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DOUGLAS ALEXANDRE

FRAMEWORK FOR ADVANCING IN ENCHYTRAEID ECOTOXICOLOGY:
USE OF COMMUNITY TESTS AND SPECIES SENSIBILITY DISTRIBUTION

LAGES
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Tese apresentada como requisito parcial para obtenção do título de doutor em Ciência do Solo pelo Programa de Pós-Graduação em Ciência do Solo do Centro de Ciências Agroveterinárias – CAV, da Universidade do Estado de Santa Catarina – UDESC.
Orientador: Prof. Dr. Osmar Klauberg Filho

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BANCA EXAMINADORA

Orientador:

Dr. Osmar Klauberg Filho
UDESC – CAV

Membros:

Dra. Cintia Carla Niva
Embrapa Cerrados

Dra. Letícia Scopel Camargo Carniel
BASF

Dr. Luís Carlos Iuñes Oliveira Filho
UDESC -CAV

Dra. Pâmela Niederauer Pompeo
UDESC -CAV

Lages, 25 de agosto de 2023.

DEDICATÓRIA

A todos aqueles que acreditam e lutam pela educação no Brasil.

Ao meu afilhado Antoni, espero estar ajudando a construir um mundo melhor para você crescer.

Dedico.

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“Every question is a cry to understand the world.”

“Toda pergunta é um grito para entender o mundo”

SAGAN, C. (1995)

FRAMEWORK PARA O AVANÇO DA ECOTOXICOLOGIA DE ENQUITREÍDEOS: USO DE ENSAIO DE COMUNIDADES E CURVAS DE DISTRIBUIÇÃO DA SENSBILIDADE DAS ESPÉCIES

RESUMO

O objetivo do presente trabalho foi desenvolver e validar um protocolo metodológico que possa ser usado para avaliar o efeito ecotoxicológico de pesticidas em comunidades naturais de enquitreídeos terrestres e uma curva de distribuição de sensibilidade de espécies (SSD) para de enquitreídeos nativos e padronizados. A fim de otimizar as metodologias de amostragem e extração de enquitreídeos e entender os efeitos das condições de laboratório nas comunidades nativas foi realizado um conjunto de estudos de campo e sob condições controladas. O método de extração não influenciou a diversidade acessada em campo, embora o método quente tenha apresentado menor esforço amostral em relação ao método frio. Ambos os métodos de extração não influenciaram na sobrevivência dos enquitreídeos pós-extração. Os resultados deste estudo demonstram que a temperatura, o tipo de substrato e a duração da exposição não influenciaram significativamente a dissimilaridade da comunidade de enquitreídeos. No entanto, a duração da exposição teve um efeito significativo na abundância de enquitreídeos, com períodos de exposição mais curtos levando a maiores abundâncias. Em testes de comunidade com o fungicida Mancozeb como modelo de pesticida. A abundância de enquitreídeos foi o atributo mais sensível (Concentração de efeito de 50% - CE_{50} = 1.310 mg kg^{-1}) seguido pela riqueza de morfoespécies (CE_{50} = 6.924 mg kg^{-1}) e dissimilaridade geral da comunidade (CE_{50} = 31,753 mg kg^{-1}). Em ensaios de reprodução em laboratório com Mancozeb a espécie nativa *Enchytraeus* sp. 1 foi a mais sensível (0.832 mg. kg^{-1}), seguido por *E. dudichi* (0.936 mg kg^{-1}), *E. crypticus* (CE_{50} = 3.078 mg kg^{-1}), *E. albidus* (CE_{50} = 3,850 mg kg^{-1}) e sendo *Enchytraeus* sp. 2 a espécie menos sensível (CE_{50} = 112.417 mg kg^{-1}). A partir destes valores foi elaborada uma curva SSD e determinado os valores de *Hazardous concentration* para um nível de proteção de 95% ($HC5_{CE50}$ = 0.1195 mg. kg^{-1}) e 50% ($HC50_{CE50}$ = 4.0417 mg.kg^{-1}).

Palavras-chave: Ecotoxicologia terrestre, Ecologia, Enquitreídeos, Agrotóxicos.

FRAMEWORK FOR ADVANCING IN ENCHYTRAEID ECOTOXICOLOGY: USE OF COMMUNITY TESTS AND SPECIES SENSIBILITY DISTRIBUTION

ABSTRACT

The objective of the present work was to develop and validate a methodological protocol that can be used to evaluate the ecotoxicological effect of pesticides in natural communities of terrestrial enchytraeids and a species sensitivity distribution curve (SSD) for native and standardized enchytraeids. In order to optimize the sampling and extraction methodologies of enchytraeids and to understand the effects of laboratory conditions on native communities, a set of field studies and under controlled conditions was carried out. The extraction method did not influence the diversity accessed in the field, although the hot method presented less sampling effort in relation to the cold method. Both extraction methods did not influence the survival of the post-extraction enchytraeids. The results of this study demonstrate that temperature, type of substrate and duration of exposure did not significantly influence the dissimilarity of the enchytraeid community. However, duration of exposure had a significant effect on the abundance of enchytraeids, with shorter exposure periods leading to higher abundances. In community tests with the fungicide Mancozeb as a pesticide model. The abundance of enchytraeids was the most sensitive attribute (Effect concentration of 50% - EC_{50} = 1.310 mg kg^{-1}) followed by morphospecies richness (EC_{50} = 6,924 mg kg^{-1}) and general dissimilarity of the community (EC_{50} = 31.753 mg kg^{-1}). In laboratory reproduction trials with Mancozeb the native species *Enchytraeus* sp. 1 was the most sensitive (EC_{50} = 0.832 mg kg^{-1}), followed by *E. dudichi* (EC_{50} = 0.936 mg kg^{-1}), *E. crypticus* (3.078 mg kg^{-1}), *E. albidus* (3.850 mg kg^{-1}) and *Enchytraeus* sp. 2 being the least sensitive species (EC_{50}= 112,417 mg kg^{-1}). From these values, an SSD curve was elaborated, and the *Hazardous Concentration* values were determined for a protection level of 95% ($HC5_{EC50}$ = 0.1195 mg. $kg^{-1)}$ and 50% ($HC50_{EC50}$ = 4.0417 mg kg^{-1}).

Keywords: Soil ecotoxicology, Ecology; Enchytraeids, Pesticides.

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SUMMARY

1 GENERAL INTRODUCTION

Enchytraeids play vital roles in ecosystems, directly or indirectly contributing to organic matter decomposition through litter feeding and interactions with soil microorganisms. These activities impact carbon and nutrient regulation, flow, and cycling in ecosystems (HENDRIX *et al.*, 1986; JÄNSCH *et al.*, 2005; AMORIM et al., 2009). Their actions also alter soil structure, enhancing porosity, reducing compaction, increasing oxygen concentration, and influencing soil aggregation and hydrology (DIDDEN, 1993; VAN VILET *et al.*, 1993; LINDEN *et al.*, 1994; ROITHMEIER & PIEPER, 2009).

Recognized as ecosystem engineers, enchytraeids modify soil structure through excavation and dietary habits involving mineral ingestion, transportation, organic matter mixing, and coprolite deposition (VAN VILET *et al.*, 1993).). In natural systems, they serve as indicators of soil biological activity, while community composition reflects changes in soil management and contaminant presence (JÄNSH *et al.*, 2005; NIVA *et al.*, 2015; PELOSI & RÖMBKE, 2016). Enchytraeids are employed as indicators in standardized ecotoxicological assays (ISO 16387, 2014).

Despite their significance, enchytraeids are not included in the pesticides Ecological Risk Assessment (ERA) frameworks of the European Union or Brazil (IBAMA,1996), even though protocols for assessing their survival and reproduction effects are available (ISO 1638, 2014). However, currently, there are no ecotoxicological protocols that can be recommended for higher-level pesticide risk assessment for this organism group.

The European Food Safety Authority – EFSA (2017) highlights the need for new methodologies to assess chemical substance effects on various soil organism groups, considering the complexity of environmental interactions, behaviors, and potential direct and indirect effects due to alterations in ecosystem food webs.

As an alternative intermediate-tier EFSA (2017) suggests utilizing Species Sensitivity Distribution (SSD) curves increasing the number of species at the first tier and conducting community-level tests as an intermediate tier. Community tests offer advantages such as simplicity, speed of execution, and result relevance.

1.1 MAIN OBJECTIVE

To develop and validate a methodological protocol that can be used to assess the ecotoxicological effect of pesticides on natural communities of terrestrial enchytraeids through a set of studies in field and under controlled conditions.

To evaluate the community test and the species sensitivity distribution curves as risk assessment schemes to enchytraeids.

1.2 SPECIFIC OBJECTIVES

1. Elaborate a review of Enchytraeids: about their morphology, taxonomy, bioecology- with focus on Brazil, sampling and extraction methods, their usefulness as indicator of soil quality, use in ecotoxicology – historic, gaps and perspectives for advance (Chapter 1).

2. Determine the number of cores needed (*sampling effort*) to sample the diversity of enchytraeids in a system, thus establishing a sample design proposal for conducting community tests (Chapter 2).

3. Compare the extraction methods of enchytraeids in terms of their effectiveness for use in community tests – considering the survival and morphological integrity of the enchytraeids after extraction (Chapter 2).

4. Define the temperature and duration (exposure time) for community test (laboratory conditions) based on the effects under life cycle, survival, and community structure of native enchytraeids (Chapter 3).

5. Validate the ecotoxicological protocol for natural enchytraeids communities with the fungicide mancozeb (Chapter 4).

6. Evaluate boric acid as a substance reference for ecotoxicological tests with native and standardized enchytraeids species (Chapter 5).

7. Generate a species sensitivity distribution (SSD) curve for the fungicide Mancozeb using native and standardized species of enchytraeids (Chapter 6).

1.3 HYPOTHESES

I. The extraction method does not influence the diversity of enchytraeids accessed in the field (Chapter 2).

II. The extraction method affects the survival of enchytraeids after extraction (Chapter 2).

III. The Sampling effort is positively related to the genus and morphospecies richness (Chapter 2).

IV. Factors such as temperature and time of exposure to pesticides influence the response of enchytraeids (life cycle, and survival) in community tests (Chapter 3).

V. Natural enchytraeids communities are sensitive and will show changes in their structure (abundance, diversity, and composition of genera/morphospecies) when exposed to pesticides, allowing their use in intermediate level in ERA (Chapter 4).

VI. Boric acid is a suitable refence substance for ecotoxicological tests with non-standardized enchytraeids species in subtropical regions (Chapter 5).

VII. By increasing the number test-species and using native enchytraeids species an SSD will help to determine whether the standard species are reliable and protective surrogates of the sensitivity to pesticides of the enchytraeids group (Chapter 6).

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2 CHAPTER 1: ENCHYTRAEIDS – AN OVERVIEW

2.1 INTRODUCTION

Enchytraeids play important roles in terrestrial ecosystems, contributing directly or indirectly to the decomposition of organic matter, given their feeding activity in the litter and through their interaction with soil microorganisms, affecting the regulation, flow and cycling of carbon and nutrients in ecosystems (HENDRIX *et al.*, 1986; JÄNSCH *et al.*, 2005; AMORIM *et al.*, 2009). The activity of the enchytraeids also contributes to alteration of the soil structure, increasing the volume of pores, thus reducing compaction, and increasing the concentration of oxygen, which may also affect soil aggregation and its hydrology (DIDDEN, 1993; VAN VILET *et al.*, 1993; LINDEN *et al.*, 1994; AMORIM *et al.*, 2009; ROITHMEIER & PIEPER, 2009).

Enchytraeids are considered ecosystem engineers, mainly due to their digging capacity and feeding habits that involve the ingestion, transport and mixing of minerals and organic particles, in addition to the deposition of coprolites (VAN VILET *et al.*, 1993;).

Below is presented a review of enchytraeids focusing on their morphology, taxonomy, bioecology, sampling, and extraction methods, their usefulness as an indicator of soil quality, use in ecotoxicology – historic, gaps and perspectives for advance.

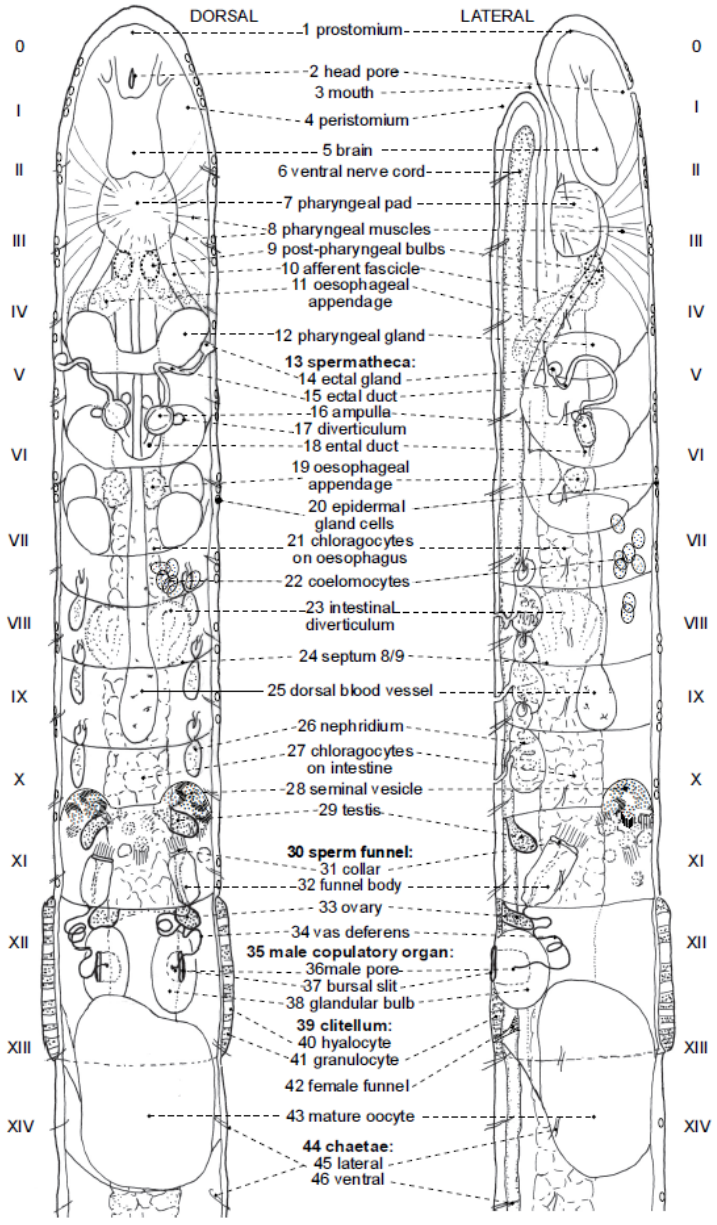
2.2 GENERAL MORPHOLOGICAL ASPECTS

Enchytraeids (Enchytraeidae, Oligochaeta, Annelida), are small whitish worms generally with inconspicuous pigmentation and popularly known as potworms. The color of the enchytraeids is usually determined by the gut content (DASH, 1983; JÄNSCH *et al.*, 2005; NIVA *et al.*, 2015) when observed by naked eyes or under a stereomicroscope. The following anatomic descriptions summarized here were compiled by Schmelz & Collado (2010) in the work "*A guide to European terrestrial and freshwater species of Enchytraeidae (Oligochaeta*)". The graphical representations of the structures are shown in Figure 1.

