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# Bioturbation and sediments of terrestrial subterranean ecosystems and their relevance to ecosystem services

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**Resumo**

O solo é o substrato da vida por todos os seres vivos dependerem dele. Este é composto por material mineral, raízes, água, gases e matéria orgânica. O solo fornece um habitat para a sobrevivência de grande diversidade de organismos. Este interage com os ambientes que o rodeiam, impactando assim a própria vida. Grutas são ecossistemas subterrâneos que variam na sua geomorfologia. Os sedimentos subterrâneos não são considerados verdadeiros solos, dada a ausência da fração vegetal em profundidade. As grutas acumulam vários tipos de sedimentos, detritos orgânicos e químicos, e os seus depósitos tendem a ficar bem preservados por longos períodos. Por isso, sedimentos de grutas possuem um grande significado geológico, arqueológico e paleontológico. A acumulação de sedimentos no interior das grutas difere por estas possuírem diferentes infraestruturas, tamanhos e relações geomorfológicas internas ou externas. Estes podem ser divididos em sedimentos clásticos, químicos e antropogénicos. Os sedimentos clásticos formam-se através do movimento mecânico dentro e fora das grutas, enquanto os sedimentos químicos são formados *in situ* e precipitados a partir da solução em água. Os sedimentos antropogénicos compreendem todos os materiais provenientes da atividade humana introduzidos em grutas. Os sedimentos subterrâneos passam por diferentes processos ao longo das suas vidas, seja por atividade química, biológica ou antropogénica. Por este motivo, os sedimentos podem alterar a sua aparência física, cor, textura e estrutura, a sua composição química, calcificação e fosfatização. Estes podem alterar o seu efeito no ciclo geológico, e numa escala maior, estes podem também afetar mudanças ambientais. Os sedimentos clásticos são os mais comuns dependendo da sua origem e de mecanismos de transporte, o seu tamanho pode variar entre um coloidal com micrómetros ou um calhau de um metro. Estes estão subdivididos em sedimentos autóctones e alóctones. Os sedimentos autóctones são formados dentro das grutas e podem ser subdivididos em travertinos, evaporitos ou fosfatos dependendo da sua composição. São compostos por detritos como areia e fragmentos de calcário, clastos de rocha derivada de dentro da gruta, levando à sua decomposição em fragmentos, e resíduos fecais de guano, principalmente de morcegos, que podem acumular-se em grandes quantidades. Por outro lado, os sedimentos alóctones são formados fora das grutas e transportados para dentro destas. São compostos por uma variedade de sedimentos dependendo do material que se encontra disponível e do tipo de rocha. Podem ser compostos de detritos estratificados da entrada da gruta, sedimentos infiltrados da superfície, sedimentos de rios, fluxos de detritos que ocorrem após um derrame ou depósitos eólicos que são transportados pelo vento. Os sedimentos das grutas são variáveis, portanto, compreender a sua composição e dinâmica é relevante pois são o substrato da vida subterrânea, um reservatório de microbiomas únicos que são fonte de matéria orgânica e nutrientes para uma ampla diversidade de organismos endémicos. Os microbiomas são altamentes dependentes de parâmetros dos sedimentos como o pH, embora se saiba que diversos animais cavernícolas que vivem nestas grutas utilizam e processam sedimentos nos seus processos biológicos. Um desses processos é a bioturbação que pode ser definida como a atividade biológica em solos e sedimentos para que haja acesso a recursos como nutrientes e água, impacta o sedimento por meio de reprocessamento de partículas, transporte de sedimentos e alterações físicas no material sedimentar resultante da produção de excrementos e constante construção de túneis/abrigos. A bioturbação é um serviço de ecossistema que altera a distribuição de matéria orgânica e nutrientes no sedimento, estimulando uma maior abundância da composição bacteriana, e produção de nutrientes. O arejamento do solo é melhorado pela distribuição de oxigénio presente nos sedimentos. Entre outras coisas, o crescimento da raíz em comprimento é estimulado, o que proporciona um aumento da matéria orgânica vegetal disponível para os organismos. Os ecossistemas subterrâneos possuem características abióticas semelhantes entre si, mas que diferem da superfície. Estas incluem a ausência de luz, alta humidade, temperaturas constantes e baixa disponibilidade de nutrientes, uma forte pressão seletiva que leva a sua fauna a adaptar-se. Essas adaptações são chamadas troglomorfismos e estão divididas em três tipos:

morfológicas, fisiológicas e comportamentais. As adaptações morfológicas incluem a perda de visão e pigmentação, a redução das asas e cutículas, e o alongamento do corpo e dos apêndices. As adaptações fisiológicas incluem baixa taxa metabólica, maior longevidade, baixa tolerância a variação térmica e fertilidade reduzida. As adaptações comportamentais incluem a perda do ciclo circadiano e mudanças nas estratégias reprodutivas. Os organismos subterrâneos podem ser classificados de acordo com a sua ocorrência no ecossistema subterrâneo e são divididos em três categorias. As espécies exclusivas deste ecossistema, não encontradas à superfície e que possuem adaptações exclusivas a este ambiente são denominadas como troglóbios. As espécies que viajam regularmente entre a superfície e o subterrâneo são denominadas como troglófilos. E por último, as espécies exclusivas da superfície e que ocorrem no subterrâneo de forma acidental são denominadas como trogloxenos. A espécie endémica portuguesa adaptada à vida nas grutas *Miktoniscus longispina* Reboleira & Taiti 2015 (Oniscidea: Trichoniscidae), foi observada a processar sedimentos de grutas, construindo túneis e abrigos para efetuar a muda, envolvendo a mobilização de matéria orgânica para o sedimento. Este estudo foca-se na caracterização de sedimentos recolhidos em 15 grutas de Portugal central e avalia o impacto do processamento e mobilização de matéria orgânica em sedimentos subterrâneos por organismos da espécie *M. longispina*. A nível do sedimento, foram feitas análises de conteúdo de matéria orgânica e carbonato de cálcio, pH, textura e distribuição do tamanho dos grãos. Os resultados mostraram que as propriedades do sedimento variam significativamente entre grutas, sendo o pH, o carbonato de cálcio e a matéria orgânica as variáveis determinantes. Os sedimentos foram caracterizados como alcalinos, compostos na sua maioria por frações finas (principalmente siltes), com um baixo percentual de matéria orgânica e uma ampla faixa de percentual de carbonato de cálcio. Quando comparados com sedimentos à superfície, estas características não se relacionam da mesma maneira, pois é comum encontrar altos valores de matéria orgânica em sedimentos alcalinos e finos. Estas variáveis são relevantes para os ecossistemas subterrâneos, uma vez que o pH controla a dinâmica microbiana, o carbonato de cálcio está presente nas conchas e exoesqueletos de artrópodes e a matéria orgânica é o fator limitante da vida. O estudo experimental foi efetuado durante dois meses onde indivíduos de *M. longispina* foram colocados em sedimento de grutas que habitam e expostos a condições nutricionais diferentes. Os animais construíram túneis e abrigos em sedimentos de grutas, com o uso das mandíbulas, maxilípedes e patas dianteiras (pereópodes). Estes organismos tendem a construí-los incorporando matéria orgânica vegetal disponível, sedimento, partículas fecais de sedimentos digeridos. Esta atividade biológica de bioturbação levou a um aumento significativo de matéria orgânica nos sedimentos das grutas. O papel destes isópodes terrestres adaptados às grutas na bioturbação dos sedimentos profundos, presta um relevante serviço de ecossistema, vital para a saúde global dos solos. Este estudo fornece a primeira evidência sobre a relevância dos serviços de ecossistema prestados pela fauna subterrânea terrestre, ponto de partida essencial para aprofundar a compreensão dos habitats subterrâneos, das espécies únicas que os habitam e dos serviços críticos que prestam a nível global, que são muitas vezes negligenciados. Estudos futuros devem incluir o aumento da diversidade de locais dentro de cada gruta através de gradientes de profundidade e de distância até às entradas da superfície, e investigar também a presença de nitratos e de fosfato que possuem uma grande influência sobre os organismos.

**Palavras-chave:** Ecossistemas subterrâneos, sedimentos, matéria orgânica, bioturbação, serviços de ecossistema

## Abstract

Soil is the substrate of life. Subterranean sediments are mainly composed of the same properties but are often overlooked and not considered soils. Caves are subterranean ecosystems with different geomorphologies sharing similar ecological characteristics, such as the absence of light, high humidity, constant temperatures, and low nutrient availability. Caves accumulate all types of materials in their sediments and depending on their origin, they can be formed autochthonously (inside) or allochthonously (outside). Cave-adapted *Miktoniscus longispina* Reboleira & Taiti 2015 (Isopoda: Trichoniscidae), endemic in Portugal, was seen in the laboratory and *in situ*, processing cave sediments. This study focuses on the characterization of cave sediments collected from 15 different caves in central Portugal and the in the role of *M. longispina* in the bioturbation of cave sediments. The results show that cave sediments were predominantly alkaline, composed of fine fractions, and low organic matter. The content in organic matter increased significantly because of the biological activity of *M. longispina* that constructed shelters and tunnels with the available sediment, vegetable organic matter, and fecal pellets. The role of these cave-adapted terrestrial isopods in the bioturbation of deep sediments provides a relevant ecosystem service, vital for global soil health.


**Keywords:** Subterranean ecosystems, cave sediments, organic matter, bioturbation, ecosystem

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### Chapter I



### Chapter II

## List of Abbreviations, Acronyms and Symbols

ANOVA: Analysis of variance
C: Carbon
$CaCO_3$: Calcium carbonate
C/N: Organic carbon and total nitrogen ratio
$CO_2$: Carbon dioxide
Corg: Organic carbon
HCl: Hydrochloric acid
ICNF: Instituto da Conservação da Natureza e das Florestas
KW: Kruskal-Wallis
LNEG: Laboratório Nacional de Energia e Geologia
OM: Organic matter
PCA: Principal component analysis
VPDB: Vienna Peedee Belemnite
$\delta^{13}C$: Stable carbon isotopes

# 1 Introduction

The subterranean is a vast domain consisting of numerous ecosystems such as caves (Fig.1 a). They are natural openings in rock and can be composed of limestones, the most common, sandstones, ice, and others (Culver & White, 2005; Gillieson 1996). Since these ecosystems are void, they accumulate various materials, including sediments (Culver & White, 2005). Cave sediments can be of three types, clastic, chemical, or anthropogenic, and are well-preserved for years (Ford & Williams, 2007). Clastic sediments are commonly found in caves and can have an autochthonous origin, inside formation, or an allochthonous origin, outside formation (Springer, 2012; White, 2007).

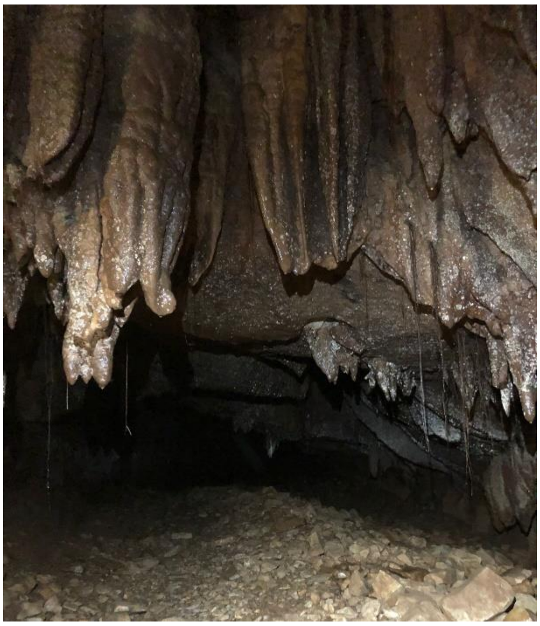

Figure 1. The inside of a karst cave (Soprador do Carvalho Cave, Sicó karst area).

Caves are found around the continent (Culver & Pipan, 2019) and share the same abiotic characteristics, such as total darkness, high humidity, low-temperature variability, and nutrient absence (Saccò et al., 2024). Because of this, the fauna present is rare, unique, and highly specialized. Subterranean species developed specific adaptations called troglomorphisms (Moldovan et al., 2018; Trajano et al., 2016). These are divided into morphological adaptations, such as loss of vision and pigment (Moldovan et al., 2018), physiological adaptations, such as low metabolic rate and reduced fertility (Hornung, 2011; Di Lorenzo & Reboleira, 2022), and behavioral adaptations, such as loss of the circadian cycle (Hornung, 2011; Moldovan et al., 2018).

Subterranean organisms are divided into three categories: troglobites, troglophiles, and trogloxenes, depending on their underground existence (Trajano, 2012). Troglobites are the species that use the subterranean domain exclusively; troglophiles are the species that can appear both at the surface and underground, and trogloxenes are the species that accidentally occur in caves (Moldovan et al., 2018).

Subterranean fauna provides ecosystem services that contribute to producing goods that help sustain human life and maintain biodiversity. These are important to sustain a healthy ecosystem (Daily, 1997; Karr, 1999). Ecosystem services can be divided into provisional, supporting, regulation, and cultural services (Van Gestel et al., 2018).

These ecosystem services are provided in sediment, which is a large part of how important sediment is. On the surface, it is known as soil, and interacting with all ecosystems impacts life (Voroney, 2007). Microorganisms help with sediment aeration and nutrient distribution (Bronick & Lal, 2005). Although their role in subterranean ecosystems is similar, little is known about it (Brevick et al., 2015).

Oniscidea is a suborder of crustaceans that have an essential role in the biogeochemical cycle by decomposing organic matter in sediment (Hornung, 2011). Troglobiont terrestrial isopods occupy a fundamental position in the web chain by being detritivores, sources of nutrients for predators, and are the underworld engineers (Hornung, 2011; Reboleira et al., 2015; Hose & Stumpp, 2019). They build shelters in the sediment to avoid predation and/or molt (Fig. 1b). This was observed with specimens from the species *Miktoniscus longispina* Reboleira & Taiti, 2015 (Fig. 1c) endemic to Portugal kept in the laboratory and potentially incorporated processed organic matter into it (Reboleira, pers. com.).

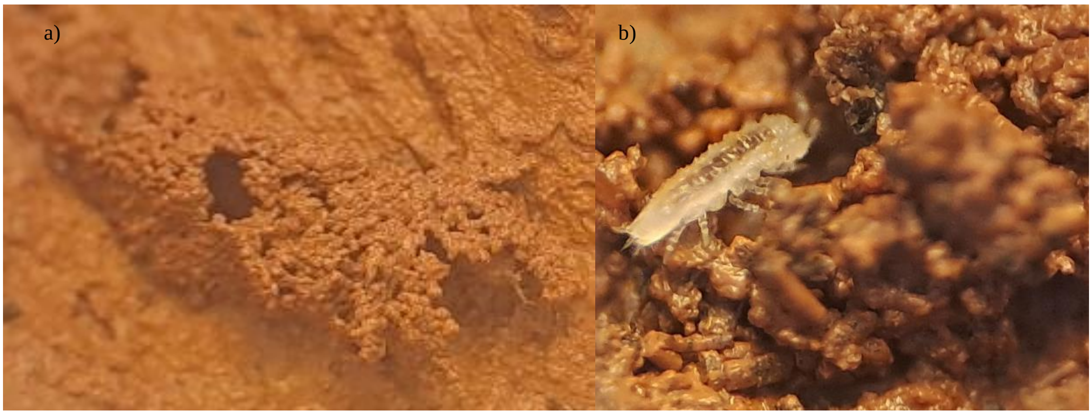


Figure 2. - a) Shelter built by *M. longispina*. b) *M. longispina* live specimen.

This work aims to characterize cave sediments from central Portugal through laboratory analysis and to understand how *Miktoniscus longispina* processes natural sediments. It also aims to determine if it mobilizes organic matter into the sediments, impacting cave sediments through bioturbation as an ecosystem service and the cave ecosystem.

