and analyzed directly from surfaces.

The extraction of *sedDNA* from soil samples is particularly challenging due to soil heterogeneity, affecting the effectiveness of DNA extraction and amplification. Current DNA extraction methods for soil rely on a combination of chemical lysis buffers and mechanical disruption techniques, such as bead-beating, to release DNA from soil particles. These methods are implemented using both commercial kits and customs and self-made protocols. However, the effectiveness of these approaches can vary significantly depending on factors such as soil type, sample volume, and environmental conditions, warranting further investigation (Deiner et al., 2017). In this context, soil fractionation can help improve DNA recovery, particularly for specific plant or microbial DNA targets. This involves separating soil samples into size and density fractions to isolate finer particles where *sedDNA* may be better preserved (Lejzerowicz et al., 2013). Furthermore, the use of chloroform-isoamyl alcohol extraction and alternative lysis buffers that target specific organisms or DNA types, such as pollen or microbial DNA, are conceivable approaches to optimize DNA extraction from soil samples (Birks and Birks, 2016).

## 5.3 Expansion of reference library

Analyzing FLM is biased toward material from humans and domestic animals, while associated (e.g., mice, rats) and co-associated animals (e.g., hyena, wolf, fox) are less frequently examined at archaeological sites. To improve fecal sourcing, the expansion of reference libraries that characterize steroid and bile acid profiles of domesticated and non-domesticated animals should be pursued. Further differentiation of organism-specific feces could be achieved by broadening the analytical spectrum of substances analyzed. According to Porru et al. (2022), mono/di/trioxo-BAs are able to distinguish between feces of wild boar and domestic pigs, while Vallejo et al. (2024) showed that the analysis of hormones such as progesterone provide insights into livestock husbandry favoring female pigs.

*sedDNA* studies often exhibit a bias toward bacterial communities and prominent taxa like domestic animals and megafauna, especially when using targeted sequencing methods. This focus can lead to an overrepresentation of specific groups while overlooking broader biodiversity. Shotgun metagenomic sequencing offers a more comprehensive, unbiased approach by capturing all DNA present, revealing greater ecological complexity and diversity (Armbrecht, 2020). However, it is resource-intensive and generates large datasets, which can complicate interpretation. Combining targeted and shotgun methods may balance the need for specificity and breadth, providing a fuller view of past environments and communities.



## 5.4 Interpretation and contamination prevention

A promising approach to interpreting FLMs in modern and ancient contexts has been successfully demonstrated by Harrault et al. (2019) in which the distributions of eleven  $5\beta$ -stanols of 90 fecal samples from 10 different species were used to conduct principal component analysis and hierarchical clustering. Extending this approach to include whole lipidomes and even *sedDNA* would allow the application of this approach to an increasingly large and diverse mixture of potential fecal sources. In order to detect fecal contamination, Grimalt et al. (1990) introduced the ratio coprostanol/(coprostanol/cholestanol). Human fecal input to the environment is argued to be reliably detected at a ratio  $\geq 0.7$ , with no fecal contamination at a ratio  $< 0.3$ . However, strict application of these thresholds is debated (Isobe et al., 2002; Nakagawa et al., 2021), especially their suitability in archaeological contexts (Birk et al., 2011, 2022; Mackay et al., 2020). A combined analysis of FLM and *sedDNA* may prove an effective means of determining whether lower threshold values can also be indicative of fecal presence. The ongoing debate surrounding the imposition of these thresholds for samples from archaeological sites may soon reach a conclusion.

For both FLM and *sedDNA* analysis, sterile sampling and laboratory conditions (sterile tools, wearing gloves, strict protocols) are required to prevent modern contamination. Specifically, for *sedDNA* analysis, highest quality assurance is additionally obligatory (e.g., cleanroom conditions, negative controls) (Cooper and Poinar, 2000).

Authentication of *sedDNA* involves independent replications, analysis of molecular damage patterns, and assessment of DNA fragment sizes to distinguish ancient DNA from modern contaminants (Kjær et al., 2022). Computational tools further enhance reliability by filtering contaminants based on reference databases and damage signatures (Kjær et al., 2022; Capo et al., 2021). Integrating *sedDNA* data with chemical proxies, such as lipid biomarkers or isotopic signatures, offers a promising interdisciplinary approach to improve the differentiation between authentic ancient DNA and modern contamination, advancing the field of environmental archaeology.

## 6 Conclusion

In our mini review we have demonstrated that a combined application of FLM and *sedDNA* gives a significant contribution to the growing field of environmental archaeology. Both approaches in tandem represent a powerful tool in the ongoing effort to understand the complexities of past human ecosystem transformation across temporal and spatial scales, offering an

innovative perspective on how ancient communities shaped, and were shaped by, the landscapes they inhabited. We hope that our review will stimulate new avenues of research in well-established disciplines, as well as inspire the combination of analytical approaches that have rarely been used in tandem to date.

## Author contributions

SF: Conceptualization, Visualization, Writing – original draft, Writing – review & editing. SS: Conceptualization, Visualization, Writing – original draft, Writing – review & editing. BK-K: Conceptualization, Visualization, Writing – original draft, Writing – review & editing.

## Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study was completed with the financial support of the Publication Fund of the Johannes Gutenberg-University.

## Acknowledgments

We are grateful to G. Janzen and M. C. A. Torbenson for critically proofreading the manuscript.

## Conflict of interest

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