Enchytraeids have a segmented body plan with bilateral symmetry, they are hermaphroditic, have a coelom, a pre-oral, pre-segmental prostomium, an anterior ventral

mouth, and a posterior post-segmental pygidium with the anus. Full-grown individuals (adults) are usually between 2 and 30 mm long and between 0.1 and 1 mm wide. In cross-section they are cylindrical. The number of segments is rarely below 20 or above 70.

Figure 1 – Enchytraeid anatomy overview.



The traits shown here represent several possibilities and do not all occur in the same specimen. **Source:** Shcmelz & Collado, 2010.

From segment two on, enchytraeids present four discrete bundles of single-pointed chaetae in each segment, two ventral bundles, and two lateral or latero-dorsal bundles. Chaetae are always absent on the prostomium, the pygidium, and on the first segment, the peristomium, which surrounds the mouth opening. The number of chaetae per bundle varies between 1 and 16 within species-specific limits and according to the body region; most often there are 2 – 8 chaetae in a bundle.

In maturity, the epidermis of segments XII and XIII thicken into secretory cells that form the *clitellum*, responsible for the formation of the cocoons. In some individuals the epidermal gland cells are visible, generally more numerous in *preclitellar* than in *postclitellar* segments.

The body segments are marked by boundaries called septa, presents from segment IV/V on; those structures are elastic, and they have clefts that allow the coelomic fluid pass back and forth across segments.

The gut extends through the entire body and is connected to the body wall by the *intersegmental* septa and by additional *intrasegmental* ventral or dorsal ligaments. The functional gut regions: pharynx, oesophagus, and intestine are not well-distinguished at light-microscopy. Oesophageal appendages are often present between segments III and IV. Their shape and position vary among genera and species. The oesophagus merges gradually or abruptly into the intestine. The transition may be marked by intestinal diverticula (23), situated in segments VI, VII or VII, varying among taxa.

The identification of specimens is performed alive through the observation of internal and external morphological traits in light microscope, in table 1 are compiled the principal traits used in the identification to genus level for the genus with occurrence in Brazil. Regarding to species identification most taxonomic literature is restricted to temperate regions, yet the first identification keys for European species were published in the mid-1950s (NIELSEN & CHRISTENSEN, 1959; 1961; 1963) and only recently updated (SCHMELZ & COLLADO, 2010; 2015). Schmelz *et al.* (2013) state that to date there is no taxonomist specialized in terrestrial enchytraeids working in Latin America, and according to Niva *et al.* (2010) a key to identifying terrestrial enchytraeid species occurring in Brazil is unavailable, in table 1 are compiled the principal morphological traits used in identification of enchytraeids genus occurring in Brazil based on unpublished workbook prepared by Schmelz & Collado (2010).

The absence of identifications keys for enchytraeids it is one of the major impediments in the advances of enchytraeids biodiversity research in Brazil.

Table 1 - General traits used in the identification of enchytraeids until genus level.

Genus	**Trais**				
	Chaetae	**Intestinal diverticulum**	**Nephridium**	**Ceolomocyte**	**Oesophageal appendage**
Achaeta	Absent	Absent	Preseptal	Mucocyte	Absente
Guaranidrilus	Always two per bundle, straight or bent	Present in segment VII	Large preseptal region, postseptal region with dorsal vesicle	Mucocyte	Present in segment VI, or absent
Tupidrilus	Absent from segment VIII	Absent	-	Mucocyte	Present in segment VI
Xetadrilus	Two per bundle absent laterally from segment VIII	Absent	-	Mucocyte	Absent
Hemienchytraeus	Always two per bundle	Absent	- Large preseptal region, postseptal region without dorsal vesicle	Mucocyte	Present, dorsally in segment III
Enchytraeus	Two to four per bundle in generally two to trhree.	Absent	Preseptal, with funnel	Mucocyte	Present between segments III-IV.
Fridericia	0-2-8 per bundle, afan-shaped	Absent	Preseptal, with nephridial body part	Mucocyte and Lenticyte	Present in segment IV posicionado ventrolaterally.

Adapted from: Schmelz and Collado, 2010.

2.3 BIOECOLOGY

Information on the bioecology of enchytraeids was first published only in 1993 by Didden (DIDDEN, 1993), where the author compiles information on population dynamics, the influence of abiotic factors (such as temperature, moisture, and pH), and biotic factors (such as issues related to food preference, competition, and relationship with predators and parasites) not specifically addressing life cycle, life span or

reproduction. Since then, progress has been made in this regard, although much remains to be explored, especially with Neotropical species.

Enchytraeids are hermaphrodites. Most species reproduce by amphimixis, with external fertilization of gametes, producing cocoons, during copulation, two adult enchytraeids fit their male copulatory organs to the spermatheca pores, to exchange sperm material that will be deposited in the spermatheca, and will be used to fertilize the eggs inside the cocoon (DASH, 1983). But some species can produce cocoons by self-fertilization or parthenogenesis without the need for mating with another enchytraeid (DASH, 1983; DÓZSA-FARKAS, 1995; SCHMELZ & COLLADO, 2010).

In reproduction by architomy (the enchytraeids do not develop *clitellum*, instead, they divide into small fragments, generally cephalic, medial and caudal portions) that develop functional mouthparts in a few days (CHRISTENSEN, 1959; NIVA *et al.*, 2012; SCHMELZ *et al.* 2013). According to Collado *et al.* (2012), reproduction by fragmentation is observed in the genus *Enchytraeus*¸ and can also in species of the genera *Buchholzia* and *Cognettia* (both do not occur in Brazil, as indicated by Schmelz *et al.*, 2013).

So far, only six species have been reported to reproduce by fragmentation (most of them in European soils): *Buchholzia appendiculata* (BUCHHOLZ, 1862), *Cognettia sphagnetorum* (VEJDOVSKÝ, 1878), *Cognettia glandulosa* (MICHAELSEN, 1888), *Enchytraeus fragmentosus* (BELL, 1959), *Enchytraeus bigeminus* (NIELSEN & CHRISTENSEN, 1963*) Enchytraeus japonenis* (NAKAMURA, 1993), and *Enchytraeus dudichi* (DÓZSA-FARKAS, 1995).

In subtropical regions, the occurrence of fragmenting species belonging to the genus *Enchytraeus* (HENLE, 1837) has been recorded in pastures and forests of the Rain Forest in the state of Paraná (RÖMBKE *et al.* 2015, 2007) and in the Amazon Forest (COLLADO et al., 2012), not yet identified at the species level. Niva *et al.* (2012) report the occurrence of the fragmenting species *E. bigeminus* and *E. dudichi* in the Rain Forest of Paraná, being the first record of these species in the South American continent.

Pioneering studies in the assessment of the life cycle of enchytraeids were conducted by Christensen (1956), who used glass observation cameras to assess cocoon production in some species. Since then, some advances have been made in this topic, in Tables 1 and 2 the results found in the literature so far are compiled. Some main points can be extracted from this compilation:

- The number of eggs per cocoon varies according to the species (1 to 35 eggs per cocoon).
- Temperature plays a key role in the reproduction of enchytraeids, studies in temperate regions indicate a range of 5 – 25 °C for their multiplication.
- For species from temperate climates, the maturation and incubation periods decrease with increasing temperature to a lethal limit between 20 – 25 °C. However, species from tropical climates can produce cocoons at higher temperatures (between 15 – 30 °C).
- The results point out a maximum longevity of one year.

Table 2 – Summary of information about reproductive aspects of terrestrial enchytraeids.

Species	Cocoon size (mm)[1]	Eggs/Cocoon	Incubation time (days)	Hatching rate (%)	Total life span (days)	Temperature for reproduction (°C)	Reference
Lumbricillus. lineatus[3]	1.18 × 0.84 (mean)	06 – 11	15.7 ± 0.9 (at 10 °C)	74	117 (at 10°C)	5 – 20	Reynoldson (1933; 1943)
Lumbricillus rivalis[3]	(–) [2]	17.1 – 47.8 (mean)	19 – 92	(–)	(–)	10 ± 1	Kirk (1971)
Enchytraeus albidus[5]	0.59 × 0.55 (mean)	2 – 6	23.8 ± 1.7 (at 10 °C)	97	68.3 (at 10°C)	5 – 25.5	Reynoldson (1943)
Enchytraeus albidus	0.5 – 1.8 (length)	1 – 35 (mean = 10)	12 (at 18°C)	(–)	261	18	Ivleva (1953)
Stercutus niveus	0.71 – 1.35 × 0.49 – 0.64	01 – 15	(–)	(–)	> 365	13 – 18	Dózsa-Farkar (1973)
Enchytraeus coronatus	(–)	5.06 ± 2.26	7 – 10	83	224	20	Rodriguez *et al* (2002)[4]
Enchytraeus variatus	(–)	5 – 20 (mean = 10.9)	14 – 30	(–)	254	18 – 22	Bouguenec & Giani (1989)
Enchytraeus. Minutus	(–)	(–)	(–)	(–)	120 – 160	(–)	Westheide *et al*. (1989)[4]
Enchytraeus bigeminus	(–)	(–)	(–)	(–)	120 – 160	(–)	Westheide *et al*. (1989) [4]
Enchytraeus. globuliferus	(–)	(–)	(–)	(–)	120 – 160	(–)	Westheide *et al*. (1989) [4]
Enchytraeus. crypticus[4]	(–)	1 – 35 (mean = 7.6)	8.3 (at 21°C)	(–)	85	(–)	Westheide & Graefe (1992)
Enchytraeus albidus	(–)	(–)	(–)	(–)	(–)	10 – 20	Dirven-van Breemen *et al*. (1994)
Enchytraeus crypticus	(–)	(–)	(–)	(–)	(–)	15 – 30	Dirven-van Breemen *et al*. (1994)
Enchytraeus. crypticus	(–)	(–)	20 (at 20 ± 2°C)	(–)	(–)	(–)	Achazi *et al*. (1999)
Enchytraeus. albidus	(–)	(–)	33 (at 18 °C) 74 (at 12 °C)	(–)	(–)	(–)	Römbke & Moser (2002)

[1]millimeters (length × width of the cocoon); [2] (–) information not available; [3] Later studies reclassified the *L. lineatus* species as *L. rivalis*; [4]Study conducted on agar.; [5]According to Albert (1975) there may have been a failure in identification and the data probably refer to the species *E. coronatus*. Source: Prepared by the author, 2023.

Table 3 – Summary of information about reproductive aspects of terrestrial fragmenting enchytraeids.

Species	Number of fragments generated	Number of segments of the new individual	Fully regeneration (days)	Reference
Enchytraeus fragmentosus	3 – 14	5	10	Bell (1959)
Enchytraeus fragmentosus	3 – 14	n.a.	10	Lavelle & Spain (2005)
Enchytraeus bigeminus	4 – 7	n.a.	6 – 7 (20 – 22 °C)	Christensen (1964; 1973)
Enchytraeus variatus	3 – 6	n.a.	n.a.	Bouguenec & Giani (1987)
Enchytraeus dudichi	7 – 18	3 –6	3 (29 – 30 °C) 7 (22 – 24 °C)	Dózsa-Farkas (1996)
Cognettia sphagnetorum	At least 3	n.a.	7 – 14	Augustsson & Runfgren (1998)
Cognettia sphagnetorum[2]	2 – 3	n.a.	8 – 26	Lavelle & Spain (2005)
Enchytraeus japonicus	n.a.[1]	n.a.	4	Inomata *et al*. (2000)
Enchytraeus dudichi	n.a.	4 –7	5 – 6	Niva *et al.* (2012)

[1](–) Information not available; [2]This species starts to fragment when individuals have more than 42 segments; Bandowet al. (2013) points out the absence of data on the life cycle of the species *E. luxuriosus a*nd E. *bulbosus.* Source: Prepared by the author, 2023.

Enchytraeids are found worldwide in soils with sufficient moisture, organic matter, and oxygen (SCHMELZ *et al.*, 2013; NIVA *et al.*, 2015).

Both the distribution and composition of species in the environment are strongly influenced by factors such as pH, organic matter, and soil use (DIDDEN, 1993; GRAEFE & SCHMELZ, 1999; JÄNSCH & RÖMBKE, 2003; JÄNSCH *et al.*, 2005).

Graeffe and Schmelz (1999) classified species of enchytraeids based on their preferred occurrence within the soil profile: Litter Dwellers (LD), Soil Dwellers (SD), and Intermediate Species (IS).