## 2 Objectives and Dissertation Structure

This work aims to study the effect on cave sediment and the processing of organic matter by specimens of the terrestrial isopod *Miktoniscus longispina*, a species endemic to caves in central Portugal. This assessment will be carried out through laboratory experiments on the cave sediment collected to study the phenomenon and its contribution as an ecosystem service.

Specific objectives:

1) Geochemical characterization of sediments from caves in central Portugal to understand the diversity and characteristics of the sediments that occurs in caves;
2) Experimental study in laboratory to understand the bioturbation role of *M. longispina* and if its activity incorporates organic matter into the cave sediment.

This dissertation is structured into an introduction, objectives, and organization, two chapters, and final remarks. The two chapters focus on:

- Chapter 1: Characterization of cave sediments from central Portugal and their significance for subterranean ecosystems.

The first chapter focuses on the geochemical study of sediments from caves and compares them with each other and between karst areas. Results show differences in the analysis performed such as grain size, pH, and calcium carbonate.

- Chapter 2: Ecological role of terrestrial cave-adapted isopods in bioturbation and nutrient distribution in subterranean sediments.

The second chapter evaluates the bioturbation role of *M. longispina* in cave sediments. Results show that the biological activity of this species includes the construction of shelters and tunnels and increases significantly the organic matter content of the sediments. This process is vital for nutrient distribution and sediment aeration and gives a first glance at the relevance of ecosystem services in terrestrial subterranean ecosystems.

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# Chapter I - Preliminary characterization of parameters of cave sediments and their significance for subterranean ecosystems

**Abstract**

Soil is a thin layer of the Earth's surface that consists of mineral material, roots, water, gases, and organic matter. Most sediment outside of it, such as cave sediments, is an understudied world. Caves are natural cavities in rocks that vary in geomorphology. They accumulate all types of sediments, being clastics the most common. Depending on their origin, clastic sediments can be formed autochthonously (inside) or allochthonously (outside). This chapter characterizes cave sediments collected in 15 different caves in Central Portugal. It analyzes color, texture, grain size, pH, calcium carbonate, and organic matter contents. The results show that sediment properties vary significantly between caves, being pH, calcium carbonate, and organic matter the driving variables. These sediments are mostly alkaline, and composed of fine fractions, mainly silts, with a low percentage of organic matter, and a wide range of calcium carbonate percentage. When compared to the surface, these characteristics do not relate in the same way. These variables are relevant for subterranean ecosystems as pH controls microbial dynamics, calcium carbonate is present in crustacean shells, and organic matter is a limited factor for life. Although the abiotic characteristics are similar (absence of light, low-temperature fluctuations, high humidity, and nutrient deficiency), in subterranean ecosystems the geochemistry of the sediments can change the environment leading to changes in the cave ecosystems and affecting their organisms.

**Keywords**: Characterization, Cave sediments, Subterranean ecosystems.

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## 1. Introduction

Soil is the naturally occurring minerals and organic matter at the surface that provides a habitat for organisms, microbiomes, and plants to survive (Voroney, 2007). It interacts with its revolving environments, thus impacting life itself (Voroney, 2007). Microorganisms are at the base of this ecosystem and sustain and support it even alone (Bronick & Lal, 2005). They help by facilitating soil aeration and, through the depletion of organic matter, which makes them rich in nutrients, while the subsoil and everything below are deprived of the same (Brevik et al., 2015).

A cave is a natural cavity in a rock that can vary in geomorphology. Several processes form these, and they can be found in limestones, sandstones, evaporites, ice, dust, and more (Gillieson, 1996). Most caves are formed in limestone, which exists on a larger scale and are larger than others on a size scale (Gillieson, 1996).

Caves accumulate all types of sediments, organic and chemical debris, and their deposits tend to be well-preserved for longer periods. Because of that, cave sediments have a geological, archeological, and paleontological significance (Ford & Williams, 2007).

Sediment accumulation inside caves differs as they have different infrastructure, size, and internal or external geomorphologic relations (Farrand, 2001). They can be divided into clastic, chemical, and anthropogenic sediments (White, 2007; Goldberg & Sherwood, 2006). Clastic sediments form through mechanical movement inside and outside caves, whereas chemical sediments are formed *in situ* and precipitated from solution in seeping, dripping, or flowing water (White, 2007; Goldberg & Sherwood, 2006). Anthropogenic sediments comprise all materials from human activity introduced in a cave (Phillipson & Butzer, 1983). The study of cave sediments enables the reconstruction of the history of cave environments and the characterization of the substrate of specialized subterranean life (Farrand, 2001).

Cave sediments undergo different processes throughout their existence, whether from chemical, biological, or anthropogenic activity. For that reason, sediments can change their physical appearance, color, texture, and structure and their chemical composition, calcification, and phosphatization. These can change their impact on the geological cycle (Goldberg & Sherwood, 2006). On a larger scale, these can also affect environmental changes (Courty & Vallverdú, 2001).

Clastic sediments are the most found in caves (Springer, 2012). The grain size of these sediments can vary depending on their origin and transport mechanisms, ranging from a micrometer colloidal to a one-meter boulder (White, 2007). Clastic cave sediments are subdivided into autochthonous and allochthonous sediments. Autochthonous sediments are formed within the cave and many are comprised of weathering detritus such as sand or limestone fragments, clasts of bedrock derived from movement inside the cave leading to its breakdown into fragments, and guano fecal waste mostly from bats that can accumulate in large quantities inside caves (White, 2007; Goldberg & Sherwood, 2006). On the other hand, allochthonous sediments are formed outside the cave and transported into it. They are composed of a variety of sediments depending on the material available and the rock type. They can be composed of stratified debris in the cave entrance, infiltrated sediments from the surface, stream sediments that are transported into the cave, debris flows that occur after a sediment spill, and aeolian deposits that are transported with the wind into the cave (White, 2007; Goldberg & Sherwood, 2006).

Color analysis is the simpler way to separate sediments. Darker colors are typically richer in organic matter, while lighter colors tend to be richer in calcium carbonate (Busch, 1991).

Textural analysis and grain size distribution permit a better understanding of the type of sediment collected. If the sample has a higher coarse fraction the sediment is mostly composed of sand, but if the sample has a lower coarse fraction the sediment is mostly composed of mud (Flemming, 2000). The mud component can be characterized by quantifying the silts and clay content in each sample.

Calcium carbonate ($CaCO_3$) is common in nature in a pure state, calcite or aragonite, and it is the principal component of limestone. It is well documented that a variety of organisms utilize calcium carbonate as a fundamental component in the construction of their hard external structures, such as exoskeletons and eggshells (Bessler & Rodrigues, 2021).

The pH measurement in sediment can describe if a sample is acidic or basic, but it also provides information about essential nutrients, if available, about toxicity and its impact on the environment and climate via gas emissions (Merl et al., 2022; Thomas, 1996). The pH is a good indicator of bacterial community composition which helps with soil fertility and is an important factor in the biogeochemical cycle in which higher pH levels have shown an increase in microbial activity and bacterial diversity (Merl et al., 2022).

Organic matter is the junction of carbon-based compounds of animal or/and plant origin. It consists of decomposed and mineralized organisms (Plaza & Senesi, 2009). In the elemental and isotopic analysis, the sources of organic matter are determined using the ratio of organic carbon and total nitrogen (C/N) and stable carbon isotopes ($\delta^{13}C$) (Khan et al., 2015). Depending on the $\delta^{13}C$ and C/N composition of a sample, the source of organic matter can vary between terrestrial plants ($C_3$ or $C_4$), bacteria, freshwater algae or macroalgae, marine algae or macroalgae, particulate organic carbon, and dissolved organic carbon (Lamb et al., 2006).

In this chapter, the characteristics of the cave sediments were determined through the analysis of samples from different caves, to understand the variances in geochemical properties of sediments between caves and between karst areas in central Portugal.

## 2. Methods and Materials

### 2.1. Study area and sampling

The sediment samples were collected in 15 caves from four karst areas (Table 1.1). The samples collected came from Cerâmica Cave from Sicó Karst Massif; Almonda, Mira D'Aire, Pinheiro, Ovelha, Ervideira, Morcegos, Salgada, and Alcobertas caves from Estremenho Karst Massif; Alvide, Assafora, and Poço Velho caves from Grande Lisboa Karst area; and Furada, Pedreiras and Utopia caves from Arrábida Karst Massif (Fig. 1.1). In Utopia Cave it was possible the collection of sediment in two different zones inside the same cave. These two samples were designated as Utopia 1 and Utopia 2 and compared separately. Therefore, the number of samples analyzed was 16.

Study area maps were produced in QGIS software (version 3.4.11) using the coordinates (EPSG:4326 - WGS 84) of each cave, geological maps at a scale of 1:50 000 (LNEG, 2024), and Google satellite images. The basic geological formation presented in Table 1.2 was compiled using geological maps for the four karst areas (Sicó, Estremenho, Lisboa, and Arrábida). This table provides a code for the geological period in which the sediment originated and provides a brief description thereof. This description is then augmented with data obtained from the explanatory texts accompanying each geological map.

Samples were collected using gloved hands. A total of 500 g of sediment was put in a plastic bag directed from the cave's ground, and then the bags were sealed and labeled before going to a thermal container. In the laboratory, all the samples were refrigerated until the beginning of the analysis, following Addesso et al. (2022).

Table 1. 1 - Studied caves, with respective coordinates (EPSG:4326 - WGS 84) and karst area in central Portugal.

| Cave | Coordinates | Karst Area |
|---|---|---|
| **Cerâmica** | 39° 55' 37.3" N, 8° 31' 4.4" W | Sicó |
| **Almonda** | 39° 30' 17.10" N, 8° 36' 54.40" W | Estremenho |
| **Mira D'Aire** | 39° 32' 24.89" N, 8° 42' 14.66" W | Estremenho |
| **Pinheiro** | 39° 30' 21.0" N, 8° 36' 59.6" W | Estremenho |
| **Ovelha** | 39° 30' 57.4" N, 8° 42' 19.0" W | Estremenho |
| **Ervideira** | 39° 32' 55.3" N, 8° 55' 23.3" W | Estremenho |
| **Morcegos** | 39° 32' 18.1" N, 8° 45' 21.6" W | Estremenho |
| **Salgada** | 39° 36' 35.0" N, 8° 38' 33.4" W | Estremenho |
| **Alcobertas** | 39° 25' 49.33" N, 8° 54' 57.13" W | Estremenho |
| **Alvide** | 38° 42' 35.60" N, 9° 25' 32.46" W | Lisboa |
| **Assafora** | 38° 54' 31.67" N 9° 25' 18.98" W | Lisboa |
| **Poço Velho** | 38° 42'1.80" N 9° 25' 16.79" W | Lisboa |
| **Furada** | 38° 25' 45.80" N, 9° 10' 28.20" W | Arrábida |
| **Pedreiras** | 38° 27' 11.90" N, 9° 4' 20.60" W | Arrábida |
| **Utopia** | 38° 26' 3.70" N, 9° 8' 50.80" W | Arrábida |

Table 1.2 describes each geological formation that is presented in Fig.1.1. These explanatory texts (Manupella et al., 1978; França & Zbyszewski, 1963; Zbyszewski & Moitinho de Almeida, 1960; Manupella et al., 2000; Ramalho et al., 1993; Ramalho et al., 2001; Manupella et al., 1999) contain information on the lithological nature of the geological formations that could give rise to the soils/sediments. All caves studied are developed in limestone rock formations (Table 1.2). Almonda and Salgada caves developed in formations that can include material described as ‘ferruginous crusts / Sandstones and Clays’, and Ovelha Cave developed on marly limestones with calcium carbonate content varying between 48 and 52% (Manupella et al., 2000). All the caves from the Arrábida Karst Massif form in the same geological formation, dolomitic breccia (Manupella et al., 1999). Assafora Cave develops in a formation where fine sandstones are referent.

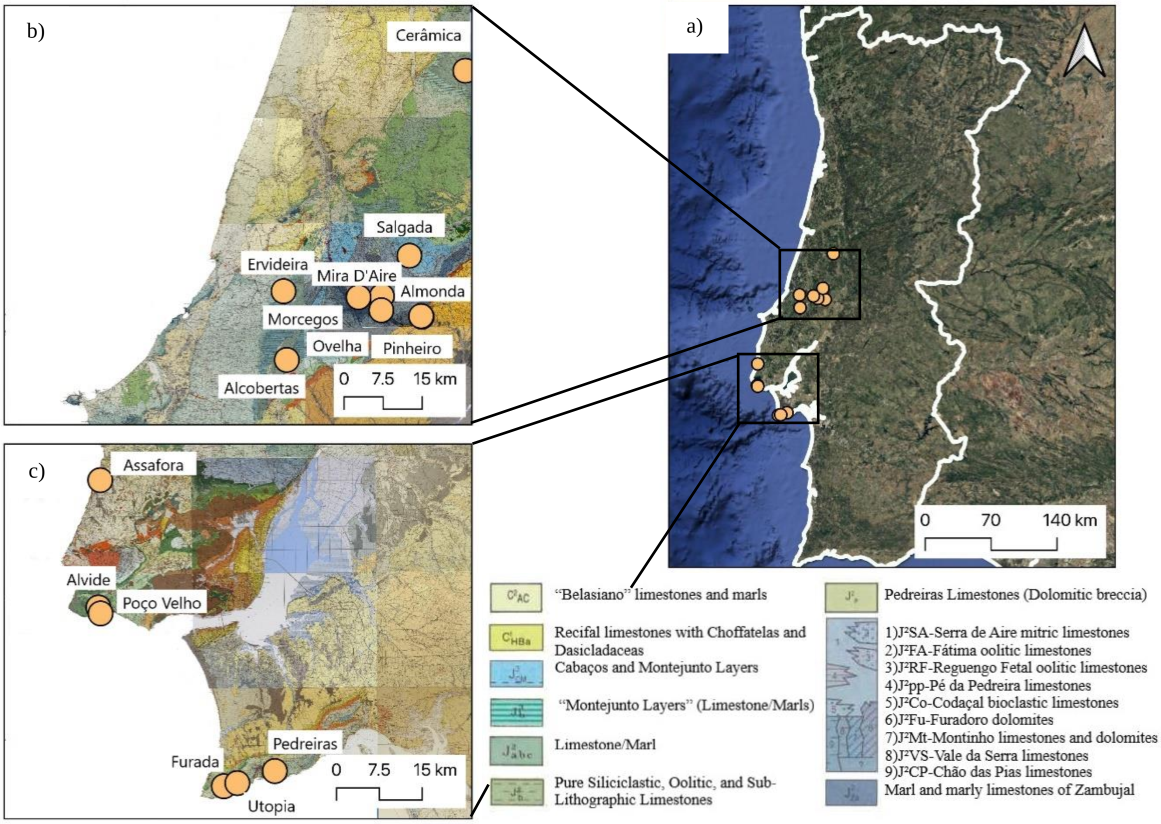


Figure 1. 1 - Location of the caves studied. a) Continental Portugal (scale 1:500 000) obtained from GeoPortal LNEG (LNEG, 2020) and added in QGIS (v. 3.4.11). b) Geological formations of Sicó Karst Massif area (Cerâmica Cave) and Estremenho Karst Massif area (Almonda, Mira D'Aire, Pinheiro, Ovelha, Ervideira, Morcegos, Salgada, and Alcobertas caves). c) Geological formations of Grande Lisboa karst area (Alvide, Assafora, and Poço Velho caves) and Arrábida Karst Massif area (Furada, Pedreiras, and Utopia caves). Geological formation legend relative to the studied karst areas.

Table 1. 2 - Studied caves, code of the geological formations where the caves developed, and a brief description of lithology obtained from a Geological map (1:50 000) downloaded from GeoPortal LNEG (LNEG, 2020). J- Jurassic period. C- Cretaceous period.

| Cave | Geological chart | Geological Code | Description |
|---|---|---|---|
| **Cerâmica** | 23A: Pombal | $J^2b$ | Pure Siliciclastic, Oolitic, and Sub-Lithographic Limestones |
| **Almonda** | 27A: Vila Nova de Ourém | $J^3CM$ | Cabaços and Montejunto Layers (Limestones / Ferruginous Crusts / Sandstones and Clays) |
| **Mira D'Aire** | 27A: Vila Nova de Ourém | $J^2SA$ | Serra de Aire Micritic Limestones |
| **Pinheiro** | 27A: Vila Nova de Ourém | $J^2Mt$ | Montinhoso limestones and dolomites (micritic and calciclastic limestones) |
| **Ovelha** | 27A: Vila Nova de Ourém | $J^2ZA$ | Marl and marly limestones of Zambujal (Carbonates between 48-52%) |
| **Ervideira** | 26B: Alcobaça | $J^3b$ | "Montejunto Layers" (Limestone/Marls) |
| **Morcegos** | 27A: Vila Nova de Ourém | $J^2SA$ | Serra de Aire Micritic Limestones |
| **Salgada** | 27A: Vila Nova de Ourém | $J^3CM$ | Cabaços and Montejunto Layers (Limestones / Ferruginous Crusts / Sandstones and Clays) |
| **Alcobertas** | 26D: Caldas da Rainha | $J^2abc$ | "Caloviano", "Batoniano" and "Bajociano" (Limestone/Marl) |
| **Alvide** | 34C: Cascais | $C^1HBa$ | Recifal limestones with Choffatelas and Dasicladaceas (Limestones, Dolomites and Marls) |
| **Assafora** | 34A: Sintra | $C^2AC$ | "Belasiano" limestones and marls (fine sandstones, clayey marls, and nodular limestones) |
| **Poço Velho** | 34C: Cascais | $C^1HBa$ | Recifal limestones with Choffatelas and Dasicladaceas (Limestones, Dolomites and Marls) |
| **Furada** | 38B: Setúbal | $J^2P$ | Pedreiras Limestones (Dolomitic breccia) |
| **Pedreiras** | 38B: Setúbal | $J^2P$ | Pedreiras Limestones (Dolomitic breccia) |
| **Utopia** | 38B: Setúbal | $J^2P$ | Pedreiras Limestones (Dolomitic breccia) |

## 2.2. Parameter Quantification

### 2.2.1. Color

To characterize the sample color, a Munsell chart (Munsell, 1929) was used. The Munsell page with the colors closest to the sample color was selected, the sample was placed on a sheet of paper and the selected page was placed on top of the sample to comparison. Whenever there was a match between the color of the sample and the color on the Munsell page, the reference was recorded. All samples have been analyzed by me to reduce operator error between observations.

### 2.2.2. Texture

The textural analysis was performed with the help of a 63 µm sieve, that separates the fine (mud) fraction (particles smaller than 63 µm), and coarse fraction (particles larger than 63 µm). All the sediment samples must be dried before this analysis using an oven or a freeze dryer, homogenized, and sub-divided to obtain a sub-sample weighing between 30 and 50 g (Gale & Hoare,1991). The sample was then sieved with tap water. Sediments were classified according to Flemming (2000). Flemming (2000) classified the sediments based on their mud content (%), with the textural classes varying from sand (<5%) to mud (>95%) passing through slightly muddy sand (5-25%), muddy sand (25-50%), sandy mud (50-75%) and slightly sandy mud (75-95%).

### 2.2.3. Grain Size Distribution

The fine fraction (<63 µm, silts and clays) was analyzed in a Malvern Mastersizer 2000 laser particle analyzer (Malvern Instruments, 2007) according to Polakowski et al. (2021).

### 2.2.4. Calcium Carbonate

To quantify calcium carbonate content, the volumetric method was performed on grounded samples using an Eijkelkamp calcimeter that measures the CO2 produced by the reaction of sample calcium carbonate with HCl 4 mol in a known sample mass. To know how much weight of a sample is needed for this analysis, a test was performed with 1g of sediment to which a 1ml of HCl (4 mol/L) was added to see the reaction. If there is a lot of effervescence the weight should be between 0.5 to 1 g but if there is none or almost none the weight should be between 3 to 5 g. The first test run is for 2 whites and 2 standards, in which the weight for the whites is zero and the weight for the standards is 0.2000 g and 0.4000 g. The subsequent runs are for the samples (Eijkelkamp, 2021).

### 2.2.5. pH

The pH of the sediment was determined using the potentiometric method, following LNEC specification E 203-1967 (LNEC, 1967). Briefly, to measure the pH 30 g of each sample were required. The sediment was put in 75 ml of boiled distilled water. The pH was measured 3 times using a pH meter WTW 730 and the 3 pH values were recorded after remaining stable for 1 min; in between measurements, the sample must be stirred. The pH value is the arithmetic mean of three measurements that do not significantly differ (differences must be lower than 0.05) and is rounded to the nearest tenth (LNEC, 1967).

### 2.2.6. Organic Matter

#### *2.2.6.1. Calcination Method*

For calcination or Loss on Ignition (LOI) method (Heiri et al., 2001) a total of 2 g of sample was put in a crucible and placed in a muffle furnace at 500 ºC for 2 h. After 2 h, the crucibles were removed and placed into the desiccator for 10-15 min to cool down. The crucibles with the calcinated samples were weighed (Heiri et al., 2001) and the organic matter content was determined by weight difference.

#### *2.2.6.2. Titration Method*

The titration method was used because values obtained by calcination seem to be higher than expected. It followed the LNEC specification E 201-1967 (LNEC E 201, 1967). The range of values for the weight of samples is between 0.2 and 5 g. For these samples, the established weight was around 3 g because their organic matter content was very low. (LNEC E 201, 1967). In an elenmeyer, 10 ml of potassium dichromate and 20 ml of concentrated sulfuric. The flask was stirred for 1 minute and then put to rest for 30 min on a non-conducting surface to allow the organic matter oxidation. A total of 20 ml of distilled water was added, as well as 10 ml of orthophosphoric acid and 1 ml of indicator. (LNEC E 201, 1967). By adding a solution of ferrous sulfate and a solution of potassium dichromate, the sample must go from blue to green, back to blue and green at the end. The final volume was registered with an approximation of 0.05 ml to make the organic matter content calculation. Samples were classified following (Costa, 1991).

#### *2.2.6.3. Isotopic analysis*

For this analysis, samples from 3 caves (Alcobertas, Pinheiro, and Morcegos) from Estremenho karst area were used. Total organic carbon (%Corg), total nitrogen (%N), $\delta^{13}C$ and $\delta^{15}N$ were determined in ground sediment sub-samples after removal of inorganic carbon using HCl 10%. Samples were processed at *Servizos de Apoio a Investigacion*, University of A Coruña, Spain. Samples were homogenized and weighed in tin capsules; capsulated samples were analysed with a FlashEA1112 combustion elemental analyser (ThermoFinnigan) coupled on-line with a Delta Plus Finnigan MAT Isotope Ratio Mass Spectrometer, Corg and N are expressed as percentages with respect to the total dry weight of the samples. Carbon isotope ratio is expressed in conventional δ notation: δ13CVPDB [(Rsample =Rstandard)] 1000, where Rsample and Rstandard are the $^{13}C/^{12}C$ isotope ratio of the sample and standard, respectively. The δ13C isotope ratio of samples was determined by comparison with a $CO_2$ reference gas standard (99.996%, δ13C VPDB =-6.317). The $\delta^{13}C$ and C/N values were compared to the literature (Lamb et al., 2006).

### 2.3. Data Analysis

The data was analyzed and processed on R software version 4.2.3 (RStudio Team, 2024). The difference in cave sediments between caves and karst areas was tested across all parameters, $\alpha<0.05$. To normality and homoscedasticity, the Shapiro-Wilk test and a Barlett or Levene test were performed, respectively. One-way analysis of variance (ANOVA) was used to test differences between the quantified parameters. If normality and homoscedasticity were not verified, non-parametric Kruskal-Wallis (KW) tests were performed. Subsequently, *a posteriori* tests were performed, the Tukey HSD test for ANOVA or the Dunn test for Kruskal-Wallis, to assess the differences between karst areas and caves in terms of sediment changes in calcium carbonate, organic matter, pH, and grain size distribution. To understand the difference between analyses and their impact on the samples, a PCA biplot was made to analyze if the cave sediments group together in function of karst areas.

## 3. Results

The samples collected are cave sediments from different karst areas of central Portugal, which could foresee characteristics markedly divergent. Their geology, however, at first look, points to a homogeneity between samples belonging to limestone karst areas. The application of geochemical analysis enabled the identification of differences at multiple levels, including the percentage of calcium carbonate and pH when comparing caves and in the coarse fraction, fine fraction (clay %), and pH when comparing karst areas.

The color analysis by cave showed a wide variety of color types (Table 1.3). In the Estremenho karst area, the prominent color is a type of brown whether it is yellowish, light, or dark. For the other karst areas, Lisboa, Arrábida, and Sicó, there is a dominance of browns, yellows, and reds.

Table 1. 3 - The color code for each sediment cave from the studied karst areas derived from the Munsell table (Munsell, 1929). Sicó karst massif area (purple). Estremenho karst massif area (blue). Grande Lisboa karst area (pink). Arrábida karst massif area (green).

| Cave | Code | Type | Color |
|---|---|---|---|
| **Cerâmica** | 5 YR | 5/8 Yellowish Red | |
| **Almonda** | 7,5 YR | 4/6 Strong Brown | |
| **Mira D'Aire** | 5YR | 6/8 Yellowish Red | |
| **Pinheiro** | 10 YR | 4/6 Dark Yellowish Brown | |
| **Ovelha** | 2,5 YR | 5/3 Light Olive Brown | |
| **Ervideira** | 10 YR | 5/4 Yellowish Brown | |
| **Morcegos** | 2,5 YR | 6/3 Light Yellowish Brown | |
| **Salgada** | 7,5 YR | 6/6 Reddish Yellow | |
| **Alcobertas** | 2,5 YR | 6/8 Light Red | |
| **Alvide** | 5 Y | 8/3 Pale Yellow | |
| **Assafora** | 2,5 Y | 6/4 Light Yellowish Red | |
| **Poço Velho** | 10 YR | 5/4 Yellowish Brown | |
| **Furada** | 10 YR | 7/4 Yellow | |
| **Pedreiras** | 5 YR | 5/8 Yellowish Red | |
| **Utopia 1** | 2,5 Y | 7/3 Pale Yellow | |
| **Utopia 2** | 10 YR | 4/3 Brown | |

In the Estremenho karst area, the caves are classified as sandy mud, with two exceptions, Salgada Cave which is slightly sandy mud, and Alcobertas Cave which is mud. In the Lisboa karst area, the Assafora Cave is the only one classified as sand while the other two are classified as sandy mud. In the Arrábida karst area, Pedreiras and Utopia 1 caves are classified as slightly muddy sand, Utopia 2 Cave as muddy sand, and Furada Cave as sand. In the Sicó karst area, the Cerâmica Cave is classified as sandy mud.

The analysis results (Table 1.4) contain the mean of every analysis, except for the coarse fraction percentage. Each analysis performed on the samples collected was replicated to ensure that no errors were committed, and standard deviations were calculated. Given that the samples were taken from disparate zones, the data was subjected to an interpretative process to facilitate analysis between each karst area and between each cave.

Table 1. 4 - Parameter Quantification Results of Cave Sediments. $CaCO_3$: Calcium Carbonate %; Titration: Organic Matter % (Titration method); Calcination: Organic Matter % (Calcination method); pH; Sand %: Coarse fraction; Silts and Clay %: Fine fraction (±) Standard Deviation.

| Karst Area | Cave | Sand (%) | Silt (%) | Clay (%) | $CaCO_3$ (%) | pH | Titration (%) | Calcination (%) |
|---|---|---|---|---|---|---|---|---|
| **Sicó** | **Cerâmica** | 38.7 | 41.2 | 20.1 | 7.30±0.06 | 7.95±0.008 | 0.52±0.02 | 6.84±0.21 |
| **Estremenho** | **Almonda** | 48.5 | 45.4 | 6.08 | 2.30±0.03 | 7.93±0.008 | 0.11±0.02 | 2.83±0.03 |
| | **Mira D'Aire** | 28.8 | 62.4 | 8.81 | 0.15±0.04 | 7.77±0.01 | 0.10±0.01 | 4.71±0.004 |
| | **Pinheiro** | 46.8 | 43.7 | 9.51 | 24.1±0.19 | 7.82±0.02 | 1.31±0.02 | 8.6±0.02 |
| | **Ovelha** | 46.6 | 42.9 | 10.5 | 14.0±0.96 | 7.84±0.01 | 0.44±0.39 | 7.60±0.56 |
| | **Ervideira** | 29.4 | 56.9 | 10.6 | 2.82±0.02 | 7.59±0.008 | 0.33±0.03 | 6.43±0.04 |
| | **Morcegos** | 33.3 | 56.1 | 10.6 | 14.9±1.41 | 7.60±0.005 | 1.64±0.13 | 9.13±0.05 |
| | **Salgada** | 8.90 | 73.4 | 17.7 | 0.40±0.14 | 7.81±0.01 | 0.14±0.01 | 5.66±0.12 |
| | **Alcobertas** | 2.76 | 63.6 | 33.6 | 1.94±0.22 | 8.12±0.01 | 0.12±0.01 | 9.81±0.19 |
| **Lisboa** | **Alvide** | 41.2 | 42.2 | 16.6 | 64.0±0.32 | 7.95±0.01 | 0.33±0.28 | 3.37±0.31 |
| | **Assafora** | 96.2 | 2.79 | 1.01 | 2.68±0.12 | 8.48±0.02 | 0.05±0.04 | 0.53±0.004 |
| | **Poço Velho** | 29.0 | 55.5 | 15.5 | 22.4±0.13 | 7.96±0.02 | 1.48±0.05 | 6.31±0.08 |
| **Arrábida** | **Furada** | 96.4 | 2.67 | 0.93 | 3.96±0.007 | 8.43±0.03 | 0.03±0.03 | 0.30±0.07 |
| | **Pedreiras** | 88.7 | 7.04 | 4.26 | 0.55±0.002 | 8.02±0.005 | 0.04±0.05 | 1.47±0.09 |
| | **Utopia 1** | 81.4 | 17.7 | 0.92 | 30.5±3.46 | 8.64±0.01 | 0.12±0.05 | 0.24±0.01 |
| | **Utopia 2** | 70.9 | 24.4 | 4.68 | 45.0±9.42 | 8.42±0.02 | 0.28±0.27 | 1.61±0.04 |

Through the grain size distribution analysis, it was possible to characterize samples in terms of their sand, silt, and clay percentages. Sand values are in the order of 30 to 50% in Sicó, Estremenho, and Lisbon except for 2 lower values in Estremenho and one higher in Lisbon. Arrábida karst area has the highest values. The higher values of sand percentage were found in that karst area, with 96.4% in Furada Cave. The minimum value was registered in the Alcobertas Cave with 2.76%. For the silt percentage, there are few significant differences between the karst areas, with two notable exceptions Furada Cave from Arrábida karst area with 2.67% for the minimum value and Pedreiras Cave from Arrábida karst area. The rest of the values do not vary much with the maximum value being 73.4 in Salgada Cave from Estremenho karst area. For the clay percentage, the values are not consistent inside each karst area with a maximum value of 33.6% in Alcobertas Cave from Estremenho karst area and a minimum value of 0.92% in Utopia 1 Cave from Arrábida karst area.

The calcium carbonate percentage in caves varied between 0.15% in Mira D'Aire Cave from Estremenho karst area and 64% in Alvide Cave in Lisboa karst area. Although these values are the minimum and the maximum, there is no consistency in karst areas.

The pH values between caves and karst areas are very similar, with a maximum of 8.64 in Utopia 1 Cave from Arrábida karst area and 7.59 in Ervideira Cave from the Estremenho karst area. In the Arrábida karst area, the values point all to basic sediments, which is normal for limestones.