Litter Dwellers (LD) commonly reproduce asexually through fragmentation. They typically feed on partially to fully decomposed plant remains and microorganisms (STANDEN, 1973; DIDDEN, 1993). Soil Dwellers (SD) are usually found in the topmost 10 cm of the soil. Their specific food preferences are not as well understood as those of litter dwellers, but they also appear to consume microorganisms and dead organic matter (SCHMIDT *et al.*, 2004). Intermediate Species (IS) inhabit both mineral soil and the organic layer. This group is heterogeneous and primarily consists of r-strategists, often residing near the soil surface regardless of the presence of a litter layer. Many intermediate species have short generation cycles due to asexual reproduction, including fragmentation. However, not much is known about their specific feeding preferences.

Regarding to food preferences, Gajda *et al.* (2017) compiled and organized information into a single existing review article, in general, nine food sources have been described: (1) plant material, (2) microalgae, (3) animal remains, (4) feces of invertebrates, (5) bacteria, (6) fungi, (7) microalgae, (8) nematodes and (9) locust eggs, and sorted the enchytraeids into two groups: Primary decomposers and Secondary decomposers/sapro-microphytophages.

It is well-established that enchytraeids exhibit clustering behavior, leading to significant differences in their horizontal distribution on a small scale (NIELSEN, 1954; PEACHEY, 1963). According to Didden (1993), they form multispecies clusters of 100-1000 cm^2 in a random distribution at arable sites. These differences in distribution may be influenced by the heterogeneous availability of resources, such as food, or specific soil parameters (CHALUPSKÝ & LEPŠ, 1985).

The vertical distribution of enchytraeids at crop sites is heavily influenced by plowing, as it transports organic matter to deeper layers (DIDDEN *et al.*, 1997).

Consequently, the typical distribution observed in non-plowed sites, with high densities near the surface gradually decreasing in deeper layers, can be altered to a more or less uniform density within the plowed layer, as long as there is available food. Vertical migration of enchytraeids is also driven by climatic factors such as temperature and moisture (LAGERLÖF *et al.*, 1989), as well as anthropogenic stressors like pesticides applied to the soil surface (RÖMBKE & FEDERSCHMIDT, 1995).

Climatic factors, mediated by soil moisture and properties (e.g., pH), play a dominant role in the occurrence and activities of enchytraeids (GRAEFE & SCHMELZ, 1999; MARALDO & HOLMSTRUP, 2010). In Central Europe, the population dynamics of enchytraeids typically follow a seasonal pattern dictated by temperature and precipitation. Peaks in population density occur in spring and autumn, while the numbers decrease during summer due to low soil moisture levels and in winter, especially in the absence of a snow cover, due to low soil temperatures (NIELSEN, 1955; DIDDEN, 1993). However, this pattern can be modified at grasslands and crop sites due to various management practices (PELOSI & RÖMBKE, 2016).

2.4 DIVERSITY ASSESSMENT – SAMPLING EXTRACTION AND TAXONOMY

The sampling of enchytraeids has an internationally standardized protocol, ISO 23611-3 (2007; 2019). The soil samples for the investigation of the enchytraeid community are collected with a soil core, measuring 5 x 5 cm. The depth depends on the soil type but usually varies between 10 cm and up to 30 cm, i.e., those layers in which the bulk of the enchytraeids are living.

Soil cores can be stored in small plastic bags and kept in a refrigerator at 4 °C to 6 °C for up to one to two weeks, but not longer than two months, as advised by Didden (1993). However, it is worth noting that these storage conditions may not be suitable for tropical regions as temperatures as low as 4 °C could be fatal to the enchytraeids, as pointed out by Niva *et al.* (2010). To ensure their survival, it is recommended to keep soil samples in cool places, but never below 12 °C.

Traditional methods used for assessing soil biodiversity, such as hand sorting of TSBF (Tropical Soil Biology and Fertility – ANDERSON, INGRANN, 2010) or hot extraction in Berlese-Tüllgren funnels (BERLESE, 1905; TÜLLGREN, 1917)

underestimates the number of individuals, due the small size or the fragility of their bodies. As a result, specific methodologies are needed to accurately assess enchytraeids communities.

Initially, the extraction of organisms from the soil was done manually, Bretscher (1904) proposed a methodology for quantitative determination of enchytraeids populations, it consists in distributing a small amount of soil in a flat bowl with water and analyzing carefully and repeatedly with a magnifying glass. The author states that besides being a time-consuming method, it delivers results that cannot be surpassed by any other method in terms of accuracy. Moszynski (1930) proposed a change in the method; the addition of a small amount of alcohol in the samples, since the alcohol forces the worm that may be still hidden to move, becoming visible to the observer.

Overgaard (1947-48) described an extraction-method for in-soil living nematodes and suggested that this method could be suitable for enchytraeids. In principle, the technique was based upon the application of heat from above to a soil sample which is spread out on a sieve under water in a funnel. The individuals will respond to this treatment by burrowing into the soil sample and eventually moving through the sieve and dropping to the bottom of the funnel which is closed by a stopcock. When the extraction is completed, the sample is removed, the stopcock opened, and it is easy to sort out the organisms among the small amount of soil particles that have fallen through the sieve.

Nevertheless, Nielsen (1952-3) states that to apply this methodology to enchytraeids, the sample size must be increased several times over that used for nematodes, and in that case, the method was found to be unsatisfactory. Yet the author points out that manual sorting of enchytraeids is too wasteful of time, the results are inaccurate and cannot in actual practice provide a satisfactory idea of population densities being necessary an automatic-extraction-method for enchytraeids.

In a short period, two techniques for large-scale extraction of enchytraeids from soil have been described the Nielsen (1952-3) and O'Connor (1995) methods. The Nielsen' technique (1952-3) consists in heating up soil cores in an earthen-ware vessel immersed in a water-bath, so the worms are driven upwards into a cooled layer of sand in the top of the soil sample. The enchytraeids are recovered by washing them from the sand. O'Connor (1955) tried to apply the Nielsen technique to access the enchytraeids community in a coniferous forest but with no success, so he proposed an adaptation to the Baermann (1917) method where the heat is applied from above, and the soil is placed in

a sieve resting in a funnel full of water. The worms move downwards through the sieve into the funnel and can be collected by releasing the spring clip.

Later, Graeffe (1984) proposed a method without the need for equipment and without temperature regulation. It can be carried out with the simple aid of a strainer and a water bowl. The soil sample is placed in the strainer, into the bowl filled with water, and the worms move downwards into the bottom of the bowl. The extraction time varies according to the site, but no lasting longer than ten days.

Römbke (1995) proposed a wet extraction apparatus based on the principle of O'Connor extractor (1955) incorporating the modifications proposed by Graeffe (1984), in which each individual sample is transferred to a sieve (diameter 15 cm, mesh size approximately 1 mm), each sieve is placed over a container filled with water until the sample is covered. The sieves and containers are cooled to about 10 – 15 °C in a water bath to prevent the individuals from dying due to lack of oxygen. Samples with high content of hummus, like form litter, remain 1 – 2 days of extracting, and soil samples with low hummus content, remain for up to 7 days of extraction. This methodology is currently recommended by the protocol ISO 23611-3 (2007).

In subtropical regions, Niva *et al.* (2015) found some difficulties with the cold wet extraction method recommended by ISO (2007), so the authors proposed a device for the hot extraction based on O'Connor's method (O'CONNOR, 1955) built with plastic funnels (19 cm diameter) coupled with sieves inside. The soil sample is placed in a sieve lined with a flannel and filled with mineral water which was heated by a lamp (75W) for 3h, with water temperature reaching 45-50 °C at the surface.

Regarding diversity assessment some works compared hot and wet extraction, Kobetičová and Schlaghamerský (2003), observed a higher number of enchytraeids when the cold method was used for 24h, while Panchenko (2006) collected twelve samples in a pine forest in Sweden, of which six were extracted by hot method and six by the cold method the author found a slightly higher abundance in cold method and Niva et al., (2015) evaluated a fragment of Mixed rain forest in the state of Paraná (n=8), found a better performance of the hot method, given the extraction time and a smaller number of damaged individuals.

2.5 BIOGEOGRAPHYCAL DISTRIBUTION (EMPHASIS ON LATIM AMERICA AND BRAZIL)

According to Schmelz & Collado (2015), there are 710 species worldwide with valid descriptions, distributed in thirty-three genera. Nine genera (*Achaeta*, *Cernosvitoviella*, *Enchytraeus*, *Hemienchytraeus*, *Fridericia*, *Guranidrilus*, *Marionina*, *Xetadrilus*,and *Tupidrilus*) have already been recorded in Latin America with sixty-two species described (SCHMELZ *et al.* 2013).

Knowledge about the ecology and diversity of enchytraeids in tropical and subtropical regions is still scarce (SCHMELZ *et al.* 2013). In Brazil, after Righi's valuable contributions to the taxonomy of enchytraeids (RÖMBKE, 2003), more recent ecological and taxonomic information was made available from the Amazon region (RÖMBKE & MELLER, 1999; SCHMELZ & RÖMBKE, 2005), Atlantic Forest in the state of Paraná (RÖMBKE et al., 2005, RÖMBKE et al., 2007, SCHMELZ et al., 2008; 2009; 2011, RÖMBKE *et al.* 2015; NIVA *et al.* 2015, DEMETRIO *et al.* 2020), Atlantic Forest in the state of Santa Catarina (ALEXANDRE *et al.*, 2022; KRAFT *et al.*, 2022), and in the Brazilian savanna Biome (NIVA *et al.*, 2021 , ALEXANDRE *et al.*, 2022).

These studies revealed relevant information such as that species composition responds to soil type, but above all revealed new species, and even a new genus (*Xetadrilus*, described by Schmelz *et al.*, 2011). Most of the data is the result of the cooperation between Brazil-Germany in the SHIFT ENV-52 and SOLOBIOMA projects, showing the importance of promoting research in the field of biodiversity. This leads us to reflect on the potential of new species and genera not yet described, also considering the hypothesis that this group originated in South America as proposed by Coates (1989).

Studies conducted in tropical and subtropical ecosystems are important to broaden the ecological database on this group of organisms. Lewinsohn *et al.* (2005) states that although Brazil is a megadiverse country, the soil fauna is still poorly known and needs more attention. A trend observed in diversity studies of enchytraeids, both in temperate regions and subtropical regions, is a prevalence of assessment in natural areas, being almost non-existent in agricultural areas (information reiterated by Pelosi & Römbke, 2016). Few studies focus on evaluating the relationship between the occurrence of genera and/or species with soil and/or litter attributes, these studies are restricted to the describe

the species composition of the evaluated sites and/or morphological description of new species/genera.

The occurrence of enchytraeids has been sporadically reported in studies of macrofauna in different land use systems in South America (SILVA *et al.* 2006; MANETTI *et a*l., 2010; PORTILHO *et al.* 2011; DOMÍNGUEZ & BEDANO 2016a; DOMÍNGUEZ & BEDANO 2016b), however, the TSBF methodology (ANDERSON & INGRAM, 1993) was used to assess these populations, with separation of organisms being done manually, may underestimate the abundance of this group, due to their size and color.

The composition of enchytraeid genera registered in Brazil is different from that observed in temperate regions, due to the occurrence of typically Latin American genera such as *Guaranidrilus*, *Hemienchytraeus*, *Xetadrilus*, and *Tupidrilus*. However, more widely occurring genera such as *Enchytraeus* and *Fridericia* also occur significantly (SCHMELZ *et al.*, 2013).

Most enchytraeids of the genus *Enchytraeus* found in agroecosystems so far are species that reproduce by fragmentation, probably due to the high adaptability that this characteristic confers to individuals in disturbed environments (SCHMELZ *et al.*, 2013; NIVA *et al.*, 2014; NIVA *et al.*, 2015; BUSSINGER, 2018; DEMÉTRIO *et al.*, 2020; NIVA *et al.*, 2021; ALEXANDRE *et al.*, 2022; KRAFT *et al.* 2022). In general, *Enchytraeus* and *Fridericia* can be very abundant and dominant in cultivated soils. *Hemienchytraeus* occurs both in native and cultivated areas and its abundance is variable with worldwide distribution (SCHMELZ *et al.*, 2013).

Guaranidrilus is predominant in soils with native vegetation and absent or inexpressive in cultivated areas, which may be indicative of soil in a better state of conservation (NIVA *et al.*, 2014; NIVA *et al.*, 2015; BUSSINGER, 2018; DEMÉTRIO *et al.*, 2020; NIVA *et al.*, 2021; ALEXANDRE *et al.*, 2022; KRAFT *et al.* 2022).

Regarding species richness in Brazil, *Guaranidrilus* presents highest richness (RÖMBKE *et al*, 2021; NIVA *et al.*, 2021) with 16 species, followed by *Achaeta* with twelve and *Hemienchytraeus* with eleven (SCHMELZ & NIVA, 2018; NIVA *et al.*, 2021), *Fridericia* has seven species, *Xetadrilus* five species, *Tupidrilus* and *Marionina* both have four species (SCHMELZ & COLLADO, 2010; SCHMELZ *et al.*, 2013).

Below are summarized some studies conducted in Brazil, using the standardized methodology of collection ISO 23611-3 (2007), and identifying the enchytraeid genera.

Table 4 – Enchytraeids diversity studies carried in Brazil.