The organic matter percentage (titration method) in caves is low in every karst area, with a maximum of 1.64% in the Morcegos Cave and a minimum of 0.03% in the Furada Cave. The maximum value,

although low, can be explained, as the name indicates, by the presence of bats who contribute to the presence of organic matter in the cave sediment. The Arrábida karst area is the poorest in terms of organic matter compared to the Estremenho and Lisboa karst areas.
The organic matter percentage (calcination method) in caves varies greatly with a minimum of 0.24% in the Utopia 1 Cave from Arrábida karst area and a maximum of 9.81% in the Alcobertas Cave from Estremenho karst area. It is possible to identify differences between karst areas given that Arrábida karst area has the lowest values of all the karst areas.

The organic carbon results (elemental and isotopic analysis) are provided in the form of C/N ratio and $\delta\ ^{13}C$ (‰). The C/N ratio ranges from 1.8 and 5.9, with the minimum value for Alcobertas Cave in Estremenho karst area and the maximum value for Morcegos Cave in Estremenho karst area. The $\delta\ ^{13}C$ (‰) values range between -22.7 and -25.7, with the maximum value for Alcobertas Cave in Estremenho karst area and the minimum value for Morcegos Cave from Estremenho karst area (Fig. 1.2).

Annex A provides the source of organic matter depending on the ranges established for the different possible sources. The elemental and isotopic values for three samples (Pinheiro, Alcobertas, and Morcegos caves) point to the same source, bacteria.

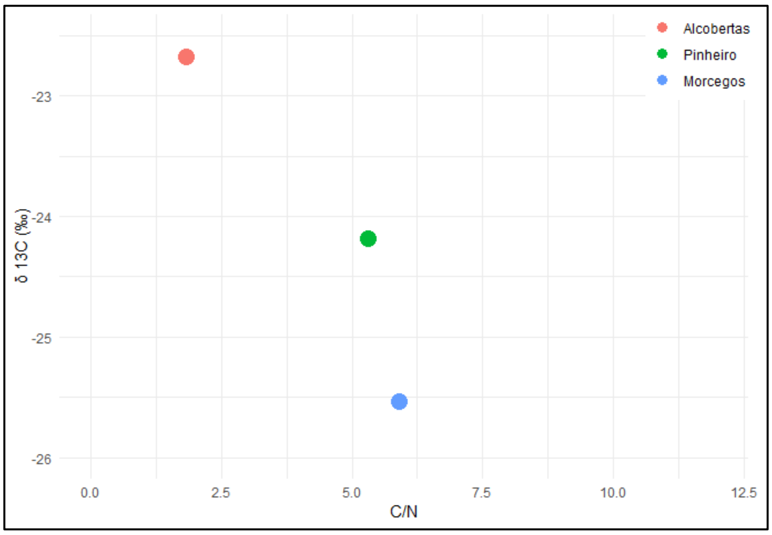


Figure 1. 2 - Comparison of $\delta^{13}C$ (‰) and C/N ranges for organic input between Alcobertas, Pinheiro, and Morcegos caves.

### 3.1. Comparison of Cave Sediment Properties

There is a significant difference between sediments from studied caves in terms of pH, calcium carbonate percentage, and organic matter percentage (calcination method) (Fig. 1.3, Table 1.5). However, there is no significant difference between caves for the organic matter percentage for the titration method analysis (Fig. 1.4; Table 1.5).

Cave sediments were significantly different in terms of pH (Table 1.6, Fig. 1.3a), calcium carbonate (Table 1.7, Fig. 1.3b), and organic matter in the calcination method (Table 1.8, Fig. 1.3c).

PCA biplot (Fig. 1.5) shows calcium carbonate strongly associated with Alvide Cave, pH associated with Arrábida karst area and Assafora Cave, organic matter percentage strongly associated with caves with bat colonies Morcegos, Pinheiro, and Ovelha caves, and Poço Velho Cave which is located bellow residential areas. Estremenho caves, and Sicó cluster away from driving variables.

Table 1. 5 - Comparison of cave sediment properties. p-value table for the normality test, and Kruskal-Wallis test for each analysis by cave. $CaCO_3$: Calcium Carbonate percentage; OM (Titration): Organic matter percentage by the titration method; OM (Calcination): Organic matter percentage by the calcination method. (*) value with a significant difference ($\alpha<0.050$).

| Analysis | Normality | Kruskal-Wallis |
|---|---|---|
| **$CaCO_3$** | < 0.001 | 0.009* |
| **pH** | < 0.001 | < 0.001* |
| **OM (titration)** | < 0.001 | 0.093 |
| **OM (calcination)** | 0.021 | 0.009* |

Table 1. 6 - p-value table for the Dunn test with only significantly different cave pairs for the calcium carbonate percentage analysis ($\alpha<0.025$).

| Cave | Alvide | Mira D'Aire | Pinheiro | Salgada | Utopia 1 | Utopia 2 |
|---|---|---|---|---|---|---|
| **Alcobertas** | 0.005 | - | - | | 0.017 | 0.010 |
| **Almonda** | 0.010 | - | - | - | - | 0.017 |
| **Assafora** | 0.017 | - | - | - | - | - |
| **Mira D'Aire** | 0.0007 | - | - | - | 0.003 | 0.001 |
| **Morcegos** | - | 0.019 | - | - | - | - |
| **Ovelha** | - | 0.024 | - | - | - | - |
| **Pedreiras** | 0.003 | - | 0.017 | - | 0.010 | 0.005 |
| **Pinheiro** | - | 0.005 | - | - | - | - |
| **Poço Velho** | - | 0.010 | - | 0.017 | - | - |
| **Salgada** | 0.001 | - | 0.010 | - | 0.005 | 0.003 |

Table 1. 7 - p-value table for the Dunn test with only significantly different cave pairs for the pH analysis ($\alpha<0.025$).

| Cave | Alcobertas | Assaforra | Ervideira | Furada | Pedreiras | Utopia 1 | Utopia 2 |
|---|---|---|---|---|---|---|---|
| **Alcobertas** | | | 0.002 | | | - | - |
| **Almonda** | - | 0.021 | - | - | - | 0.010 | - |
| **Alvide** | - | - | 0.024 | - | - | - | - |
| **Assafora** | - | - | 0.0002 | - | - | - | - |
| **Furada** | - | - | 0.0006 | - | - | - | - |
| **Mira D'Aire** | 0.009 | 0.0009 | - | 0.003 | 0.019 | 0.0003 | 0.003 |
| **Morcegos** | 0.004 | 0.0003 | - | 0.0009 | 0.007 | 0.0001 | 0.001 |
| **Ovelha** | | 0.007 | - | 0.017 | | 0.003 | 0.020 |
| **Pedreiras** | - | - | 0.005 | - | - | - | - |
| **Pinheiro** | | 0.004 | - | 0.010 | | 0.002 | 0.0012 |
| **Poço Velho** | - | - | 0.017 | - | - | - | - |
| **Salgada** | | 0.003 | - | 0.009 | | 0.001 | 0.011 |
| **Utopia 1** | - | - | 0.0001 | - | - | - | - |
| **Utopia 2** | - | - | 0.0007 | - | - | - | - |

Table 1. 8 - p-value table for the Dunn test with only significantly different cave pairs for the organic matter percentage analysis by the calcination method (α<0.025).

| Cave | Alcobertas | Assafora | Furada | Morcegos | Pinheiro | Utopia 1 |
|---|---|---|---|---|---|---|
| **Almonda** | 0.017 | - | - | - | - | - |
| **Assafora** | 0.001 | - | - | - | - | - |
| **Cerâmica** | - | - | 0.011 | - | - | - |
| **Ervideira** | - | 0.003 | 0.020 | - | - | - |
| **Furada** | 0.001 | - | - | - | - | - |
| **Morcegos** | - | 0.002 | 0.002 | - | - | - |
| **Ovelha** | - | 0.008 | 0.007 | - | - | 0.018 |
| **Pedreiras** | 0.010 | - | - | 0.017 | - | - |
| **Pinheiro** | - | 0.004 | 0.004 | - | - | - |
| **Poço velho** | 0.005 | - | - | 0.010 | 0.010 | - |
| **Utopia 1** | 0.003 | - | - | 0.005 | 0.017 | - |

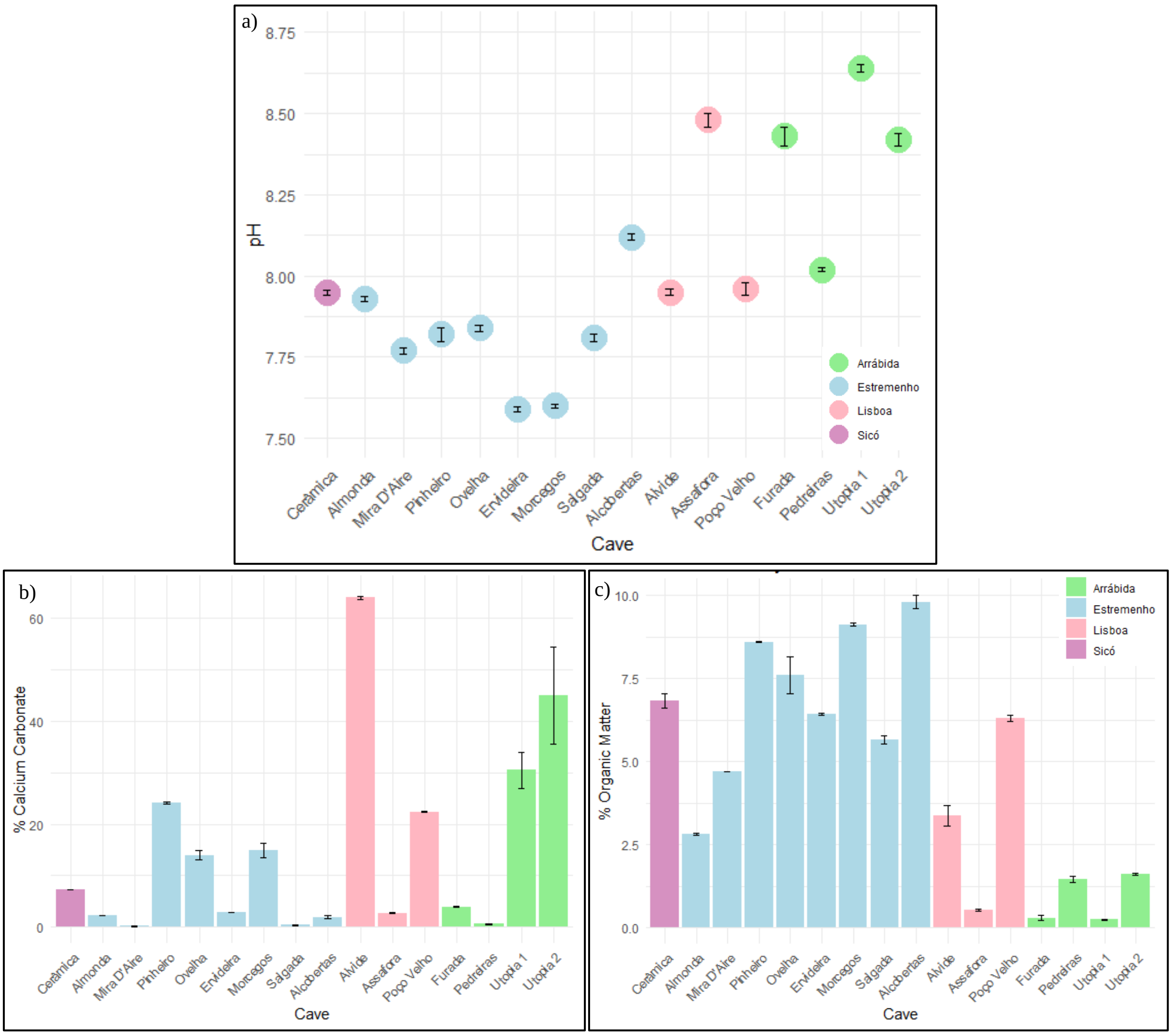


Figure 1. 3 - Comparison of parameters between cave sediments. a) pH; b) calcium carbonate percentage; c) organic matter percentage (calcination method).

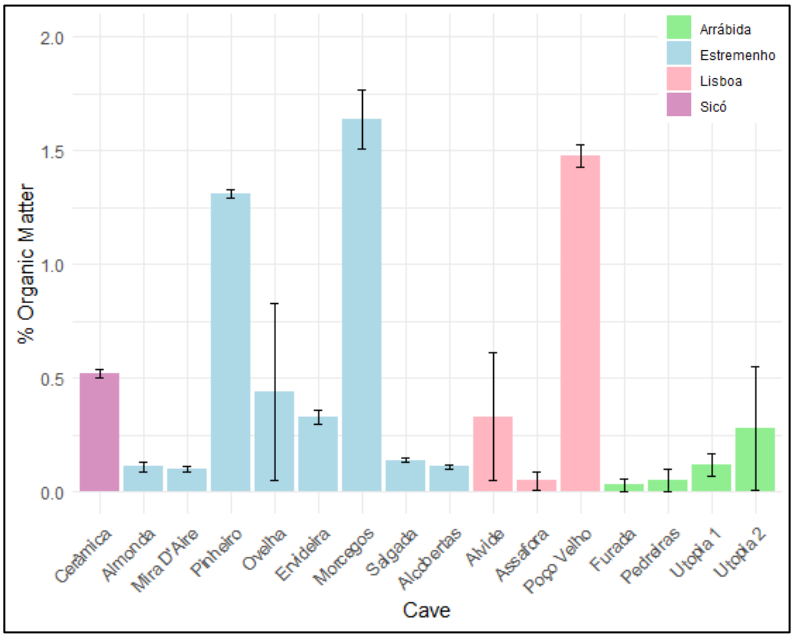


Figure 1. 5 – Organic matter percentage (titration method) from sediments between caves.

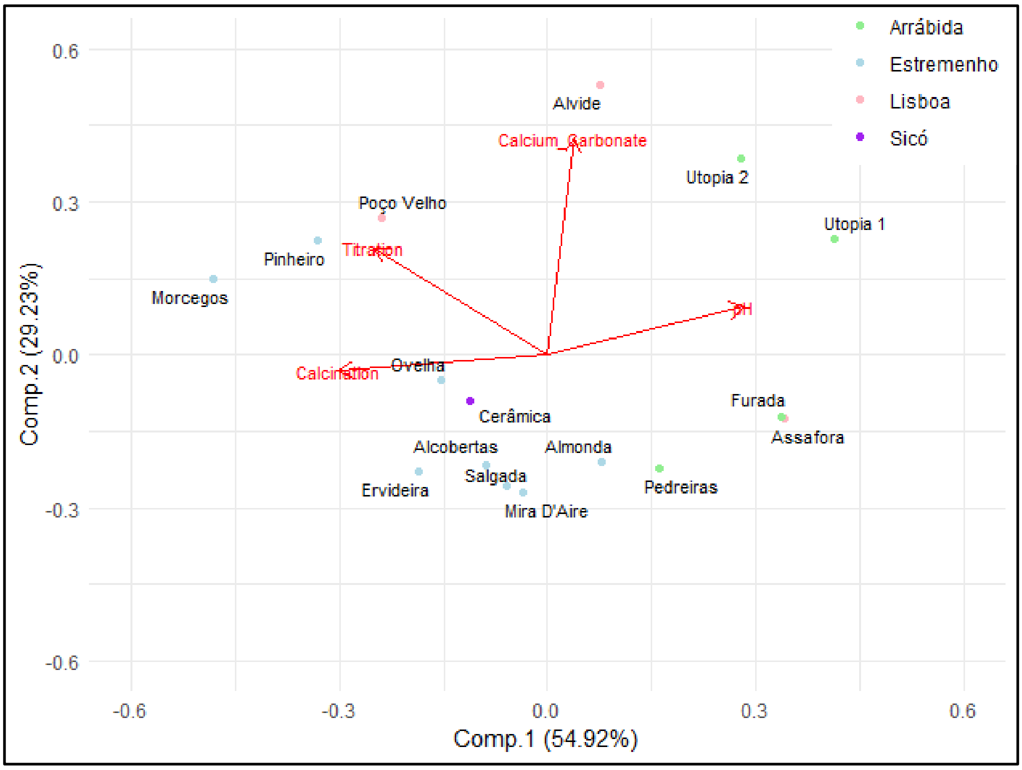


Figure 1. 4 - Principal component analysis (PCA) biplot comparing the analysis performed (calcium carbonate, pH, and organic matter-titration and calcination method) between cave sediments, and its karst area.