Reference	Site	Biome	Ecosystem	Density (ind.m²)	Soil	Sampling method	Extraction method	Species identified	Genus occurrence
Römbke & Meller, 1999	Manaus (AM)	Amazon	Florets plantation (Polyculture system)	4.5	Xanthic Ferrsol[1]	n.a	GRAEFE & RÖMBKE (1995)	Yes	(A)(E)(G)(H)
	Manaus (AM)	Amazon	Native vegetation (Secondary forest)	4.6	Xanthic Ferrsol[1]	n.a	GRAEFE & RÖMBKE (1995)	Yes	(A)(E)(G)(H)
	Manaus (AM)	Amazon	Native vegetation (Primary forest)	3.85	Xanthic Ferrsol[1]	n.a	GRAEFE & RÖMBKE (1995)	Yes	(A)(E)(G)(H)
Schmelz & Römbke, 2005	Manaus (AM)	Amazon	n.a	n.a	Xanthic Ferrsol[1]	n.a	GRAEFE & RÖMBKE (1995)	Yes	(H)
Römbke et al., 2005	Antonina (PR)	Atlantic Forest	Pasture	80	Cambissolo[2]	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)
	Antonina (PR)	Atlantic Forest	Pasture	62.4	Gleissolo[2]	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)
	Antonina (PR)	Atlantic Forest	Native vegetation	191.2	Cambissolo[2]	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)
	Antonina (PR)	Atlantic Forest	Native vegetation	106.7	Gleissolo[2]	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)

	Antonina (PR)	Atlantic Forest	Native vegetation	142.8	Cambissolo²	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)
	Antonina (PR)	Atlantic Forest	Native vegetation	31.2	Gleissolo²	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)
	Antonina (PR)	Atlantic Forest	Native vegetation	53.6	Cambissolo²	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)
	Antonina (PR)	Atlantic Forest	Native vegetation	106.1	Gleissolo²	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)
	Antonina (PR)	Atlantic Forest	Native vegetation	53.8	Cambissolo²	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)
	Antonina (PR)	Atlantic Forest	Native vegetation	n.a	Gleissolo²	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)
Römbke et al., 2007	Antonina (PR)	Atlantic Forest	Pasture	2416	Cambissolo²	ISO (2004)	Cold-wet	Yes	(A)(E)(F)(G)(H)(M)
	Antonina (PR)	Atlantic Forest	Pasture	1534	Gleissolo²	ISO (2004)	Cold-wet	Yes	(A)(E)(F)(G)(H)(M)
	Antonina (PR)	Atlantic Forest	Native vegetation	2001	Cambissolo²	ISO (2004)	Cold-wet	Yes	(A)(E)(F)(G)(H)(M)
	Antonina (PR)	Atlantic Forest	Native vegetation	1555	Gleissolo²	ISO (2004)	Cold-wet	Yes	(A)(E)(F)(G)(H)(M)

	Antonina (PR)	Atlantic Forest	Native vegetation	2354	Cambissolo²	ISO (2004)	Cold-wet	Yes	(A)(E)(F)(G)(H)(M)
	Antonina (PR)	Atlantic Forest	Native vegetation	1596	Gleissolo²	ISO (2004)	Cold-wet	Yes	(A)(E)(F)(G)(H)(M)
	Antonina (PR)	Atlantic Forest	Native vegetation	1648	Cambissolo²	ISO (2004)	Cold-wet	Yes	(A)(E)(F)(G)(H)(M)
	Antonina (PR)	Atlantic Forest	Native vegetation	1711	Gleissolo²	ISO (2004)	Cold-wet	Yes	(A)(E)(F)(G)(H)(M)
	Antonina (PR)	Atlantic Forest	Native vegetation	3898	Cambissolo²	ISO (2004)	Cold-wet	Yes	(A)(E)(F)(G)(H)(M)
	Antonina (PR)	Atlantic Forest	Native vegetation	n.a	Gleissolo²	ISO (2004)	Cold-wet	Yes	(A)(E)(F)(G)(H)(M)
Römbke et al, 2005	Antonina (PR)	Atlantic Forest	Native vegetation	n.a	Cambisol Gleysol¹	ISO (2007)	ISO (2007)	Yes	(A)(G)(H)
Römbke et al, 2015	Antonina and Guaraqueçaba (PR)	Atlantic Forest	Pature	2003 - 2008	Gleissolo²	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)(X)
	Antonina and Guaraqueçaba (PR(	Atlantic Forest	Native vegetation	2003 - 2008	Gleissolo²	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)(X)

	Antonina and Guaraqueçaba (PR)	Atlantic Forest	Native vegetation	2003 - 2008	Gleissolo²	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)(X)
	Antonina and Guaraqueçaba (PR)	Atlantic Forest	Native vegetation	2003 - 2008	Gleissolo²	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)(X)
	Antonina and Guaraqueçaba (PR)	Atlantic Forest	Pasture	2003 - 2008	Cambissolo²	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)(X)
	Antonina and Guaraqueçaba (PR)	Atlantic Forest	Native vegetation	2003 - 2008	Cambissolo²	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)(X)
	Antonina and Guaraqueçaba (PR)	Atlantic Forest	Native vegetation	2003 - 2008	Cambissolo²	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)(X)
	Antonina and Guaraqueçaba (PR)	Atlantic Forest	Native vegetation	2003 - 2008	Cambissolo²	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)(X)
	Antonina and Guaraqueçaba (PR)	Atlantic Forest	Native vegetation	2003 - 2008	Cambissolo²	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)(X)
	Antonina and Guaraqueçaba (PR)	Atlantic Forest	Native vegetation	2003 - 2008	Cambissolo²	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)(X)

	Antonina and Guaraqueçaba (PR)	Atlantic Forest	Native vegetation	2003 - 2008	Cambissolo²	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)(X)
	Antonina and Guaraqueçaba (PR)	Atlantic Forest	Native vegetation	2003 - 2008	Cambissolo²	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)(X)
	Antonina and Guaraqueçaba (PR)	Atlantic Forest	Native vegetation	2003 - 2008	Cambissolo²	n.a	n.a	Yes	(A)(E)(F)(G)(H)(T)(X)
Niva et al. 2015	Colombo (PR)	Atlantic Forest	Native vegetation (Araucaria Mixed Forest)	202.667	Cambissolo²	ISO (2007)	ISO(2007); O'CONNOR (1955)	No	(A)(E)(F)(G)(H)
	Colombo (PR)	Atlantic Forest	Native vegetation (Araucaria Mixed Forest)	141.575	Cambissolo²	ISO (2007)	ISO(2007); O'CONNOR (1955)	No	(A)(E)(F)(G)(H)
	Colombo (PR)	Atlantic Forest	Native vegetation (Araucaria Mixed Forest)	61.192	Cambissolo²	ISO (2007)	ISO(2007); O'CONNOR (1955)	No	(A)(E)(F)(G)(H)

Demetrio et. al, 2020	Quitandinha (PR)	Atlantic Forest	Native vegetation (Secondary ombrophilous mixed forest in advanced stage of regeneration)	3845	Acrisoils[1]	ISO (2007)	O'CONNOR (1955); NIVA et al., (2010); NIVA et al., (2015)	No	(E)(F)(G)(H)
	Quitandinha (PR)	Atlantic Forest	Agriculture (Cabbage and carrots, Potatoes)	18931	Acrisoils[1]	ISO (2007)	O'CONNOR (1955); NIVA et al., (2010); NIVA et al., (2015)	No	(E)(F)(G)(H)
	Quitandinha (PR)	Atlantic Forest	Agriculture (mayze/soybean, wheat/black oat)	8270	n.a	ISO (2007)	O'CONNOR (1955); NIVA et al., (2010); NIVA et al., (2015)	No	(E)(F)
	Quitandinha (PR)	Atlantic Forest	Agriculture (Potato, Squash)	20589	Acrisoils[1]	ISO (2007)	O'CONNOR (1955); NIVA et al., (2010); NIVA et al., (2015)	No	(E)(F)
Römbke et al., 2021	Manaus (AM)	Amazon	Forestry plantation	5200	Xantic Ferrasoil[1]	ISO (2007)	RÖMBKE (1995); ISO (2007)	Yes	(A)(E)(G)(H)(M)

	Manaus (AM)	Amazon	Forestry plantation	4300	Xantic Ferrasoil[1]	ISO (2007)	RÖMBKE (1995); ISO (2007)	Yes	(A)(E)(G)(H)(M)
	Manaus (AM)	Amazon	Native vegetation (Secondary Forest)	6300	Xantic Ferrasoil[1]	ISO (2007)	RÖMBKE (1995); ISO (2007)	Yes	(A)(E)(G)(H)(M)
	Manaus (AM)	Amazon	Native vegetation (Primary Forest)	5600	Xantic Ferrasoil[1]	ISO (2007)	RÖMBKE (1995); ISO (2007)	Yes	(A)(E)(G)(H)(M)
Kraft et al., 2022	Chapecó (SC)	Atlantic Forest	Native vegetation (transition from Mixed Ombrophilous Forest to Semi Deciduous Seasonal Forest)	6451	n.a	ISO (2007)	O'CONNOR (1955); NIVA et al., (2010); NIVA et al., (2015)	No	(A)(E)(F)(G)(H)
	Faxinal dos Guedes (SC)	Atlantic Forest	Native vegetation (transition from Mixed Ombrophilous Forest to Semi Deciduous Seasonal Forest)	6451	n.a	ISO (2007)	O'CONNOR (1955); NIVA et al., (2010); NIVA et al., (2015)	No	(A)(E)(F)(G)(H)

	Campo Erê (SC)	Atlantic Forest	Agriculture (Plantations under No-Tillage Systems cultivated with soybean)	4966	n.a	ISO (2007)	O'CONNOR (1955); NIVA et al., (2010); NIVA et al., (2015)	No	(A)(E)(F)(H)
	Chapecó (SC)	Atlantic Forest	Agriculture (Plantations under No-Tillage Systems cultivated with soybean)	4966	n.a	ISO (2007)	O'CONNOR (1955); NIVA et al., (2010); NIVA et al., (2015)	No	(A)(E)(F) (H)
	Faxinal dos Guedes (SC)	Atlantic Forest	Agriculture (Plantations under No-Tillage Systems cultivated with soybean)	4966	n.a	ISO (2007)	O'CONNOR (1955); NIVA et al., (2010); NIVA et al., (2015)	No	(A)(E)(F)(H)

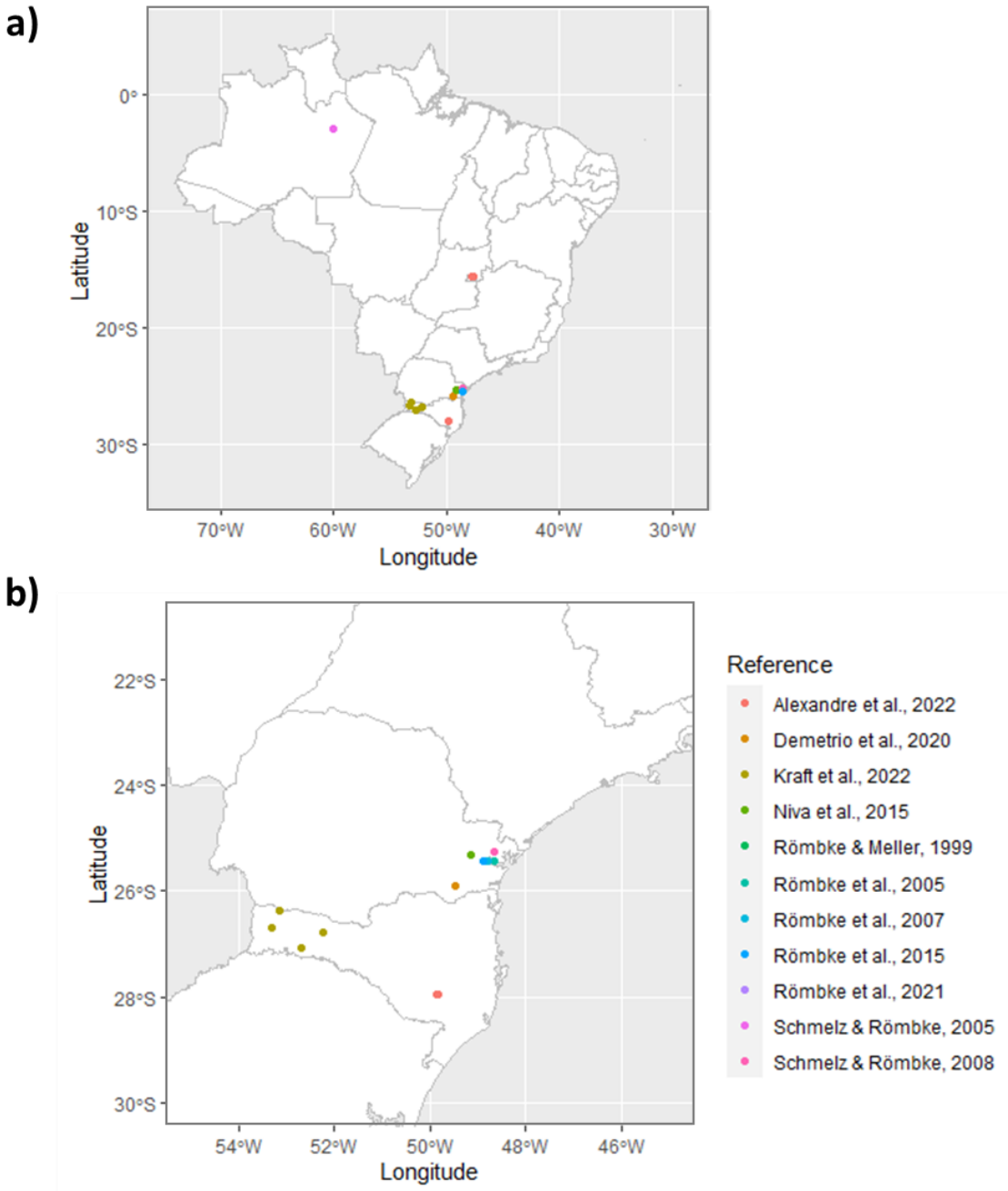
	Tigrinhos (SC)	Atlantic Forest	Agriculture (Plantations under No-Tillage Systems cultivated with soybean)	4966	n.a	ISO (2007)	O'CONNOR (1955); NIVA et al., (2010); NIVA et al., (2015)	No	(A)(E)(F)(H)
Alexandre et al., 2022	Planaltina (DF)	Cerrado	Native vegetation	18.844	Oxisols[1]	ISO (2007)	O'CONNOR (1955); NIVA et al., (2010); NIVA et al., (2015)	No	(A)(E)(G)(H)
	Planaltina (DF)	Cerrado	Pasture	2.036	Oxisols[1]	ISO (2007)	O'CONNOR (1955); NIVA et al., (2010); NIVA et al., (2015)	No	(A)(E)(H)
	Urupema (SC)	Atlantic Forest	Native vegetation	9.677	Entisoil[1]	ISO (2007)	O'CONNOR (1955); NIVA et al., (2010); NIVA et al., (2015)	No	(E)(F)(G)(H)

	Urupema (SC)	Atlantic Forest	Pasture	13.242	Inceptisol[1]	ISO (2007)	O'CONNOR (1955); NIVA et al., (2010); NIVA et al., (2015)	No	(A)(E)(G)(H)

[1]FAO classification; [2] Brazilian Classification; (A) *Achaeta;* (E) *Enchytraeus;* (H) *Hemienchytraeus;* (G) *Guaranidrilus;* (M) *Marionina;* (T) *Tupidrilus;* (X) *Xetadrilus.* Source: Prepared by the author, 2023.