### 3.2. Comparison of Cave Sediment Properties Between Karst Areas

A significant difference in cave sediments between Estremenho and Arrábida karst area was found for the coarse fraction percentage (sand %), silts %, pH, and organic matter percentage by the calcination method (Table 1.9; Table 1.10; Fig. 1.6, Fig. 1.7). No significant difference was found between cave sediments in karst areas in clay %, calcium carbonate %, and organic matter % by the titration method (Table 1.9, Fig. 1.8).

Table 1. 9 - Comparison of cave sediment properties between karst areas (Sicó, Estremenho, Lisboa, and Arrábida). P value table for the normality test, homoscedasticity test, ANOVA test, or Kruskal-Wallis test for each analysis performed. $CaCO_3$: Calcium Carbonate percentage; OM (Titration): Organic matter percentage by the titration method; OM (Calcination): Organic matter percentage by the calcination method. Sand: Coarse fraction percentage; Silts and Clay: Fine fraction percentage (*) value with a significant difference (α<0.05).

| Analysis | Normality | Homoscedasticity | ANOVA | Kruskal-Wallis |
|---|---|---|---|---|
| **Sand** | 0.208 | 0.492 | 0.008* | - |
| **Silts** | 0.145 | 0.389 | 0.004* | - |
| **Clay** | 0.078 | 0.819 | 0.130 | - |
| **$CaCO_3$** | 0.002 | - | - | 0.438 |
| **pH** | 0.128 | 0.833 | 0.009* | - |
| **OM (titration)** | < 0.001 | - | - | 0.304 |
| **OM (calcination)** | 0.233 | 0.188 | 0.005* | - |

Table 1. 10 - Comparison of significantly different karst areas (Sicó, Estremenho, Lisboa, and Arrábida). P value table for the Tuckey's HSD test for each analysis with significant differences. OM (Calcination): Organic matter percentage by the calcination method. Sand: Coarse fraction percentage; Silts: Fine fraction percentage. (α<0.05).

| Analysis | Estremenho-Sicó | Lisboa-Sicó | Arrábida -Sicó | Lisboa-Estremenho | Arrábida-Estremenho | Arrábida-Lisboa |
|---|---|---|---|---|---|---|
| **Sand** | 0.982 | 0.891 | 0.242 | 0.322 | 0.004* | 0.299 |
| **Silts** | 0.798 | 0.968 | 0.363 | 0.179 | 0.003* | 0.313 |
| **pH** | 0.932 | 0.894 | 0.354 | 0.198 | 0.006* | 0.489 |
| **OM (calcination)** | 1 | 0.551 | 0.128 | 0.151 | 0.004* | 0.475 |

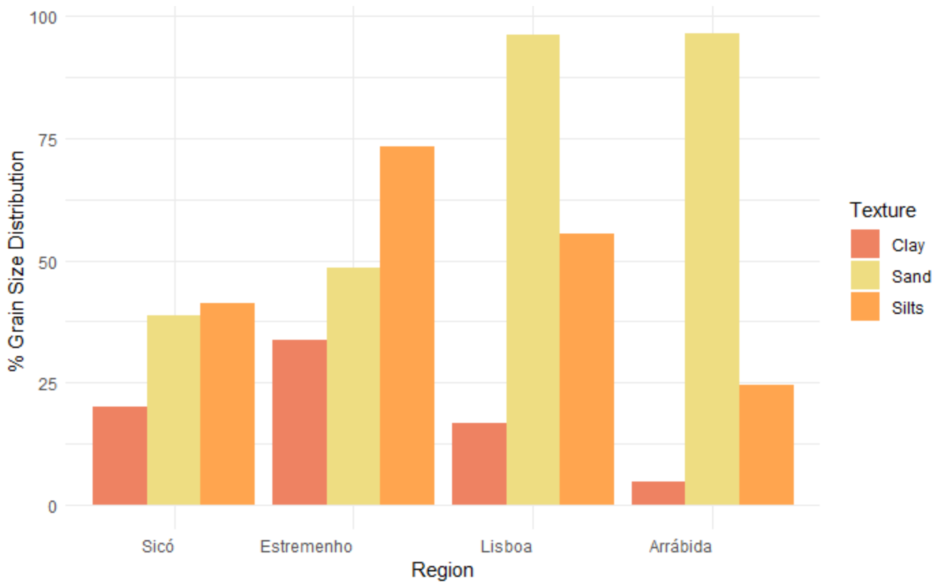


Figure 1. 6 - Grain size distribution of cave sediments (sand, silts, and clay percentage) between karst area (Sicó, Estremenho, Lisboa, and Arrábida).

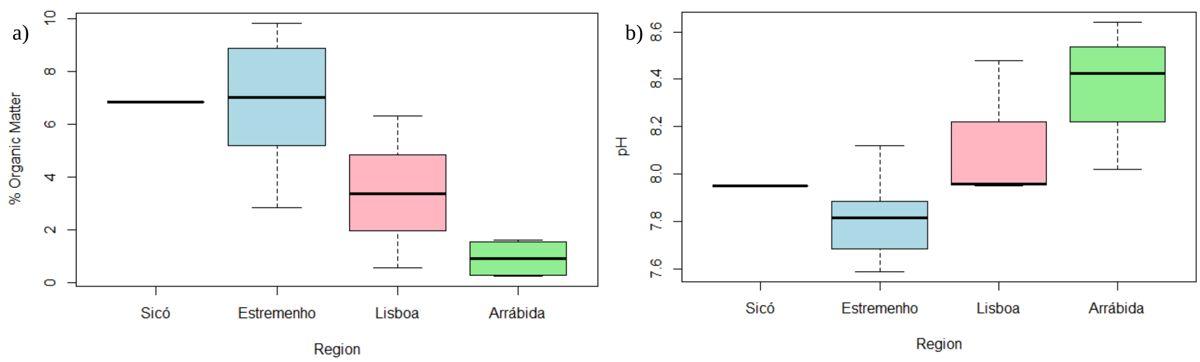


Figure 1. 7 - Boxplots with significant differences between karst areas. a) Organic matter percentage (calcination method) from cave sediments between karst areas (Sicó, Estremenho, Lisboa, and Arrábida). b), and pH from cave sediments between karst areas (Sicó, Estremenho, Lisboa, and Arrábida).

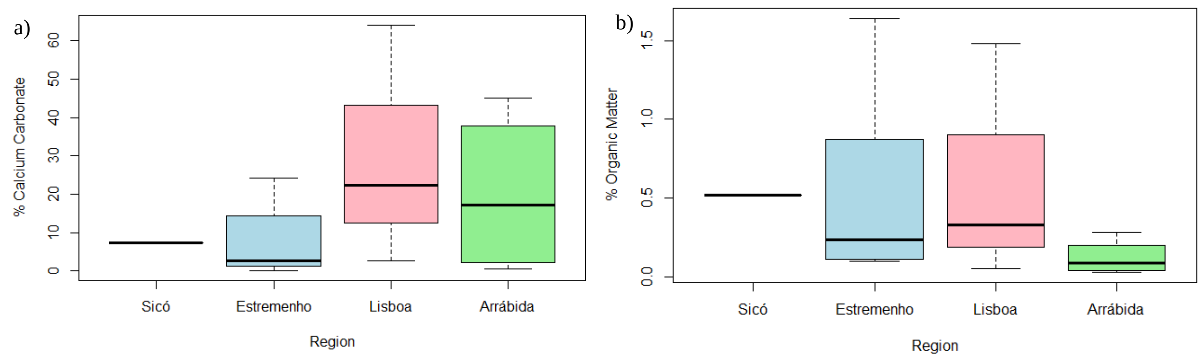


Figure 1. 8 - Boxplots with no significant differences between karst areas. a) Calcium carbonate percentage from cave sediments between karst areas (Sicó, Estremenho, Lisboa, and Arrábida). b) Organic matter percentage (titration method) from cave sediments between karst areas (Sicó, Estremenho, Lisboa, and Arrábida).

## 4. Discussion

The cave sediments from Central Portugal preliminarily characterized in this study are alkaline, composed mainly of fine fractions (clay and silts), have a low organic matter %, and have a wide range of $CaCO_3$ values. These characteristics are commonly unrelated, and the most prevalent combination at the surface is fine soils with alkaline pH are more likely to have a high $CaCO_3$ content and higher organic matter percentage (Bronick & Lal, 2005; Akça et al., 2005; Voroney, 2007).

The calcium carbonate and organic matter contents in sediments at the surface are directly associated with its color. Therefore, high concentrations of organic matter produce an increase in color darkness, and high concentrations of calcium carbonate produce a decrease in color darkness (Busch, 1991). The cave sediment sample with a lighter color is from Alvide Cave in Grande Lisboa karst area with 64% $CaCO_3$, which positively correlates with values known from soil samples (Busch, 1991). However, when analyzing cave sediment samples with darker colors, Almonda and Pinheiro caves from Estremenho karst area, the results obtained are inconsistent with the studies done at the surface (Busch, 1991). This can be explained by the nutrient scarcity in subterranean habitats, expressed in low organic matter content (Bodawatta et al., 2023).

Cave sediment samples were mainly classified as sandy mud, except for the Arrábida karst area, in which the sand content is higher up to 50-75%, i.e., higher composition of fine fractions with clay and silts as components. Silt-rich sediment systems are mainly estuaries and rivers, types of ecosystems with high biodiversity and nutrient rates (Slaa et al., 2013; Attrill et al., 1996). Clay soils are present in fields, crop sites, or near seawater and can retain water and nutrients, making a greater quantity available to organisms (Samuel et al., 2023; Bardgett, 2005). Their presence in cave sediments is necessary to assist with nutrient retention, including organic matter, which are essential sediment properties but very scarce (Alakukku et al., 2009; Culver & Pipan, 2019).

Calcium carbonate is present in all the samples collected. It varies between 0.15% and 64%, implying differences present in the cave sediments. This inorganic salt is present in exoskeletons, eggshells, and coral reefs (Bessler & Rodrigues, 2008) but it also accumulates in cliffs, artic soils, beneath glaciers, or precipitates in ocean waters (Ford & Williams, 2007). Carbonate sands are frequently found in beaches and deltas but in this case, the Arrábida karst area samples must have carbonate precipitation from the cave itself (Ford & Williams, 2007; Freitas, pers. com.). Their presence in sediment not only enables a higher advantage for organisms' survival and their increase in population numbers but also influences the pH, leading to values around 8 as is the case with the collected samples (Bressler & Rodrigues, 2008; Akça et al., 2005).

The pH values of the studied cave sediments, ranged between 7.6 and 8.5, which according to the Pratolongo scale (Costa, 1991), are classified as subalkaline. These values are favorable to microbe growth (Bardgett, 2005) and affect soil solubility and the ionization of inorganic and organic constituents present within the soil solution (Voroney, 2007). Soil pH, at the surface, has an impact on vegetal growth and clay dispersion. These fine particles of sediment tend to react when there is an increase in pH, the particles create a repulsion between them (Bronick & Lal, 2005). The pH values in the karst areas are in pair with other pH studies in caves (Bodawatta et al. 2023; De Paula et al., 2020) since sediments tend to be alkaline.

The organic matter was measured by two methods, calcination and titration. In the calcination method, the organic matter is measured by weight loss after the samples are heated (Heiri et al., 2001). The values ranged between 0.24%-9.13%, giving the impression of high differences between samples. However, this method does not consider the presence of iron oxide resulting in skewed values (Heiri et al., 2001). Because of this, the organic matter percentage by the calcination method is discarded because it overestimated the real organic matter content. The performance of the titration method consists of oxidizing organic matter of chromic acid in the presence of sulphuric acid. This is followed by chromic acid titration with a solution of ammonium ferrous sulfate (Costa, 1991). This leads to organic matter values more consistent with those expected and they range between 0.03%-1.64%. Organic matter content depends on chemical composition and impacts soil structure ability, prevents its erosion, and is efficient in retaining water. Since the samples collected have low values of organic matter, the quantity of carbon (C) and nutrients available are going to impact negatively on the biota presence in subterranean habitats (Bardgett, 2005).

Significant differences were registered in pH and calcium carbonate percentage when comparing caves and in silts, sand, and pH when comparing karst areas. The caves of Alvide, Mira D'Aire, and Utopia 2 demonstrate several significant differences from others, hinting at distinct characteristics influencing the $CaCO_3$. While the Ervideira Cave shows differences in pH with multiple other caves (i.e., Alcobertas, Alvide, Assafora, Furada, and Poço Velho), indicating it may have a distinct pH environment relative to others. The significant differences in pH between caves suggest possible variations in geological composition affecting the alkalinity in these environments. A significant distinction between Ervideira and Utopia 1 caves, indicates that the presence of $CaCO_3$ may have an impact on the pH values (Akça et al., 2005). The significant differences between karst areas were registered between Estremenho and Arrábida for the three analyses. This points to higher values of sand

and pH in Arrábida karst area and higher values of silts in Estremenho karst area, resulting in opposite environments.

Cave sediments are variable, therefore understanding their composition and dynamics is relevant as they are the substrate to subterranean life, a reservoir of unique microbiomes that are a source of organic matter and nutrients to a wide diversity of endemic organisms (Culver & Pipan, 2019). Microbiomes are highly dependent on sediment parameters such as pH and nutrient availability (Reboleira et al., 2022; Bodawatta et al. 2023), while diverse cave animals living in these caves are known to use and process sediments in their biological processes (Reboleira et al., 2015; Reboleira & Enghoff, 2016). This study is a preliminary characterization of cave sediments parameters. Further research should include increasing the diversity of sites within each cave, across gradients of depth and distance to surface entrances, and investigating also the presence of phosphate and nitrates that influence organisms (Levin & Crooks, 2011; Reboleira et al., 2022).

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# Chapter II - Ecological role of terrestrial cave-adapted isopods in bioturbation and nutrient redistribution in subterranean sediments


## Abstract

A healthy ecosystem provides services that benefit humankind. Subterranean ecosystems differ from the surface in their abiotic characteristics, including the absence of light, high humidity, low-temperature amplitude, and nutrient deficiency. Consequently, subterranean organisms underwent morpho-physiological adaptations, giving them a rare and unique status with great conservation importance. The cave-adapted Portuguese endemic species *Miktoniscus longispina* Reboleira & Taiti, 2015 (Oniscidea: Trichoniscidae) was observed processing cave sediments, constructing tunnels and molting shelters, involving the mobilization of organic matter into the sediment. This chapter evaluates its impact on processing and mobilizing organic matter in subterranean sediments, and studied different nutritional conditions for two months, which included behavioral observations. Organic matter and calcium carbonate content, pH, texture, and grain size distribution were studied at the sediment level. Individuals of *M. longispina* built tunnels and shelters in cave sediments using their mandibles, maxillipeds, and front legs (pereopods). They tended to construct them by incorporating available organic vegetable matter, sediment, and fecal pellets of digested sediment. The biological activity of *M. longispina* leads to a significant increase in organic matter in the cave sediment. This shows that terrestrial cave-adapted isopods provide bioturbation, a vital ecosystem service in subterranean ecosystems leading to better nutrient distribution in sediments and better aeration, contributing to potentially higher microbial activity. This study provides the first insight into the relevance of ecosystem services provided by terrestrial cave habitats, which is essential to deepen our understanding of these habitats, the unique species they host, and the critical services they provide globally, which are often overlooked.



**Keywords**: Subterranean ecosystem, Organic matter process, Bioturbation.



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## 1. Introduction

Under the surface, there is a vast domain of subterranean ecosystems. Cave habitats are part of the subterranean ecosystem and can be formed into distinct types of rocks, the most common ones developed by the dissolution of calcite or dolomite (Culver & White, 2005). Caves can vary from large and deep down to more than 2000 meters below the surface, and they can be small and shallow (Sendra & Reboleira, 2012). These can be classified as superficial subterranean habitats, deep caves, or meso and macro fissures (Moldovan et al., 2018). A cave can be considered a natural opening on a rock, with an area bigger than a few millimeters, and total darkness. They can be found on all continents except in Antarctica and as well the various countries and their islands on the continents (Culver & Pipan, 2019). Caves share ecological features such as the absence of light, high humidity, low-temperature variability, and scarcity of nutrients, hosting exclusive and highly specialized fauna that differ between them by their origin, inherited traits, organizational degree, and time since colonization (Moldovan et al., 2018; Saccò et al., 2024).

Subterranean species evolved with strong convergent environment pressure, developing specific adaptations, called troglomorphisms, that can be divided into physiological, morphological, and behavioral (Trajano et al., 2016). Morphological adaptations include the loss of vision and pigment, the reduction of wings and cuticles, and the elongation of the body, legs, and antennae (Moldovan et al., 2018). Physiological adaptations include low metabolic rate, longer lifespan, low thermal acclimation, and reduced fertility (Hornung, 2011; Di Lorenzo & Reboleira, 2022). Behavioral adaptations include loss of circadian cycle and changes in mating strategies (Hornung, 2011; Moldovan et al., 2018).

Subterranean organisms can be classified depending on their occurrence in the underground and are divided into three categories according to Trajano (2012). Species that are exclusive to the subterranean domain, are not found on the surface, and have adaptations exclusive to this environment, are known as troglobites for terrestrial or stygobites for aquatic. Species that travel between the surface and the underground on a regular basis are known as troglophiles or stygophiles. And last, species that are exclusive to the surface and occur in the subterranean in an accidental way are known as trogloxenes or stygoxenes (Moldovan et al., 2018). Troglobitic species have a K strategy, meaning that they invest in fewer eggs, but more resources per egg, and great longevity due to the energy saved by their slow metabolism, permitting a higher adult survival rate (Reboleira et al., 2010; Hornung, 2011).

Ecosystem services are the conditions and processes provided by the species that live in that ecosystem (Daily, 1997; Karr, 1999). They sustain human life, are free of cost, maintain biodiversity, and contribute to the production of services and goods. In addition to services, ecosystems contribute life-support functions such as cleaning, recycling, and renewal, as well as cultural and aesthetic benefits (Daily, 1997). According to Van Gestel et al. (2018), we can divide ecosystem services and functions into four categories: 1) provisional services include the production of materials such as sustenance; 2) supporting services that provide habitat and maintain biodiversity, 3) regulation services that appear in the form of regulating agents, and 4) cultural services which are benefits provided in the form of mental and physical well-being. In a subterranean ecosystem, the services and functions provided are 1) the formation of sediment through the retention and accumulation of organic matter, 2) water purification, water supply, natural attenuation, and bioturbation, 3) water storage, energy, drought and flood buffering, refuge, and water balance, and 4) sport, tourism, thermal spas, and religion/spiritualism (Castaño-Sanchez et al., 2020; Millennium Ecosystem Assessment, 2005).

Oniscidea is a suborder of crustaceans with great biodiversity (Hornung, 2011). They can be found in various ecological niches, ranging from supralittoral zones to sea level, mountains, and caves (Hornung, 2011). Oniscidea plays a crucial role in the decomposition of organic matter in sediment, contributing to biogeochemical cycles on Earth (Hornung, 2011). Therefore, these organisms can serve as bioindicators of sediment quality (Van Gestel et al., 2018). In subterranean ecosystems, they play a crucial role as detritivores, occupying an elemental position in the food chain of caves. Troglobiont terrestrial isopods serve as a source of food for predators and are typically associated with stable, and undisturbed areas (Reboleira et al., 2015). Terrestrial isopods have a capillary conduction system that allows them to extract water from the environment to keep the branchia wet, and even to excrete and reuse water from urine, evaporating nitrogenous waste in the form of gaseous ammonia before reabsorbing water (Hornung, 2011). Other peculiarities of these organisms are that they consume oxygen mainly through their abdominal appendages, they seek shelter to avoid predation and/or to molt, their dietary choices favor quality over quantity and their fertility rate is very low such as the number of descendants (Hornung, 2011).

Terrestrial isopods are considered beneficial organisms because they recycle nutrients by fragmenting decomposing plant material and contribute to the decomposition of detritus directly through consumption and assimilation, or indirectly by fragmenting and increasing the surface area available for the colonization of saprotrophic microbiota (Marín & Tiunov, 2023; Waller & Verdi, 2017). They are also considered bioindicators because they are sensitive to the responses of different environmental variables, can accumulate heavy metals through feeding, and can survive in areas polluted by industrial waste, making them potential bioindicators of sediment quality (Waller & Verdi, 2017). Therefore, they have been widely used to determine sediment quality by conducting ecotoxicity tests (Van Gestel et al., 2018).

Mainland Portugal is one of the subterranean biodiversity hotspots in the circum-Mediterranean region (Reboleira et al., 2022). The region is home to several species adapted to caves, with the most diverse being the terrestrial isopods. There are fifteen troglobitic species of terrestrial isopods, with the Trichoniscidae family being the most diverse, comprising twelve species (Reboleira et al., 2015). Previous observations of specimens of cave-adapted trichoniscids endemic to Portugal kept in the laboratory showed that they build shelters and tunnels in the sediment and potentially incorporate processed organic matter into the sediment (Reboleira, pers. com.). This chapter aims to understand the bioturbation made by an endemic species of cave-adapted trichoniscid, how it processes natural sediments from caves, and if it mobilizes organic matter into the sediments. Bioturbation is an important ecosystem service, so far unknown in terrestrial cave habitats.

## 2. Materials and methods

### 2.1. Model Species and study area

Specimens of the terrestrial isopod *Miktoniscus longispina* Reboleira & Taiti, 2015, were collected in Cerâmica Cave, Sicó karst massif (39°55'37.3' N; 8°31'4.4' W) in January and April of 2024. Organisms were collected using brushes and are stored in vials, under the appropriate license provided by the Institute for Nature Conservation and Forests (ICNF). Simultaneously, cave sediment was collected *in situ* and transported to the laboratory.

*Miktoniscus longispina* (Fig. 2.1) is a troglobiont terrestrial isopod of the family Trichoniscidae, endemic to caves of central Portugal, in Sicó and Cesaredas karst areas with a maximum size of 3.5mm (Reboleira et al., 2015).

Figure 2. 1 - The troglobiont terrestrial isopod *Miktoniscus longispina* Reboleira & Taiti, 2015, endemic from caves of Portugal.

The Sicó massif is situated in central-western Portugal, approximately five km South of Coimbra, and encompasses an area of 400 km² (Cunha et al., 2020). The Serra de Sicó massif reaches an elevation of approximately 560 meters and is part of the Serra de Sicó inter-municipal association, which encompasses the municipalities of Alvaiázere, Ansião, Condeixa-a-Nova, Penela, Pombal and Soure (Matos & Fonseca, 2019). The massif represents one of the carbonate landscapes of the Lusitanian basin, comprising a series of dolomitic hills, depressions, mountains, and limestone plateaus. These elements collectively delineate a distinctive regional morphological unit elevated to the surrounding plain (Cunha et al., 2020). The climate of the Sicó massif is typically Mediterranean, with dry summers and rainy winters, and an average annual temperature of 15.6°C. As this area is relatively undisturbed, it supports a diverse range of dominant natural vegetation, including meadows, rupicolas communities, scrubland, woodlands, and ruderal communities (Matos & Fonseca, 2019). The Sicó massif is home to approximately 300 caves, exhibiting a range of typologies, dimensions, and speleogenetic attributes. These caves are predominantly concentrated within the limestone mountains and plateaus of the northwestern block of the mountain (Cunha et al., 2020).

This study will focus on the sediments of Cerâmica Cave, located in the municipality of Ansião/Pombal near Monte das Barreirinhas (39°55'37.3' N; 8°31'4.4' W), with an elevation of 355 m, a development of 120 m and a depth of 21 m (Nóbrega et al., 1984). The cave is a natural cavity with two entrances in the ceiling. Analysis of ceramic fragments found within the cave indicates a date range of the Bronze Age (Nóbrega et al., 1984). This cave was selected for its exceptional specific richness, which places it at the forefront of caves in the center of Portugal (Reboleira et al., 2015).

### 2.2. Experimental Design

Specimens of *M. longispina* were installed in plastic vials, 120 ml, with the bottom covered with 1 cm of cave sediment and placed in total darkness in a VWR INCU-Line temperature-controlled chamber at 17°C, the cave’s temperature (Medina et al. 2023). Acclimatization to lab conditions lasted 30 days.

#### 2.2.1. Experiment 1

The experiment comprises four groups, two of which included specimens of *M. longispina*. Consequently, 50 organisms were gathered so that each group could have five replicates and five organisms per vial. Before the sediment went into the vials, it was determined that 20 g of sediment per vial was necessary to construct the experiment. From the cave sediment collected, 400 g of sediment was weighed and then sterilized in a Memmert autoclave at 120°C for 20 minutes to eliminate any contamination present. The group experiment was designed in the following form (Fig. 2.2):

Group 1: Sediment + Organisms + Organic Matter (*Quercus* spp leaves)

Group 2: Sediment + Organisms

Group 3: Sediment + Organic Matter (*Quercus* spp leaves)

Group 4: Sediment (Control)

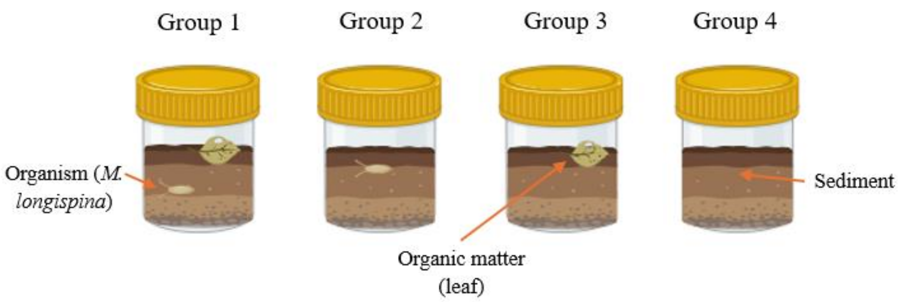


Figure 2. 2 - Experimental Design for Experiment 1. Group 1: Sediment + Organisms + Organic Matter (Quercus spp leaves). Group 2: Sediment + Organisms. Group 3 Sediment + Organic Matter (*Quercus spp* leaves). Group 4: Sediment (Control).

The vials were assembled in the VWR INCU-Line temperature-controlled chamber at 17°C, the cave's temperature, and the experiment was conducted lightless. After one month each week, a group was selected to transfer the organisms visible to another vial with sediment and *Quercus* spp leaves, and the vials with sediment were put in the freezer.

#### 2.2.2. Experiment 2

For the second experiment, a total of 15 live specimens of *M. longispina* were placed per vial. The increase in individuals was to attempt to understand whether this increase would also be registered in the organic matter incorporated into the sediment by the organisms. This experiment had a total of 3 replicas for each group, where group 1 was constituted by sediment, organisms, and organic matter while group 2 was constituted by only sediment, the control group (Fig. 2.3). Food in the form of roots that pend from the cave ceiling was administrated *ad libidum* to the vials with *M. longispina*. The amount of sediment present in this experiment was the same as the previous one, with the only difference being this one was not sterilized. This decision was made with the intention of preserving the quality of sediment.

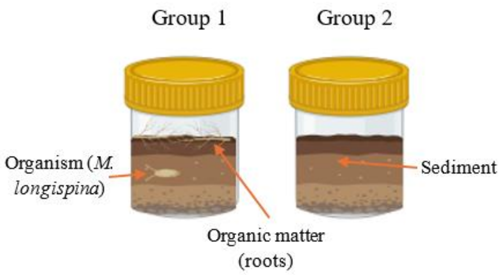


Figure 2. 3 - Experimental Design for Experiment 2. Group 1: Sediment + Organisms + Organic Matter. Group 2: Sediment (Control)

The vials were put together in the VWR INCU-Line temperature-controlled chamber at 17°C, the cave's temperature, and the experiment was conducted lightless, except for the observations made in the stereomicroscope.

### 2.3. Behavioral observation

During the first month of the experiment, data was collected with observations made of the organisms in a stereomicroscope Leica E24. Observations were made every 2 days, and it was registered the number of organisms present in each vial, the number of shelters and tunnels constructed, and whether the organisms consumed the organic matter present (leaves and fine roots). Every 48 hours, water was added to each vial, to the leaves in group 1 (experiment 1), the sediment in group 2 (experiment 1), and the roots in group 1 (experiment 2).
Observations were made in groups 3 and 4 (experiment 1), and group 2 (experiment 2) mainly to report any changes in the sediment and leaves.

### 2.4. Sediment quantification

The frozen sediment from the experiments was transferred to the geology laboratory where it underwent chemical and physical analysis to help characterize its type. The analysis included color, texture, grain size, calcium carbonate (gasometric method), pH, and organic matter percentage (titration method).

#### 2.4.1. Color

For this analysis, a Munsell table is required which has a coded color scheme with holes to compare the colors to the samples. This analysis associates a color from the chart with the sample. A page of the Munsell chart was selected with the colors that resemble the most with the sample. The sample is then placed on top of a white paper and compared with all the colors from that page until one is selected. The code, the page, and the designated color were written down. This process is repeated for all the samples (Munsell, 1929). I analyzed all the samples to decrease the error between them.

#### 2.4.2. Texture

To physically analyze sediments a textural analysis is needed to divide it into two. First, the coarse fraction, called sand, with dimensions superior to 63µm and second, the fine fraction, called silts or clays, with dimensions inferior to 63µm (Flemming, 2000). The samples were dried and divided into samples with 30-50 g (Gale, & Hoare,1991). Water was added to the samples and left to settle down until the next day. The samples were stirred and then poured on top of a 63µm sieve with a wash bucket underneath. The sample on top, coarse fraction, of the sieve was put to dry on a sand bath at 60ºC. The sample on the bottom, fine fraction, was ground and put on a bag. Sediments were classified according to Flemming (2000).

### 2.4.3. Grain Size Distribution

For this analysis, the samples were collected from the textural analysis and performed on the fine fraction (silts and clay) (Polakowski et al., 2021). The samples were analyzed in a mastersizer 2000 laser particle analyzer (Malvern Instruments, 2007) following Polakowski et al. (2021).

### 2.4.4. Calcium carbonate

The volumetric method is used to determine the quantification of calcium carbonate. Before the analysis it is necessary to weigh the sample and ground them. To determine the exact quantity of sample needed it is recommended to add 1ml of HCl to a sample and see its reaction. Depending on the reaction, small or big effervescent, the sample weighs between 0.5 and 5g. The first run is for the whites and patterns, in which the weight for the whites is zero and the weight for the patterns is 0.2000 and 0.4000 for 1 and 2, respectively of $CaCO_3$. The rest are the same for the samples which consist of adding 20ml of distilled water to the erlenmeyer and adding 7ml of HCl (4mol/L) into small flat-bottomed flasks which are put inside the erlenmeyers. (Obtained from the calcimeter manual: Eijkelkamp, 2021).

### 2.4.5. pH

To quantify the pH the potentiometric method was used, following LNEC specification E 203-1967 (LNEC, 1967). For this analysis, a total of 30 g of sample was used. The sediment was disaggregated and then put on a glass flask. 75 ml of boiled distilled water was added to each glass flask and then stirred for a minute. The samples were left overnight to rest (LNEC E 203,1967). For each sample, an electrode of a pH meter WTW 730 was put in suspension. Each sample was measured 3 times and in between measurements, the sample was stirred. The 3 readings, to be registered, were constant by a minute and did not differ over 0.05 units. The pH value is the arithmetic mean of three measurements and was rounded to the nearest tenth (LNEC, 1967).