So far, eleven studies were conducted in Brazil (Figure 2), 6 in Paraná state, 3 in Amazonas, 2 in Santa Catarina and 1 in Federal District. Atlantic Forest is the Biome most studied presenting 8 studies, followed by Amazon with 3 and Cerrado (Brazilian Savanah) with 1.

Figure 2 - Geographical location of the enchytraeids biodiversity studies conducted in Brazil.



a) View of Brazil; b) detail on Santa Catarina and Paraná sates. Source: Prepared by the author, 2023.

Most studies were conducted in natural forest and pastures sites, Demetrio *et al.* (2020) and Kraft *et al.* (2022) were the only studies conducted in agricultural systems.

2.6 USE OF ENCHYTRAIEDS IN ECOTOXICOLOGY

2.6.1 Historical perspective: The use of enchytraeids in ecotoxicology

Enchytraeids have played a significant role in the field of ecotoxicology, contributing to providing insightful information on how pollutants affect the health of ecosystems and soil organisms.

In this historical overview the evolution of using enchytraeids in ecotoxicological studies is highlighted, from early observations to their establishment as important bioindicators.

2.6.1.1 Early observations

Römbke *et al.* (2017) indicated that enchytraeids, despite their relevance, took a long time to be included as indicators in ecotoxicological assays due to the lack of taxonomic information and the difficulty in their cultivation and maintenance in the laboratory.

The first report of the use of enchytraeids in ecotoxicological tests in the laboratory dates from the late 1960s (WEUFFEN, 1968), before the establishment of standardized protocols, enchytraeids were tested not only in soil, but also in water (RÖMBKE & KNACKER, 1989) and agar (WESTHEIDE *et al.*, 1991). Early studies noted that Enchytraeids showed visible responses, such as altered behavior and reduced reproduction, when exposed to pollutants. These first findings provide the basis for further investigations into their potential as bioindicators.

2.6.1.2 Development and standardization of Enchytraeid-Based Toxicity Tests

In the following years, efforts were made for the development of standardized tests using enchytraeids. Toxicity tests were developed, incorporating endpoints such as

survival, growth, reproduction, and avoidance behavior of enchytraeids under laboratory-controlled conditions.

The need for reproducible and reliable methods to evaluate the effects of contaminants on enchytraeids lead to the conduction of several tests throughout 90's and early 2000s.Those ring-tests involving researchers from around 15 countries (RÖMBKE & MOSER, 1999; 2002), Researchers conducted comparative studies, assessing the sensitivity and reproducibility of Enchytraeid tests across different laboratories and soil types. These validation studies helped refine the test protocols, ensuring consistent and meaningful results.

Those efforts culminated in the standardization of lethality and reproduction tests, under controlled laboratory conditions, by international intuitions: ASTM E1676 (2004), ISO 16387 (2002) and OECD 220 (2004). These tests provided a useful instrument for evaluating the toxicity of diverse substances.

There have been attempts to standardize a test of enchytraeids avoidance behavior, initially proposed by Achazi *et al.* (1996), and some sparse reports in the following years (PANNECK, 2000; WAGNER-VASKE 2000; AMORIN *et al.* 2005), but no regulations have been established so far. When compared to effects on reproduction, the endpoint in question appears to be of low relevance and difficult to standardize.

2.6.1.3 Effects of pesticides on enchytraeids - standardized tests

Research was conducted in the main databases of the Web of Science using the keywords *Ench**, *Potworm**, *Ecotox**, *Toxicology**, *Pesticid**, *Herbicid**, *Fungicid** and *Inseticid** with the boolean operators: AND and OR for the last twenty tree years (2000 – 2023).

The results of the review are compiled in Table 2. Were extracted from the articles The active ingredient used, the class to which it belongs and the form in which it was tested (formulated product or pure active ingredient), the normative used (detail for articles published before the ISO and OECD standards indicated as *draft,* for conducting the tests based on the normative in development) In addition, are presented the substrate used as well as the species used as an indicator.

The endpoints highlighted were the Lethal concentration to 50% of the population in test (CL_{50}); Non observed effect concentration – Reproduction ($NOEC_R$); Effective concentration – 50% Reproduction ($EC_{50\text{-}R}$); Effective concentration – 50% Avoidance ($CE_{50\text{-}A}$)

Table 5 – Ecotoxicological effects of pesticides on enchytraeids in laboratory tests.

Class	Pesticide	Formulation	Guideline	Test species	Substrate	*Endpoint*	CL_{50}	$NOEC_R$	EC_{50-R}	EC_{50-A}	Reference
(F)	Carbendazim	Derosal	OECD – Draft	*E. coronatus*	OECD	(L) (R)	>321.8	10.17.	14.09	n.a.	Arrate et al. 2002
(I)	Lindane	γ-HCH	OECD 220	*E. albidus*	OECD	(L) (R)	107	3.29	9.68	n.a.	Lock et al. 2002
(I)	Lindane	γ-HCH	OECD 220	*E. albidus*	Filed	(L) (R)	384	32	41.9	n.a.	
(I)	Lindane	γ-HCH	OECD 220	*E. albidus*	Field	(L) (R)	76.7	n.a.	n.a.	n.a.	
(H)	Phenmedi-pham	Betosyp (Betanal)	ISO 16387	*E. albidus*	OECD	(L) (R)	>100	n.a.	52	n.a.	Amorim et al. 2005a
(H)	Phenmedi-pham	Betosyp (Betanal)	ISO 16387	*E. albidus*	LUFA 2.2	(L) (R)	50	n.a.	24	n.a.	
(H)	Phenmedi-pham	Betosyp (Betanal)	ISO 16387	*E. albidus*	Natural soil	(L) (R)	103	n.a.	17	n.a.	
(H)	Phenmedi-pham	Betosyp (Betanal)	ISO 16387	*E. albidus*	Natural soil	(L) (R)	59	n.a.	28	n.a.	
(H)	Phenmedi-pham	Betosyp (Betanal)	ISO 16387	*E. luxuriosus*	OECD	(L) (R)	118	n.a.	45	n.a.	
(H)	Phenmedi-pham	Betosyp (Betanal)	ISO 16387	*E. luxuriosus*	LUFA 2.2	(L) (R)	51	n.a.	22	n.a.	
(H)	Phenmedi-pham	Betosyp (Betanal)	ISO 16387	*E. luxuriosus*	Natural soil	(L) (R)	86	n.a.	28	n.a.	
(H)	Phenmedi-pham	Betosyp (Betanal)	ISO 16387	*E. luxuriosus*	Natural soil	(L) (R)	41	n.a.	1	n.a.	
(H)	Phenmedi-pham	Betosyp (Betanal)	ISO 16387	*E. luxuriosus*	Natural soil	(L) (R)	7	n.a.	5	n.a.	
(F)	Benomyl	Benlate	ISO – Draft	*E. albidus*	OECD	(A)	n.a.	n.a.	n.a.	46.8	Amorim et al. 2005b
(F)	Benomyl	Benlate	ISO – Draft	*E. albidus*	LUFA 2.2	(A)	n.a.	n.a.	n.a.	>32,0	
(F)	Carbendazim	Derosal	ISO – Draft	*E. albidus*	OECD	(A)	n.a.	n.a.	n.a.	>32.0	

(F)	Carbendazim	Derosal	ISO – Draft	*E. albidus*	Natural soil	(A)	n.a.	n.a.	n.a.	7.9	
(H)	Phenmedi-pham	Betosyp (Betanal)	ISO – Draft	*E. albidus*	OECD	(A)	n.a.	n.a.	n.a.	252.2	
(H)	Phenmedipham	Betosyp (Betanal)	ISO – Draft	*E. albidus*	LUFA 2.2	(A)	n.a.	n.a.	n.a.	50.7	
(H)	Phenmedipham	Betosyp (Betanal)	ISO – Draft	*E. albidus*	Natural soil	(A)	n.a.	n.a.	n.a.	45.9	
(H)	Phenmedipham	Betosyp (Betanal)	ISO – Draft	*E. albidus*	Natural soil	(A)	n.a.	n.a.	n.a.	<1	
(H)	Phenmedipham	Betosyp (Betanal)	ISO – Draft	*E. albidus*	Natural soil	(A)	n.a.	n.a.	n.a.	34.2	
(I)	Toxaphene	Toxaphene	OECD 220	*E. albidus*	OECD	(L) (R)	>620	620.	n.a.	n.a.	Bezchlebova et al. 2007
(I)	Toxaphene	Toxaphene	OECD 220	*E. crypticus*	OECD	(L) (R)	>620	620	n.a.	n.a.	
(I)	Chlorpyriphos	Chlorpyri-phos	ISO – Draft	*E. albidus*	LUFA 2.2	(A)	n.a.	n.a.	n.a.	933	
(I)	Dimethoate	Dimethoate	ISO – Draft	*E. albidus*	LUFA 2.2	(A)	n.a.	n.a.	n.a.	58.3	
(I)	Lindane	γ-HCH	ISO Draft	*E. albidus*	LUFA 2.2	(A)	n.a.	n.a.	n.a.	172.5	
(F)	Carbendazim	Derosal	ISO - Draft	*E. albidus*	LUFA 2.2	(A)	n.a.	n.a.	n.a.	8.0	Amorim et al. 2008
(F)	Pentachloro-phenol	Pentachloro-phenol	ISO 16387; ISO – Draft	*E. albidus*	LUFA 2.2	(L) (A)	136	n.a.	n.a.	703	
(H)	Atrazine	Atrazine	ISO Draft	*E. albidus*	LUFA 2.2	(A)	n.a.	n.a.	n.a.	38.0	
(H)	Phenmedipham	Betosyp (Betanal)	ISO – Draft	*E. albidus*	LUFA 2.2	(A)	n.a.	n.a.	n.a.	7.0	
(I)	Alpha-cyper-methrin	Alpha-cyper-methrin	OECD 220	*E. crypticus*	Natural soil	(L) (R)	31.4	2.51	4.91	n.a.	Hartnik et al. 2008
(I)	Dimethoate	Dimethoate	ISO – Draft	*E. albidus*	LUFA 2.2	(A)	n.a.	n.a.	n.a.	2.0	
(H)	Atrazine	Atrazine	ISO 16387	*E. albidus*	LUFA 2.2	(L) (R)	12.0	1.0	2.0	n.a.	Novais et al. 2010
(H)	Phenmedipham	Betosyp (Betanal)	ISO – 16387	*E. albidus*	LUFA 2.2	(R)	n.a.	10.0	28.0.	n.a.	

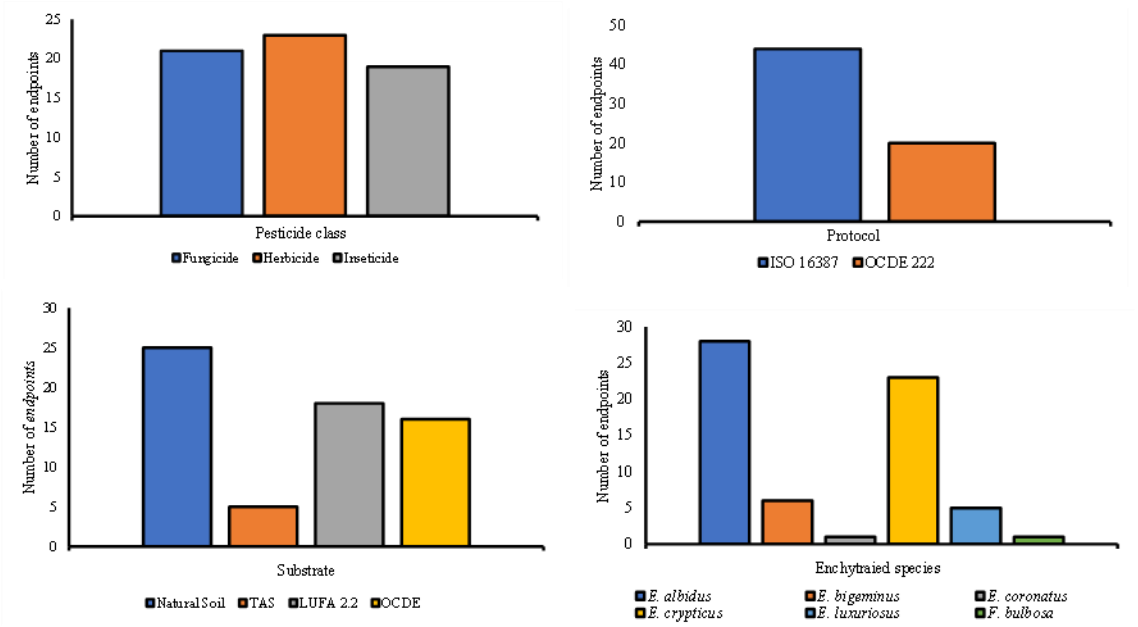
(F)	Carbendazim	Derosal	ISO 16387	*E. crypticus*	LUFA 2.2	(L) (R)	>32	n.a.	<1	n.a.	Castro-Ferreira et al. 2012
(F)	Carbendazim	Derosal	ISO 16387	*E. albidus*	LUFA 2.2	(R)	n.a.	n.a.	0.5.	n.a.	Novais et al. 2012
(I)	Carbofuran	Furadan 350SC	ISO 16387	*E. crypticus*	Natural soil	(L) (R)	>1.7	n.a.	0.74	n.a.	Chelinho et al. 2012
(I)	Diazinon	Pinorel 60 EC	ISO 16387	*E. crypticus*	Natural soil	(R)	n.a.	> 16	n.a.	n.a.	Natal-da-Luz et al. 2012
(H)	Bromoxynil	n.a.	OECD 220	*F. bulbosa*	OECD	(L)	2.41	n.a.	n.a.	n.a.	Yang et al. 2012
(I)	Lambda-cyhalothrin	Karate Zeon™	OECD 220 (30% CRA)	*E. bigeminus*	OECD	(R)	n.a.	0.21	1.34	n.a.	Bandow et al. 2013
(I)	Lambda-cyhalothrin	Karate Zeon™	OECD 220 (50% CRA)	*E. bigeminus*	OECD	(R)	n.a.	1.06	3.79	n.a.	
(I)	Lambda-cyhalothrin	Karate ZeonTM	OECD 220 (70% CRA)	*E. bigeminus*	OECD	(R)	n.a.	1.12	4.77	n.a.	
(F)	Pyrimethanil	Scala	OECD 220 (30% CRA)	*E. bigeminus*	OECD	(R)	n.a.	85.6	437	n.a.	
(F)	Pyrimethanil	Scala	OECD 220 (50% CRA)	*E. bigeminus*	OECD	(R)	n.a.	220	499	n.a.	
(F)	Pyrimethanil	Scala	OECD 220 (70% CRA)	*E. bigeminus*	OECD	(R)	n.a.	598	829	n.a.	
(F)	Chlorotha-lonil	Chlrotha-lonil	ISO 16387	*E. crypticus*	Natural soil	(R)	n.a.	n.a.	112.9	n.a.	Leitão et al. 2014
(I)	Ethoprophos	Ethoprophos	ISO 16387	*E. crypticus*	Field	(R)	n.a.	n.a.	68.5	n.a.	
(F)	Azoxystrobin	Azoxystro-bin	ISO 16387	*E. crypticus*	Natural soil	(R)	n.a.	n.a.	99.2	n.a.	
(I)	Chlorantrani-liprole	Chlorantrani-liprole	OECD 220	*E. crypticus*	LUFA 2.2	(L) (R)	>1000	n.a.	>1000	n.a.	Lavtizar et al. 2016
(F)	Difenoconazole	SCORE®	OECD 220	*E. crypticus*	Natural soil (Brasil – MG)	(L) (R)	n.a.	100	125	n.a.	De Menezes Oliveira et al. (2018)
(I)	Abamectin	KRAFT® 36 EC	OECD 220	*E. crypticus*	Natural soil (Brasil – MG)	(L) (R)	n.a.	2.5	2.80	n.a.	
(F)	Mancozeb	Dithane® NT	ISO 16387	*E. crypticus*	Natural soil (Oxisol, Brasil – SC)	(L) (R)	6.97	0.2	3.56 (1.39 – 5.74)	n.a.	Carniel et al. (2019)

(F)	Mancozeb	Dithane® NT	ISO 16387	*E. crypticus*	Natural soil (Ultisol – Brasil -SC)	(L) (R)	280.21	<15	29.67 (16.10 – 43.25)	n.a.	
(I)	Abamectin	Kraft® 36 EC	OECD 220	*E. crypticus*	Natural soil	(R)	n.a.	n.a.	n.e..	n.a.	Da Rocha et al (2020)
(F)	Difenoconazole	SCORE®	OECD 220	*E. crypticus*	Natural soil	(R)	n.a.	n.a.	s.r	n.a.	
(H)	Metsulfuron-methy	Ally®	ISO 16387	*E. crypticus*	TAS	(R)	n.a.	n.a.	5.73 (1.76 - 12.23)	n.a.	De Santo et al. (2019)
(H)	Metsulfuron-methy + mineral oil	Ally® + Assist®	ISO 16387	*E. crypticus*	TAS	(R)	n.a.	n.a.	>33.33	n.a.	
(I)	Clothianidin	Inside FS	ISO 16387	*E. crypticus*	TAS (5%)	(R)	n.a.	20	21.3 (3.40-39.2)	n.a.	Bandeira et al. (2021)
(F)	Fosetyl-Al	Fosetyl-Al	OECD 220	*E. crypticus*	LUFA 2.2.	(R)	n.a.	n.a.	1250	n.a.	Barreto et al. (2021)
(H)	2,4-D	DMA® 806 BR	ISO 16387	*E. crypticus*	TAS	(R)	n.a.	n.a.	n.e.	n.a.	Triques et al. (2021)
(H)	2,4-D	DMA® 806 BR	ISO 16387	*E. crypticus*	Natural soil (Brasil – SP)	(R)	n.a.	n.a.	n.e.	n.a.	
(F)	Fipronil	Regent® 800 WG	ISO 16387	*E. crypticus*	TAS	(R)	n.a.	n.a.	n.e.	n.a.	
(F)	Fipronil	Regent® 800 WG	ISO 16387	*E. crypticus*	Natural soil (Brasil – SP)	(R)	n.a.	n.a.	n.e.	n.a.	

Insecticide (I); Fungicide (F); Herbicide (H); Lethality (L); Reproduction (R); Non-available data (n.a.); No effect (n.e.).
The following values are expressed in mg a.i. kg. $soil^{-1}$: Lethal concentration to 50% of the population in test (CL_{50}); Non observed effect concentration – Reproduction ($NOEC_R$); Effective concentration – 50% Reproduction ($EC_{50\text{-}R}$); Effective concentration – 50% Avoidance ($CE_{50\text{-}A}$).

A similar number of studies can be observed with all classes of pesticides (Figure 3), the highest number of endpoints recorded was for the molecule Phenmedipham (14), followed by Carbendazin (6), Lindane (4), Pyrimethanil (3) and Lambda-cyhalothrin (3). A prevalence of studies using the ISO 16387 protocol, and with natural soil as substrate. The standardized species E. *albidus* is the most used, followed by *E. crypticus*.

Figure 3 - Number of endpoints evaluated for enchytraeids by pesticide class, protocol substrate and species.



Font: Prepared by the author, 2023.

The first study evaluating effects of pesticides on enchytraeids in Brazil was Menezes-Oliviera *et al* (2018), in which the effects of Difenoconazole and Abamectin in natural soil from Minas Gerais state towards *E. crypticus* were accessed. Followed by Carniel *et al* (2019) evaluating effects of Mancozeb on lethality and reproduction of *E. crypticus* in two natural soils from Santa Catarina and Triques *et al.* (2021) accessing effects of Fipronil and 2,4-D in the reproduction of *E. crypticus* in Tropical Artificial Soil (TAS) and a Natural soil from São Paulo. Other studies were conducted in Brazil, but they did not used natural soil, using artificial soil instead (DE SANTOS *et al.*, 2019; BANDEIRA *et al*, 2021).

2.6.1.4 Use of enchytraeids in ecotoxicological semi-fields tests

Some semi-field methods have been tested over the years, such as microcosms, which are small pots with soil kept in the laboratory under controlled conditions; all components of the system are controlled by the experimenter, including the organisms (MOTHES-WAGNER *et al.*, 1992; SCHAEFFER *et al.*, 2010).

According to Jensen and Scott-Fordmand (2012) microcosms are suitable for specific issues, such as the influence of pesticides on standardized invertebrate combinations, but so far, no standardization of microcosms is available. Martikainen *et al.* (1998) highlighted the value of microcosm experiments over laboratory testing when studying complex issues.

There are more complex models of ecotoxicological evaluations in semi-field, the so-called Terrestrial Model Ecosystem (TMEs) (KNACKER *et al.*, 2004; FÖRSTER *et al.*, 2006; MOSER & RÖMBKE, 2007; MOSER *et al.*, 2007; SCHOLZ - STARKE *et al.*, 2013; BANDOW *et al.*, 2006).TMEs are undisturbed soil columns collected in the field and containing the original community of soil organisms, there are indoor and outdoor schemes, Schaeffer *et al.*, (2010) have proposed a test guideline for the OECD, so far without a published standard.

The first record of a study evaluating the effects of pesticides on enchytraeids in a TME system is by Römbke *et al.* (1994), in which researchers evaluated the effects of Parathion and the herbicide formulation Ustinex (containing Amitrol + diuron) on an enchytraeid community in a pasture in Germany. The number of enchytraeid species and the total abundance were not negatively affected, except in the treatment with the highest concentration of Parathion.

Moser *et al.* (2007) evaluated TMES collected from pasture soils in Germany, Great Britain, Holland and Portugal, six doses of Carbendazim were tested. In all locations, the genus *Fridericia* was the most negatively affected by the pesticide, followed by species of the genus *Henlea*. Many species of *Achaeta* and *Enchytraeus* did not decline or partially increased.

Bandow *et al.* (2016) found that the fungicide Pyrimethanil did not affect the composition of the community (in the experiment in question composed of *Enchytraeus buchholzi, E. bulbosus, E. dichaetus, Fridericia bulboides, F. pretoriana, F. tuberosa* and another species of the genus *Fridericia*) in a TME experiment carried out in Portugal soil.

2.6.1.5 Use of enchytraeids on ecotoxicological field tests

Regarding experiences of toxicological assessments in the field, the first record evaluating the effects of pesticides on enchytraeids was carried out in northern Germany by Weber (1953). In the following years Edwards *et al.* (1968) and Edwards & Lofty (1971) described the effects of various pesticides on enchytraeids in agricultural areas.

Voronova (1968) studied the effects of the insecticide Sevin on enchytraeids in Russia (data may be underestimated, given that the specimens were extracted by hand sorting).

When evaluating the effect of the pesticide Fenithrothion on the abundance of enchytraeids in a New Zealand pasture at field-relevant concentrations Martin (1975) found no effect. The same trend was reported by McColl (1984) when studying the effects of Benomil and Fenamiphos on enchytraeids in New Zealand pastures. Popovici et al. (1977) reported effects of Atrazine on enchytraeids, the authors further report that despite a rapid decrease in the number of enchytraeids at both concentrations 1 month after application the values increased after 4 months.

Römbke *et al.* (1994) studied the effect of Parathion and or a mixture of Amitrol + Diuron at the highest recommended application rate and a 5-fold higher concentration rate. No effects were observed on the enchytraeids when exposed to Parathion, however in relation to Amitrol + Diuron there was an increase in the abundance and biomass of enchytraeids at the lowest rate, but the high rate reduced the evaluated parameters by 50%.

According to Kattwinkel *et al.* (2015) no studies were reported on the recovery of enchytraeid communities in agricultural systems after exposure to pesticides, the published studies are restricted to forest systems.

2.6.1.6 Advances in enchytraeids molecular and cellular ecotoxicology

In the last decades, progress can be observed in research towards molecular and biochemical tools. Recent studies use molecular and biochemical mechanisms of enchytraeids to assess the response to pesticide exposure using differential gene expression, and biomarkers (HOWCROFT *et al.*, 2011; NOVAIS *et al.*, 2014).

Goncalves *et al.* (2015) proposed adaptations in reproduction tests to assess embryotoxicity by measuring parameters such as embryonic development, number of embryonic structures, and hatching success combined with macroscopic monitoring, and histological and immunohistochemical analysis. However, so far only data for cadmium is available.

Novais *et al.* (2012) using the microarray tool developed for *E. albidus*, showed that exposure to Phenmedipharm triggered the expression of a different set of genes compared to exposure to copper metal. Therefore, the two groups of chemicals affected distinct biological functions, reproduction was affected only by pesticides, and lipid metabolic processes were affected only by metals.

2.6.2 Gaps and perspectives on Enchytraeids ecotoxicology

Advances in studies with enchytraeids are necessary, there are still several information gaps that still need to be accessed, both in simpler issues such as taxonomic advances aimed at expanding the number of described species and available keys, both in more complex aspects such as the establishment of new methods, increasing in the understanding of the bioecology, physiology, reproductive physiology of enchytraeids, which can help in understanding the routes of exposure of enchytraeids to pesticides.

Improve the already established and standardized assays trying to insert new genus more representative for subtropical regions. Standardize semi-field tests and field tests, to increase the reliability of the results and allow them to be directly compared.

2.6.3 Lack of enchytraeids on ERA scheme and advanced approaches

The EU follows a tiered approach to the Plant Protection Products (PPPs) Ecological risk assessment (ERA) to in-soil fauna non-target organisms (EC 2009), this process should address the risk with higher realism, going from a more restrictive *low tier* to a more realistic *high tier*.

In TIER 1- preliminary laboratory tests are conducted, in which the effects in the worst possible scenario are evaluated and if this stage indicates a risk of the substance for 5% of the population under test, the evaluation follows to the second tier. At the TIER 2 - *semi-field* tests are carried out in mesocosms, this method was developed to combine the

advantages of laboratory and field conditions, aiming at an exposure closer to reality, if risk continues to be observed, we move to the third tier. On the TIER 3 - field tests are carried out to prove the toxicity of products to organisms.

The *semi-field* tests, despite being an interesting approach from the point of view of the complexity of interrelations and possibilities of variables to be evaluated, demand for a high workload and resources. So, it would be interesting to have an intermediate stage between laboratory and *semi-field* tests that would provide more information reducing the uncertainties of laboratory tests and the high load of semi-field tests.

The European Food Safety Authority - EFSA (2017) signals the need for new methodologies that allow the evaluation of the effects of chemical substances on the most varied groups of soil organisms, considering the complexity of interactions in the environment, their behavior, and potential direct and indirect effects due to changes in the food web in ecosystems. In this sense, they have pointed out some lacks in the PPPs ERA, after the last improvements (EC 2009; EU 283, 2013; EU 284, 2013).

For instance, increase the number of species used in TIER 1 (*low tier*) and establish some methodologies to *intermediate tiers* – such the use of the Species Sensitivity distribution (SSDs) approach, since testing alternative species in soil ecotoxicology started recently and is still unclear how to develop the SSDs until more data is available, EFSA (2017) suggested also another possible *intermediate tier* – the use of community tests.