### 2.4.6. Organic matter

To determine the organic matter, the samples were quantified using the titration method and following the LNEC specification E 201-1967 (LNEC E 201, 1967). The samples were weighed, and depending on their organic matter content, between 0.2 to 5 g. (LNEC E 201, 1967). A total of 10 ml of potassium dichromate and 20 ml of concentrated sulfuric acid was added into an Erlenmeyer flask. Then, 20 ml of distilled water was added, with 10 ml of orthophosphoric acid and 1 ml of indicator (LNEC E 201, 1967). For the final part, the sample is turned from blue to green with a solution of ferrous sulfate, and a solution of potassium dichromate (LNEC E 201, 1967). Following Costa (1991), the samples were classified on their level of organic matter.

## 2.5. Data Analysis

To prove the organic matter process made by the organisms, the data was analyzed and processed on R software version 4.2.3, RStudio, and Jamovi software version 2.3.28 (RStudio Team, 2024; The Jamovi project, 2024). To test the normality ($\alpha<0.10$), the Shapiro-Wilk test and a Levene test for homoscedasticity. For two samples, a t-student test was performed to test differences between quantified parameters. If normality and homoscedasticity were not verified, non-parametric Mann-Whitman tests were performed. For more than two samples, one-way analysis of variance (ANOVA) was used to test for differences between the quantified parameters. If normality and homoscedasticity are not verified, non-parametric Kruskal-Wallis (KW) tests were performed. Subsequently, *a posteriori* tests, Tukey test for parametric distribution or Dunn test for non-parametric distribution, will be performed to assess the differences between groups in the experiment in terms of sediment changes. The tests were performed on the four-group design but to have full comprehension of the difference between groups it is necessary to test between the groups with organisms and organic matter, for group 1, only organisms, for group 2, and only organic matter for group 3, with the control group which only has sediment.

## 3. Results

The troglobiont terrestrial isopod *Miktoniscus longispina* constructs subterranean tunnels and shelters within the sedimentary deposits of its subterranean habitat, after a week in the vials (Fig. 2.4 a). These are built using both the mandibles, maxillipeds, and front legs (pereopods), using sediment available and incorporating fecal pellets and vegetable organic matter provided (Table 2.1). These are also seen *in situ* inside caves (Fig. 2.4 b). They seem to have minimal interaction with other specimens. When no organic matter is provided, they use only the available sediment, incorporating fecal pellets of digested sediment into the constructions (Fig. 2.4 c). Significant differences were found in the percentage of organic matter in both treatments, between groups 1 and 4 in experiment 1 (Table 2.3; Fig. 2.6) and experiment 2 (Table 2.5; Fig. 2.7).

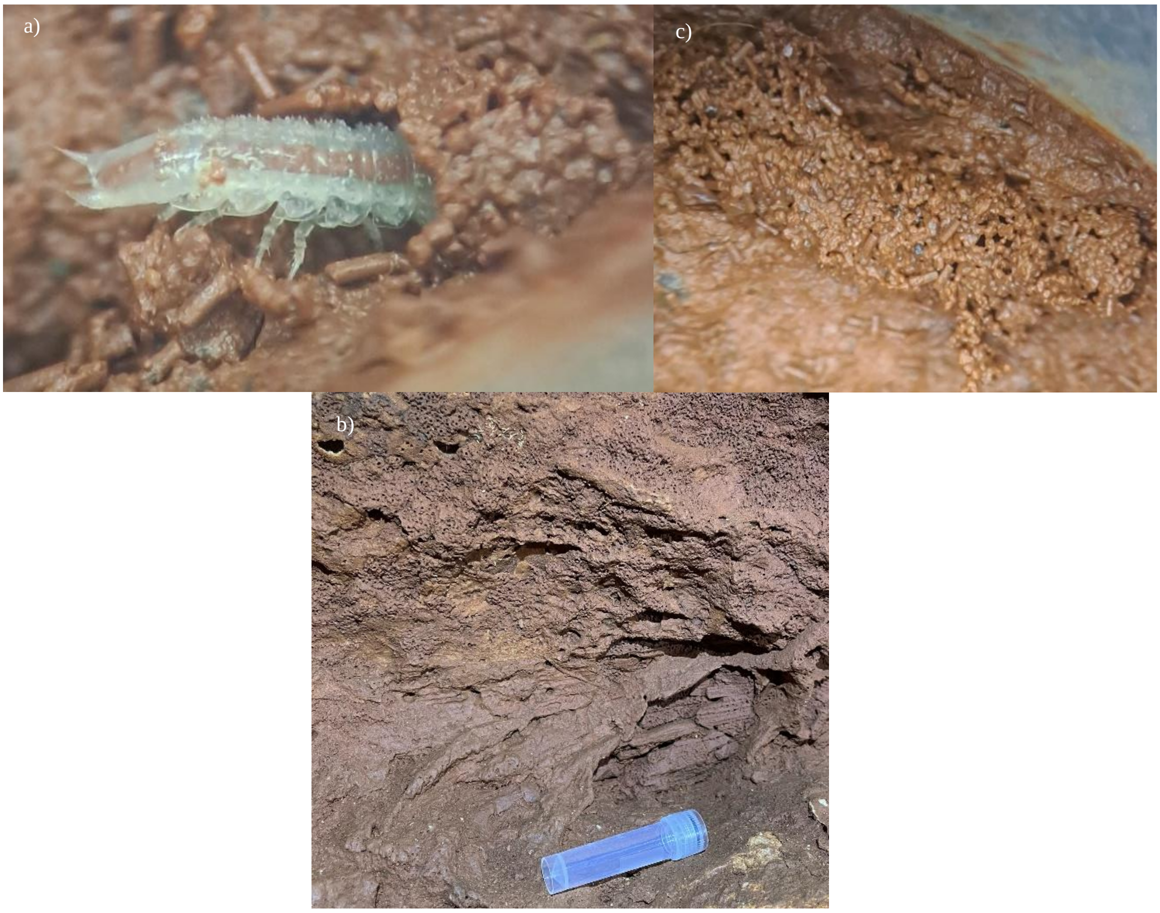


Figure 2. 4 - Behavioral observations a) *Miktoniscus longispina* entering a molt shelter. b) Bioturbation *in situ* inside of a cave. c) Molting shelter constructed by *M. longispina* in cave sediment.

### 3.1. Experiment 1

#### 3.1.1. Behavioral observation

Experiment one was designed with four groups with two of them containing *M. longispina*. The registers (Table 2.1) were made for the first two groups and in each vial individually. In the weekly observations, it was always possible to observe these organisms at the surface, and within the first week, it was observed organisms with the molting made, and the construction of shelters and tunnels in the sediment. These observations were registered during the two months of the experiment.

Table 2. 1 - Weekly behavioral observation with organisms, shelters, and tunnel registers. Group 1: Sediment + Organisms + Organic Matter (Leaves). Group 2: Sediment + Organisms.

| Date | Registers | Observations |
|---|---|---|
| **7 days** | Group 1: 19 organisms, 7 shelters, 6 tunnels<br><br>Group 2: 9 organisms, 19 shelters, 11 tunnels | Shelter construction with the mandible and front legs<br><br>Minimal interaction between organisms |
| **14 days** | Group 1: 9 organisms, 16 shelters, 6 tunnels<br><br>Group 2: 10 organisms, 23 shelters, 11 tunnels | Guidance through their antenna<br><br>Reduced movement in group 2 |
| **21 days** | Group 1: 13 organisms, 20 shelters, 6 tunnels<br><br>Group 2: 12 organisms, 25 shelters, 11 tunnels | Construction of shelters with available sediment and fecal pellets |
| **28 days** | Group 1: 18 organisms, 23 shelters, 7 tunnels<br><br>Group 2: 14 organisms, 19 shelters, 17 tunnels | More shelters than tunnels in both groups<br><br>Leaves were fed along their veins |
| **35 days** | Group 1: 11 organisms, 19 shelters, 5 tunnels<br><br>Group 2: 7 organisms, 20 shelters, 12 tunnels | Withdrawal of organisms from vial number 1 of each group and freeze the sediment<br><br>Group 1: 2 organisms (leaves removed)<br><br>Group 2: 3 organisms |
| **42 days** | Group 1: 9 organisms, 17 shelters, 3 tunnels<br><br>Group 2: 9 organisms, 16 shelters, 13 tunnels | Withdrawal of organisms from vial number 2 of each group and freeze the sediment<br><br>Group 1: 1 organism (removal of leaves)<br><br>Group 2: 1 organism |
| **49 days** | Group 1: 4 organisms, 8 shelters, 1 tunnel<br><br>Group 2: 4 organisms, 9 shelters, 11 tunnels | Withdrawal of organisms from vial number 3 of each group to freeze the sediment<br><br>Group 1: 3 organisms (leaves removed)<br><br>Group 2: 3 organisms |
| **56 days** | Group 1: 3 organisms, 4 shelters, 0 tunnels<br><br>Group 2: 1 organism, 5 shelters, 8 tunnels | Withdrawal of organisms from vial number 4 and freezing of the sediment<br><br>Group 1: 3 organisms (removal of leaves with fungi)<br><br>Group 2: 5 organisms |

| | | |
|---|---|---|
| **63 days** | *No observations were made except an organism count from their withdrawal | Withdrawal of the organisms from vial number 5 of each group and freezing them<br><br>Group 1: 3 organisms (removal of leaves)<br><br>Group 2: 2 organisms |

Between groups 1 and 2, it was observed that there was visible higher movement within the first group. The number of shelters and tunnels had a minimal variation between groups, but the number of shelters was always higher than the number of tunnels.

After the first month, organisms were withdrawn in a vial of each group every week until there was no vial left. It was registered which vial (number) and how many organisms were possible to withdraw before freezing the sediment (Table 2.1).

Groups 3 and 4 were observed in terms of sediment and leaf change. These two groups did not register any changes until the third week when a white tread was observed in the leaves of group 3 and sediment of group 4, which was then identified as a fungus. The fungus also appeared in the first two groups and was monitored until the end of the experiment, and its presence did not affect the organisms in terms of mortality.

### 3.1.2. Sediment Quantification Results

Table 2.2 corresponds to the sediment quantification results for experiment 1, which includes the percentage of calcium carbonate, organic matter, pH, and grain size distribution with the coarse fraction (sand) and the percentage of fine fraction (silts and clay).

Table 2. 2 - Sediment Quantification Results table for experiment 1. 1. Sed+Org+Org Mat: group 1 (sediment + organisms + organic matter). 2. Sed+Org: group 2 (sediment + organisms). 3.Sed+Org Mat: group 3 (sediment + organic matter). 4. Sed (Control): group 4 (control group with sediment). Cerâmica Cave (Sicó karst Massif): purple color.

| Group | Calcium Carbonate (%) | Organic Matter (%) | pH | Coarse Fraction: Sand (%) | Fine Fraction: Silts (%) | Fine Fraction: Clay (%) |
|---|---|---|---|---|---|---|
| 1.Sed+Org+Org Mat | 2.08 | 0.56 | 8.19 | 26.8 | 44.8 | 28.4 |
| | 1.89 | 0.48 | 8.14 | 27.1 | 44.1 | 28.8 |
| | 1.60 | 0.36 | 8.19 | 27.1 | 44.1 | 28.8 |
| | 1.73 | 0.54 | 8.18 | 27.8 | 43.4 | 28.8 |
| | 1.23 | 0.52 | 8.18 | 28.1 | 45.4 | 26.5 |
| 2.Sed+Org | 1.62 | 0.40 | 8.17 | 27.1 | 44.3 | 27.8 |
| | 1.67 | 0.48 | 8.21 | 27.9 | 44.5 | 27.6 |
| | 1.35 | 0.45 | 8.17 | 26.7 | 46.4 | 26.9 |
| | 1.18 | 0.45 | 8.10 | 27.7 | 45.7 | 26.6 |
| | 1.64 | 0.46 | 8.25 | 26.7 | 45.2 | 28.1 |
| 3.Sed+Org Mat | 1.71 | 0.51 | 8.17 | 27.2 | 44.1 | 28.8 |
| | 1.48 | 0.39 | 8.20 | 24.9 | 46.7 | 28.4 |
| | 1.54 | 0.38 | 8.17 | 27.2 | 43.7 | 29.1 |
| | 1.55 | 0.47 | 8.13 | 27.2 | 45.6 | 27.2 |
| | 1.41 | 0.46 | 8.23 | 27.4 | 46.2 | 26.4 |
| 4.Sed (Control) | 1.60 | 0.45 | 8.18 | 26.9 | 45.3 | 27.8 |
| | 1.76 | 0.45 | 8.18 | 27.1 | 46.2 | 26.7 |
| | 1.67 | 0.38 | 8.25 | 27.5 | 45.2 | 27.3 |
| | 1.60 | 0.34 | 8.16 | 26.8 | 45.7 | 27.5 |
| | 1.68 | 0.31 | 8.17 | 26.9 | 46.3 | 26.8 |

The calcium carbonate percentage was low between the groups of the Cerâmica Cave sediment. The maximum registered value was 2.08 % in group 1 (sediment + organism + organic matter). For the minimum value, it was registered in group 2 (sediment + organism) with 1.18%.
The organic matter analysis demonstrated results with a “very low” classification (Costa, 1991). There are still differences observed in the groups, with the maximum value, 0.56%, being from group 1 (sediment + organism + organic matter) and the minimum value, 0.31%, being from group 4 (sediment + control). This can mean organic matter was indeed processed but to confirm this it is necessary to process the data in terms of the significance difference between groups.
The pH values by group of the Cerâmica Cave differed between 8.10 and 8.25, the last one being registered both in group 2 (sediment + organism) and group 4 (sediment-control). These values conform to the values registered in limestones, basic values up to 9.9.
The grain size distribution analysis consists of the division of coarse fraction (sand %) and fine fraction (silts % and clay %). Both fractions are consistent within the groups. For the percentage of sand, the maximum value of 28.1% is from group 1 (sediment + organisms + organic matter) and the minimum value of 24.9% is from group 3 (sediment + organic matter). For the silt percentage, the maximum value of 46.7 % is from group 3 (sediment + organic matter), and the minimum value of 43.4 % is from group 1 (sediment + organisms + organic matter). For the clay percentage, the maximum value of 29.1 % is from group 3 (sediment + organic matter), and the minimum value of 26.4 % is from group 3 (sediment + organic matter).

### 3.1.3. Data Analysis Results

There was a significant increase in organic matter content in the sediment in the vials with sediment + organisms + organic matter (group 1), due to the biological activity of *Miktoniscus longispina* (Fig 2.5), when compared to the control (group 4), which only had sediment. Nevertheless, there were no significant differences in organic matter percentage when comparing all together the four groups (Fig. 2.5, Table 2.3).

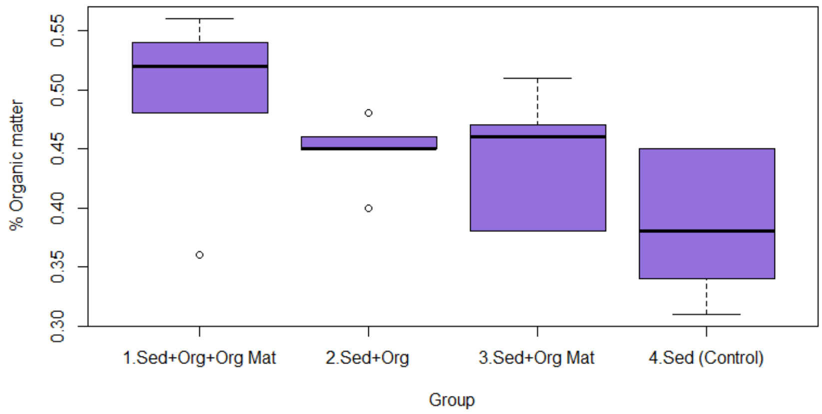


Figure 2. 5 - Organic matter percentage between groups. Sed+Org+Org Mat: group 1 (sediment + organisms + organic matter). Sed+Org: group 2 (sediment + organisms). Sed+Org Mat: group 3 (sediment + organic matter). Sed (Control): group 4 (control group with sediment).