Another point raised is that, despite the ecological relevance of enchytraeids they are not included in the PPPs ERA schemes, even though standardized ecotoxicological protocol being available to access effects on enchytraeids survival and reproduction when exposed to contaminants (ISO 16387, 2014; OECD 220, 2004), intermediate and advanced protocols are not yet available. The absence of enchytraeids in ERA scheme in Europe already has been criticized by the European Food Safety Authority in previous documents (EFSA, 2007).

2.6.3.1 Use of alternative species of enchytraeids:

EFSA (2017) states that the use of alternative species in soil ecotoxicology is important to estimate the toxicity of PPPs and calibrate trigger values. According to Römbke et al. (2017) the main criteria used in the selection of new species are the ease of identification, cultivation, low generation time and the sensitivity of the organism.

The current guidelines for ecotoxicological tests with enchytraeids ISO 16387 (2014) and OECD 220 (2016) describe methodologies for obtaining acute and chronic effects of contaminated soils on adult enchytraeids of the genus *Enchytraeus*. According to the documents, species of this genus are ecologically relevant and can be used in laboratory, semi-field and field tests. Considering that they are species of easy handling, creation, and their generation time is shorter than that of earthworms.

The document also provides an annex, in which it indicates the procedures for using other species of the genus *Enchytraeus*, in addition to the initially proposed *E. albidus* (Henle, 1873), such as – the species *E. crypticus* (Westheide and Graefe, 1992), *E. buchholzi* (Vejdovsky, 1879), *E. luxuriosus* and *E. bulbosus* (Nielsen and Cristensen, 1963). However, the document does not indicate the use of species from other enchytraeid genera.

Originally, *E. albidus* was the most used organism, but *E. crypticus* became more popular due to the shorter duration of the test, and greater number of juveniles (KUPERMAN *et al.*, 2006). Over the years other species have been proposed, most of them from the genus *Enchytraeus*.

Bandow et al (2013) proposed *E. bigeminus* that reproduces asexually via fragmentation. There are records of reproduction tests with *E. coronatus* (ARRATE *et al.*, 2002), and with *E. doerjesi* (KARAMARZ *et al.*, 2005; VOUA OTOMO *et al.*, 2013.). There are also reports of testes with species of the genus *Enchytraeus* with pending description, as in Assis (2015) and Morais et al. (2016).

According to Niva et al. (2010) and Schmelz et al. (2013) species of the genus *Enchytraeus* tend to be easy to grow on a large scale in unnatural conditions, which may be one of the main factors for the prevalence in the use of species from this group. Schmelz et al (2013) also point out that some species of the genus *Enchytraeus* reproduce primarily by fragmentation, which confers high adaptability.

In relation to other genera: Augustsson and Runfgren (1998) discuss the advantages and disadvantages of using the fragmenting species *Cognettia sphagnetorum* in the risk assessment, given its relevance in soil of temperate regions (The genus in question, *Cognettia*, was not registered in South America [SCHMELZ *et al.* 2013]), however its use is not recommended by the authors. Species of the genus *Fridericia* have been evaluated for their suitability for ecotoxicological tests, such as *F. ratzeli* (RÖMBKE & FEDERSCHMITT, 1995), *F. peregrinabunda* (AN & YANG, 2009) and

F. bulbosa (ZHU *et al.* 2008; YANG *et al.*, 2012). Typical Latin American genera such as *Hemienchytraeus, Xetadrilus, Tupidrilus* and *Guaranidrilus* have not been reported in ecotoxicological tests so far.

2.6.3.2 Use of enchytraeids species sensitivity distribution (SSD)

The SSD approach assumes that the variation of sensitivity among a set of species to a certain compound or mixture fits in a probability function. The SSD curve is estimated from a sample of toxicity data obtained from ecotoxicological laboratory tests with multiple species (i.e., $LC_{50,}$ EC_{50} or NOEC) and plotted as a cumulative distribution function. The use of SSD allows the establishment of threshold value (Hazardous Concentration: HC). (POSTHUMA *et al.* 2002).

One of the major issues in using this approach to in-soil organisms is the absence of a dataset which an acceptable number of test species to estimate the hazardous concentrations (HC) (FRAMPTON *et al.*, 2006).

2.6.3.3 Enchytraeids Community tests

Community-tests were initially proposed for arthropods (CHELINHO *et al.*, 2014), with records with oligochaetes communities. Community tests have several advantages such as simplicity and speed in execution, and the relevance of their results. When compared to field and semi-field, community tests present less demand of time, space, and resources. Hence it is still not standardized, and few information is available (CHELINHO *et al.* 2014; CARNIEL, 2019; MATHIEU *et al.* 2021), more information is necessary to improve and standardize the methods to be useful in the ERA.

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3 CHAPTER 2: METHODOLOGICAL OPTIMIZATION FOR ECOTOXICOLOGICAL COMMUNITY TESTS WITH ENCHYTRAIEDS - EXTRACTION PROCEDURES AND SAMPLING EFFORT

3.1 INTRODUCTION

Enchytraeids are small organisms with a worm-like body, usually without coloration, ranging from 2 to 40 mm in length (JÄNSCH *et al.*, 2005; NIVA *et al.*, 2015). Enchytraeids play important roles in ecosystems, contributing directly or indirectly to the decomposition of organic matter, given the feeding activity in the litter and the interaction with soil microorganisms, affecting the regulation, flow and cycling of carbon and nutrients in ecosystems (HENDRIX *et al.*, 1986; JÄNSCH *et al.*, 2005; AMORIM *et al.*, 2009).

Despite their ecological relevance, enchytraeids are commonly neglected in soil biodiversity assessments, since the most used methods such as TSBF (ANDERSON, INGRANN, 1993), in which the selection is done by hand sorting, underestimate the number of individuals, due the small size of their bodies, or the soil cores (ISO 23611-2, 2007) subjected to hot extraction in Berlese-Tullgren funnels known to be inefficient because the enchytraeids dry up before they get out of the samples or during the passage from the sample to the collecting tube, being necessary the use of specifical methodologies to access enchytraeids communities.

Initially, the extraction of organisms from the soil was done manually, (BRETSCHER, 1904), or depended on complex apparatus (NIELSEN, 1952-53) currently Römbke's methodology is recommended by the ISO protocol (ISO 23611-3, 2007), the methodology consists in the extraction of enchytraeids by the wet-method without the use of a heat source, nevertheless, the O'Connor methodology with adaptations from Niva *et al.* (2010, 2015) has been widely applied in enchytraeids diversity studies in subtropical regions, which consists in the extraction of individuals by wet method with application of a temperature gradient through an external heat source.

To develop a methodology to access the effects of pollutants on natural communities of enchytraeids, some methodologies require optimization. Considering that there is no consensus on the extraction method and sampling effort of enchytraeids, the main goal of this work is to evaluate the performance of the two extraction methods in

relation to the structural diversity of the enchytraeid community accessed; and determine the sampling effort for each extraction method; also evaluate the survival of the specimens subjected in a *post-extraction* lethality assay.

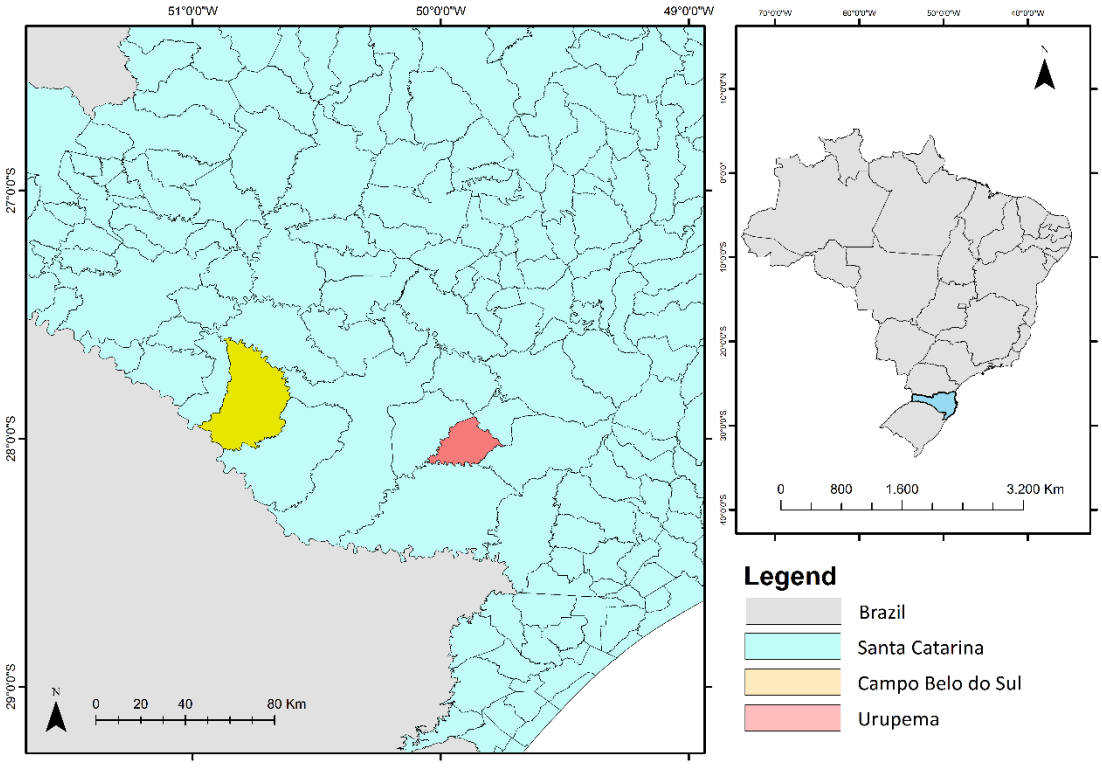
We hypothesize that: (i) The extraction method does not influence the diversity of enchytraeids accessed in the field; (ii) The sampling effort is positively related to the genus and morphospecies richness; (iii) the extraction method affects the survival of organisms after extraction; (iv) the extraction method determines the soil sampling effort of enchytraeids.

3.2 MATERIAL AND METHODS

3.2.1 Characterization of study areas and sampling scheme

To conduct the study, one area with natural pasture were selected in two municipalities in the plateau of the Santa Catarina State, Brazil (Figure 4).

Figure 4 – Location of the sampled municipalities: Urupema e Campo Belo do Sul.



Prepared by the author, 2023.

In the municipality of Urupema (URP) the sampling was carried out in an area of Cambisolo (Table 4) with coverage of native pasture dominated by pastures of the genera *Andropogum* and *Paspalum* (27°57'44.24" S 49°50'67" W). The climate in Urupema is subtropical type Cfb, according to the Köppen classification, with rainfall distributed throughout the year, an average rainfall of 1,789 mm and an altitude of 1,540 m asl. It is considered one of the coldest places in Brazil, the average annual temperature is 13 °C, and snow is common in the period between July and August.

In Campo Belo do Sul (CBS) the sampling was carried out in an area of Nitossolo Bruno (Table 6) with coverage of native pasture with predominance of pastures of the genera *Plantago* and *Axonopus* (27°53'42.5" S 50°40'18.4" W). The climate in Campo Belo do Sul is subtropical type Cfb according to the Köppen classification, rainy and with mild winters and summers, and altitude of 923 m asl.

Table 6 – Physicochemical attributes of the Soils.

Soil attribute[a]	Nitisol	Cambisol
Clay (%)	41	39
pH_{water}	4,4	4,9
P (mg/dm^3)	3,7	6,9
K (mg/dm^3)	120	104
Organic matter content (%)	5,5	6,7
Al ($cmol_c/dm^3$)	4,7	1,3
Ca ($cmol_c/dm^3$)	1,7	3,7
Mg ($cmol_c/dm^3$)	1,6	2,2

[a] Determined according to Tedesco et al. (1995)

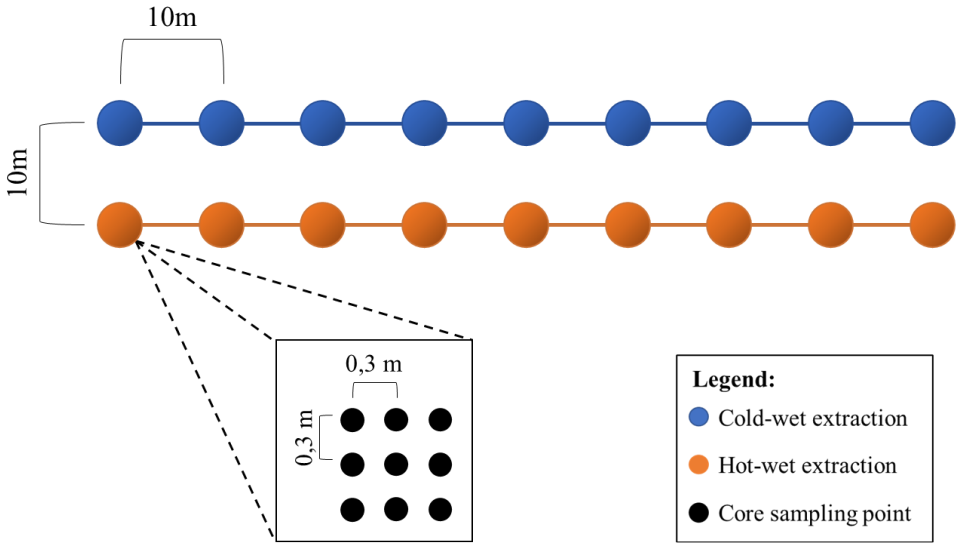
3.2.2 Sampling and extraction

In each area, two transects were established (Figure 5) one for each extraction method: (i) wet-hot extraction method; and (ii) wet-cold extraction method); each transect contained 9 sampling points, at each point 9 cores were collected (n=81 per extraction method). The nine cores were collected in a grid of 3 x 3 subpoints, spaced 0,3 m.

The enchytraeids were collected following the ISO 23611-3 (2007) which consists of collecting not deformed soils samples in a metallic ring measuring 5 x 5 cm. The

samples were transported to the laboratory and kept in a temperature-controlled room (16 ± 2 °C) until further processing.