Table 2. 3 - Comparison of organic matter percentage in cave sediment between groups. P-value table for the normality test, homoscedasticity test, and T-student test in group 1 (sediment + organisms + organic matter), group 2 (sediment + organisms), and group 3 (sediment + organic matter) when compared to group 4 (control group with sediment). (*) value with a significant difference (α>0.05).

| Groups | Normality Test | Homoscedasticity Test | T student |
|---|---|---|---|
| **Group 1-Group 4** | 0.233 | 0.798 | 0.048* |
| **Group 2-Group 4** | 0.610 | 0.069 | 0.083 |
| **Group 3-Group 4** | 0.144 | 0.843 | 0.197 |

## 3.2. Experiment 2

### 3.2.1. Behavioral observation

Like in the first experiment, biological activity was observed within the first week of experiment 2, including the construction of shelters and tunnels (table 2.4). These shelters were equally made with both the mandible and front legs (pereopods). The shelters were made from the sediment available and fecal pellets. Once more, it was observed that these organisms guided themselves through their antennae and had minimal interaction between organisms. The construction of tunnels at the surface level was almost nonexistent and the possibility of the tunnels being located under the branches and/or shelters is high because its presence was observed at the bottom of the flasks.

Table 2. 4 - Weekly behavioral observation with organisms, shelters, and tunnel registers. Group 1: Sediment + Organisms + Organic Matter (Branches).

| Date | Registers | Observations |
|---|---|---|
| **7 days** | Group 1: 14 organisms, 12 shelters, 0 tunnels | Shelter construction with the mandible and front legs<br><br>Minimal interaction between organisms |
| **14 days** | Group 1: 12 organisms, 14 shelters, 0 tunnels | Guidance through their antenna |
| **21 days** | Group 1: 18 organisms, 15 shelters, 0 tunnels | Construction of shelters with available sediment and fecal pellets |
| **28 days** | Group 1: 24 organisms, 11 shelters, 0 tunnels | More shelters than tunnels<br><br>Feeding: bark of the branches |
| **35 days** | Group 1: 23 organisms, 11 shelters, 0 tunnels | Constant observation of molting |
| **42 days** | Group 1: 17 organisms, 12 shelters, 0 tunnels | Shelters built under branches |
| **49 days** | Group 1: 19 organisms, 10 shelters, 2 tunnels | Small distinction between flasks |
| **56 days** | Group 1: 18 organisms, 12 shelters, 1 tunnel | Observation of high energy in organisms |
| **63 days** | Group 1: 25 organisms | Withdrawal of organisms from Group 1 flasks and freezing them<br><br>Group 2: freezing |

Since there was a higher number of organisms by vial, 15, there was a constant observation of molting taking place every week in the vials. The organisms behaved similarly in all 3 vials of the group leading to almost no distinctions between them.

At the 2-month mark, group 1 and group 2 were frozen without the organisms, they were withdrawn and counted. Group 2 was observed in terms of sediment and branch change. This group did not register any changes until the end of the experiment.

### 3.2.2. Sediment Quantification Results

Table 2.5 corresponds to the sediment quantification results for experiment 2, which includes the percentage of calcium carbonate, organic matter, pH, and grain size distribution with the coarse fraction (sand) and the fine fraction (silts and clay).

Table 2. 5 - Sediment Quantification Results table for experiment 2. Sed+Org+Org Mat: group 1 (sediment + organisms + organic matter). Sed+Org: group 2 (sediment + organisms). Sed+Org Mat: group 3 (sediment + organic matter). Sed (Control): group 4 (control group with sediment). Cerâmica Cave (Sicó karst Massif): purple color.

| Group | Calcium Carbonate (%) | Organic Matter (%) | pH | Coarse Fraction: Sand (%) | Fine Fraction: Silts (%) | Fine Fraction: Clay (%) |
|---|---|---|---|---|---|---|
| 1.Sed+Org+Org Mat | 4.81 | 0.65 | 7.83 | 45.1 | 36.4 | 18.5 |
| | 5.54 | 0.65 | 7.75 | 43.8 | 37.2 | 19.0 |
| | 5.75 | 0.61 | 7.75 | 48.9 | 33.8 | 17.2 |
| 2.Sed (Control) | 4.09 | 0.34 | 7.83 | 47.1 | 34.3 | 18.6 |
| | 4.80 | 0.51 | 7.78 | 45.3 | 36.7 | 18.8 |
| | 5.55 | 0.54 | 7.75 | 47.0 | 35.3 | 17.7 |

The calcium carbonate percentage was inconsistent between the groups of the Cerâmica cave sediment. The maximum value registered was 5.75% in group 1 (sediment + organisms + organic matter). For the minimum value, it was registered in group 2 (sediment-control) with 4.09%.

The organic matter analysis demonstrated results with a “very low” classification (Costa, 1991). There are still differences observed in the groups, with the maximum value, 0.65%, being from group 1 (sediment + organism + organic matter) and the minimum value, 0.34%, being from group 2 (sediment-control). This can mean organic matter was indeed processed but to confirm this it is necessary to process the data in terms of the significance difference between groups.

The pH values by group of the Cerâmica Cave differed between 7.75 and 7.83, both being registered in both groups. These values conform to the values registered in limestones, basic values up to 9.9.

The grain size distribution analysis consists of the division of coarse fraction (sand %) and fine fraction (silts % and clay %). Both fractions are consistent within the groups. For the percentage of sand, the maximum value of 48.9% is from group 1 (sediment + organisms + organic matter) and the minimum value of 43.8% is from group 1 (sediment + organisms + organic matter). For the silt percentage, the maximum value of 37.2 % is from group 1 (sediment + organisms + organic matter), and the minimum value of 33.8 % is from group 1 (sediment + organisms + organic matter). For the clay percentage, the maximum value of 19.0% is from group 1 (sediment + organisms + organic matter), and the minimum value of 17.2 % is from group 1 (sediment + organisms + organic matter).

#### 3.2.3. Data Analysis Results

There was a significant difference in organic matter percentage between group 1 (sediment + organisms + organic matter) and group 2 (control group) (Fig. 2.6), due to the biological activity of *M. longispina*.

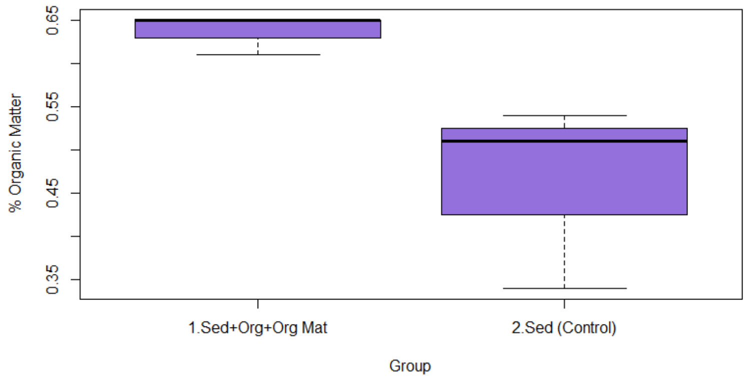


Figure 2. 6 - Organic matter percentage between groups. 1. Sed+Org+Org Mat: group 1 (sediment + organisms + organic matter). 2. Sed (Control): group 2 (sediment + organisms).

## 4. Discussion

The study shows that *Miktoniscus longispina* constructs tunnels and shelters in cave sediments using their mandibles, maxillipeds, and front legs (pereopods). Depending on availability, the species builds shelters using organic matter, sediment, and fecal pellets, incorporating available organic matter and own fecal pellets of digested sediment in the constructions. Its biological activity contributed significantly to the increase in organic matter % in cave sediments. With larger numbers in the population, shelter and tunnel numbers increase. Extensive areas of this processed cave sediments can be observed *in situ*, in the caves where the species is distributed, and there is evidence of extensive lab and field bioturbation processes promoted by this species.

Other species are known to process and use cave sediments for molting shelters in caves of Portugal, as the millipede *Lusitanipus alternans* (Reboleira & Enghoff, 2016), and other trichoniscids have been observed to build the same kind of tunnels (Reboleira pers. Com.). Previous research in a Brazilian Cave in the Bahia State with the cave-adapted terrestrial isopod species *Iuiuniscus iuiuensis* Souza, Ferreira & Sena, 2015, showed that they also built shelters, but *M. longispina* individuals show minimal interaction between individuals of the same species, contrary to *I. iuiuensis* which cohabitates with individuals of the same species and with one other species (Souza et al., 2015). Shelter-building behavior in caves has been related to avoid dissection and/or predation (Souza et al., 2015; Reboleira & Enghoff, 2016).

Bioturbation can be defined as the biological activity in soils and sediments to access resources such as nutrients and water (Ruiz et al., 2023), impacts the sediment through particle reworking, sediment transportation, and physical alterations to sedimentary material resulting from fecal pellets production, and tunnel construction (Shull, 2009). Those physical alterations can appear in various forms such as grain size, porosity, and permeability, which can impact sediments' formation, stability, and erosion (Shull, 2009; Mazik & Elliott, 2000). Since the organisms are observed feeding sediment, it was expected to see some change in their grain size distribution but like sediment-feeding groundwater invertebrates (Hose & Stumpp, 2019), the sediment redistribution by excretion does not seem to change sediment parameters.

Bioturbation activity in caves has been studied in the aquatic compartments, inducing changes in the sediment and in the biological communities inside the cave. It also induces an increase in biodiversity that typically inhabits oceanic waters (Brito et al., 2009).

Bioturbation is an ecosystem service that changes organic matter distribution and nutrient availability in sediments, leading to a higher abundance of bacteria composition which then leads to higher microbial production (Shull, 2019). Soil aeration is enhanced by the distribution of oxidants in sediments, including oxygen, nitrates, and iron. It also leads to the reduction of nitrates, and sulfates, and contributes to ammonium oxidation (Levin & Crooks, 2011). Among other things, root growth in length is prompted which provides an increase in vegetable organic matter available for organisms (Kumar et al., 2004). The role of terrestrial cave-adapted communities in bioturbation, the relevance of its contribution to global ecosystem services and its contribution to global health of soils remains to be understood.

This chapter shows that terrestrial cave-adapted isopods endemic from caves of Central Portugal, provide bioturbation, a vital ecosystem service in subterranean ecosystems leading to better nutrient distribution in sediments and better aeration, contributing to potentially higher microbial activity. This study provides the first insight into the relevance of ecosystem services provided by terrestrial cave habitats, which is essential to deepen our understanding of these habitats, the unique species they host, and the critical services they provide globally, which are often overlooked.

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**Final Remarks**

The cave sediment samples analyzed in this study from 15 caves in central Portugal are characterized as alkaline, composed of mainly silts, have a low organic matter content, and a wide variety of $CaCO_3$ content.

Significant differences were found between caves and between karst areas, having commonly pH as a driving variable in both comparisons. Estremenho and Arrábida were the karst areas with significant differences between them, the former having a higher silt content, whereas the latter had a higher pH and sand content.

The terrestrial cave-adapted species *Miktoniscus longispina* constructs tunnels and molt shelters in cave sediments. The specimens use their maxillipeds, mandibles, and front legs to process the sediment and incorporate available organic matter (plant leaves and fine roots), and fecal pellets into the constructions. This species promotes sediment aeration and increases the organic matter content in the cave sediments. The bioturbation resulting from its biological activity is classified as an ecosystem service. Furthermore, bioturbation helps the distribution of organic matter, nutrients, and oxidants and enhances microbial production, root growth is prompted, increasing organic matter availability in soils.

Cave sediments are fundamental to subterranean fauna, possess unique microbiomes, and are a source of organic matter and nutrients to all organisms. Further research should focus on deeper understanding of the role of bioturbation and its impact on soil health.

# A. Annex

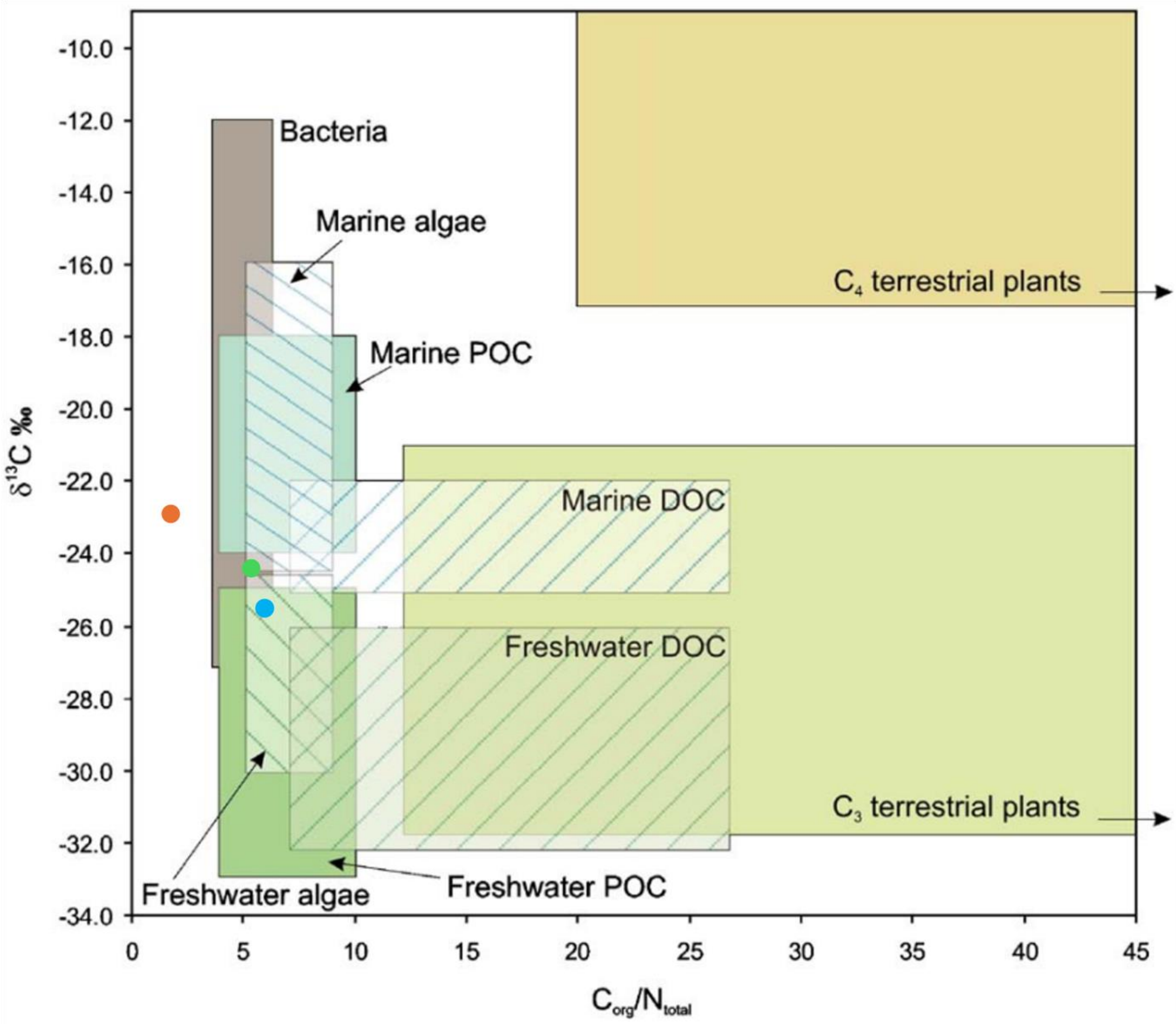


Figure A. 1 – Typical $\delta^{13}C$ (‰) and C/N ranges for organic inputs to coastal environments (Lamb et al., 2006).