Figure 5 – Representation of the enchytraeids sampling scheme.



Prepared by the author, 2023.

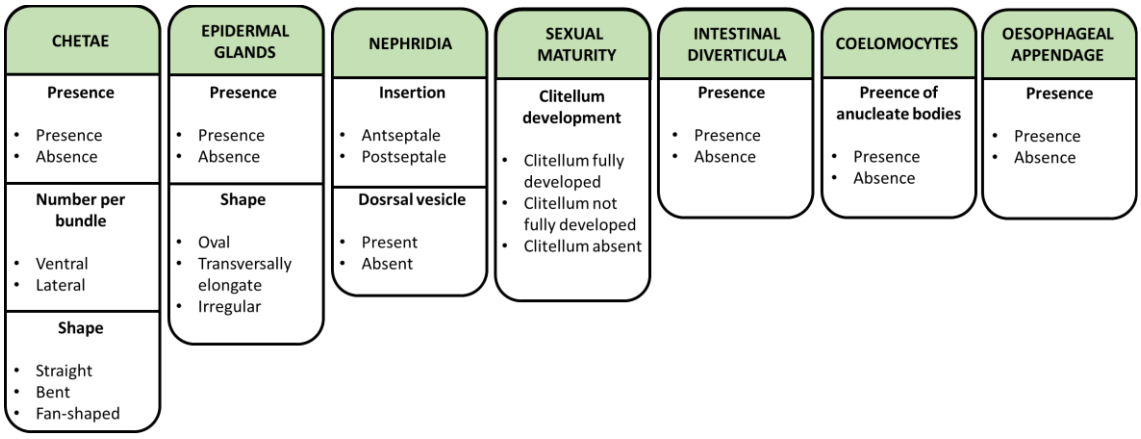
Individuals were extracted by two extraction methods: (i) the hot- wet extraction method (O'CONNOR, 1955) with adaptation from Niva *et al.* (2010, 2015). For that, the soil rings were placed in plastic sieves (15 cm diameter), lined with a porous flannel in a plastic funnel (19 cm diameter) with a hose and a valve attached to its end, the set being filled with water. The sample was heated by a lamp so that the surface temperature of the water reached between 40-50° C. The heat gradient produced by the lamp causes the enchytraeids to move downwards from the soil to the water, falling through the connected valve in the lower end of the funnel. After 2.5 hours of heating, the valve was opened and the water and enchytraeids were collected in containers with a capacity of 1.5 liters. After allowing the sample to decant for about 10 minutes, the excess water was carefully discarded, avoiding the loss of any sediment. The decanted material was transferred to petri dishes and visualized under a stereoscopic microscope to count the enchytraeids. In the (ii) cold-wet extraction method (RÖMBKE, 1995) recommended by ISO 23611-3 (2007), the samples were deposited on a flannel attached to a container with dimensions of 20 cm in diameter x 10 cm in height, filled with water, the organisms will migrate to

the bottom of the container along with the sediments and evaluated daily, with water reposition , during a period of seven days.

3.2.3 Diversity assessment

Live individuals with preserved morphological integrity were identified to the genus level according to Schmelz and Collado (2010) and classified in morphospecies according to seven chosen morphological traits (Figure 6). The identification was carried out *in vivo* through the observation of internal and external morphological characteristics of the enchytraeids under an optical microscope.

Figure 6 – Morphological traits of enchytraeids used in the attribution of morphospecies.



Prepared by the author, 2023.

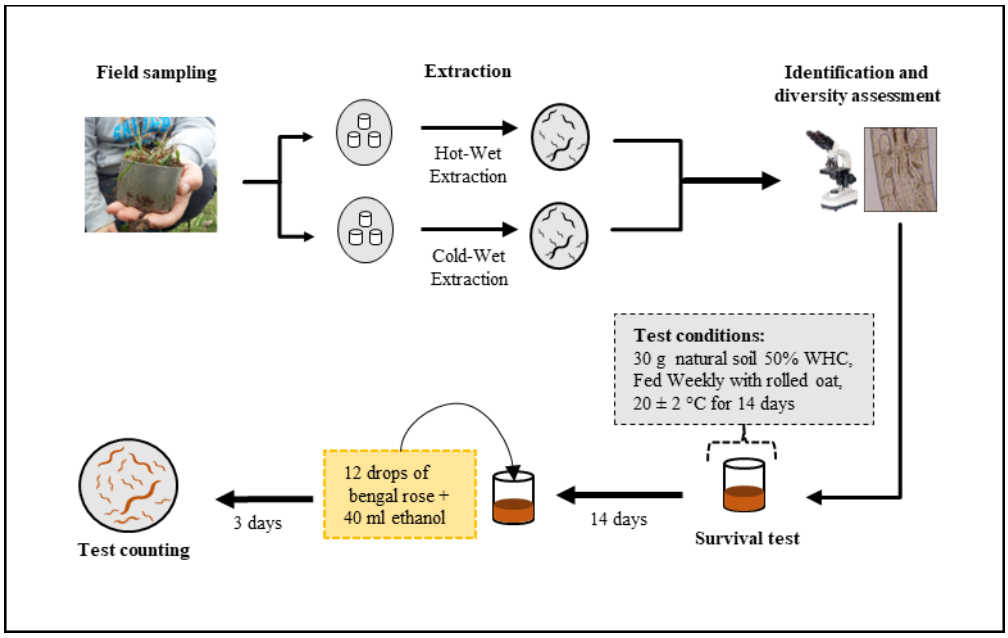
3.2.4 Post-extraction survival-test

After extraction and identification, the individuals were placed in a container containing 30 g of soil from their site of origin, sifted defauned and with 50% of the WHC, placed in a room with controlled temperature (20 ± 2 °C), photoperiod of 16:8h light: dark, provided feeding (rolled oat) and correction of moisture (distilled water) weekly, after 14 days the recovery rate (survival) of the enchytraeids was evaluated (Following the guidelines of lethality test ISO 16387, 2004) as shown in figure 7.

After the test period of tests with enchytraeids 30 mL of alcohol (95%), and twelve drops of Bengal rose stain (1% in ethanol) were added in the test containers to preserve

and color the organisms. After a minimum of 72 h, the organisms were counted using a stereomicroscopic microscope (60× of magnification).

Figure 7 – Framework for the post-extraction survival test with native enchytraeids communities.



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3.2.5 Statistical analysis

For the derivation of sampling effort, species abundance matrices were first imported to EstimateS 9 (Colwell, 2013), with the purpose of calculating rarefaction curves. This way, it is possible to estimate the number of species expected per number of samples, based on the total number of species found in all the samples. Empty samples were not considered for the analysis. The obtained results were then imported to STATISTICA 7 (Statsoft Inc., 2004), where a species accumulation function - in this case, the linear dependence model was applied to all the sites for the estimation of the parameters a and b, as given by the equation **S(t) = a/b * [1-EXP(-b*t)]**,

Where: **t** is a measure of sampling effort, **S(t)** is the predicted number of species at t, **a** is the rate of species increase, **b** a parameter related to the shape of the accumulation of new species with increasing sampling, and **a/b** is the asymptote (estimated maximum richness) (SHIU & LEE, 2003). The remaining procedures to estimate sampling effort

were completed in Excel, by applying the linear dependence model derived equation tq = -1/b ln (1 - q),

Where **tq** is the sampling effort and **q**, a proportion of the asymptote (Shiu and Lee, 2003), to obtain the number of samples required for estimating 60%, 70%, 80% and 90% of diversity for each site. The average number of samples for all the sites was then calculated, as well as the mean for each site.

To evaluate the spatial scale dependence of enchytraeids community diversities, distance–decay models of beta pairwise Bray–Curtis dissimilarities (from 0 to 90 m) using the function "decay.model," in the "vegan" R package were performed.

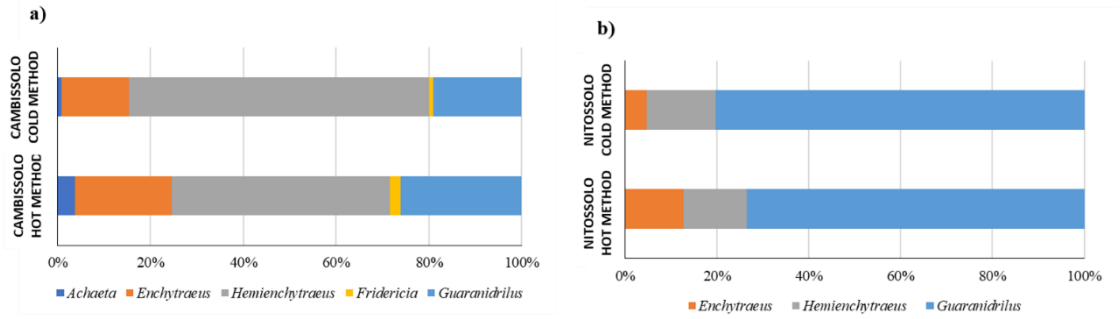
3.3 RESULTS

The enchytraeids were identified until genus and morphospecies level. Five genera were found in Cambissolo (*Achaeta*, *Encytraeus*, *Hemienchytraeus*, *Fridericia* and *Guaranidrilus*) while in Nitossolo only three were found (*Enchytraeus*, *Hemienchytraeus* and *Guaranidrilus*). No difference was found regarding the genus diversity accessed between the extraction method in booth sites (Figure 8).

In Cambissolo 137 individuals were found in hot-method, while 110 were found in cold-method. In Nitossolo, 111 and 98 individuals were found by hot and cold wet methods, respectively. No statistical difference was found in the abundance accessed for each method in both sites.

When evaluating the morphospecies richness and abundance, in Cambissolo 14 morphospecies were found in both methods, while in Nitossolo even with a lower genus richness, a similar morphospecies richness were found, presenting 11 morphospecies in both extraction methods (Table 7).

Figure 8 - Relative abundance of enchytraeid genera for hot and cold extraction method at the evaluated sites in a) Cambissolo and b) Nitossolo.



Prepared by the author, 2023.

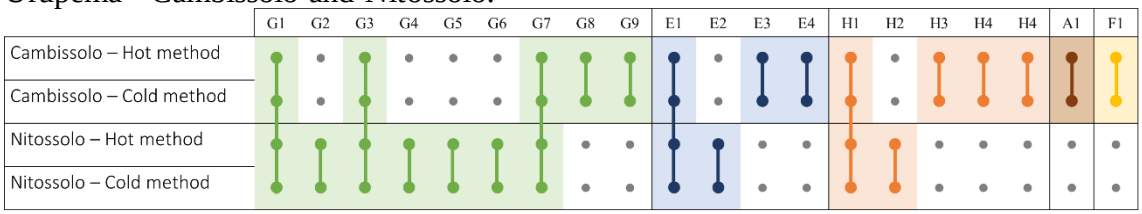
Table 7 – Enchytraeids morphospecies abundance, morphospecies and genus richness in Cambissolo and Nitossolo in Hot and Cold extraction.

Morphospecies	Cambissolo		Nitossolo	
	Hot method	Cold method	Hot method	Cold method
GUA1	8	5	24	23
GUA2	0	0	19	15
GUA3	5	2	12	8
GUA4	0	0	5	3
GUA5	0	0	12	18
GUA6	0	0	3	1
GUA7	1	1	4	12
GUA8	4	4	0	0
GUA9	15	8	0	0
ENC1	24	13	7	2
ENC2	0	0	6	4
ENC3	3	2	0	0
ENC4	3	1	0	0
HEM1	24	36	14	9
HEM2	0	0	5	3
HEM3	10	4	0	0
HEM4	4	4	0	0
HEM5	28	28	0	0
ACH1	5	1	0	0
FRI1	3	1	0	0
Morphospecies richness	14	14	11	11
Genus richness	5	5	3	3
Total abundance	137	110	111	98

Prepared by the author, 2023.

A diagram of occurrence was elaborated observe if the not only the morphospecies richness were the same in both methods, but also the same morphospecies are occurring in both methods in each site (Figure 9).

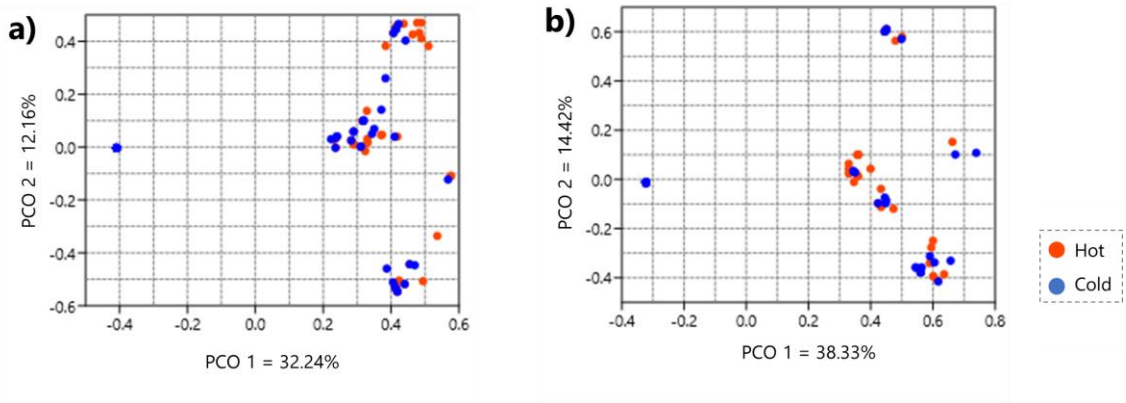
Figure 9 – Diagram of occurrence of morphospecies in each collection method in Urupema - Cambissolo and Nitossolo.



(G) *Guaranidrilus* morphospecies; (E) *Enchytraeus* morphospecies; (H) *Hemienchytraeus* morphospecies; (A) *Achaeta* morphospecies and (F) *Fridericia* morphospecies. Prepared by the author, 2023.

No differences were found when observing the occurrence of morphospecies in each site regardless of extraction method. To compare if the extraction method poses significant effect on the community structure accessed a PCoA analyses were performed (Figure 10).

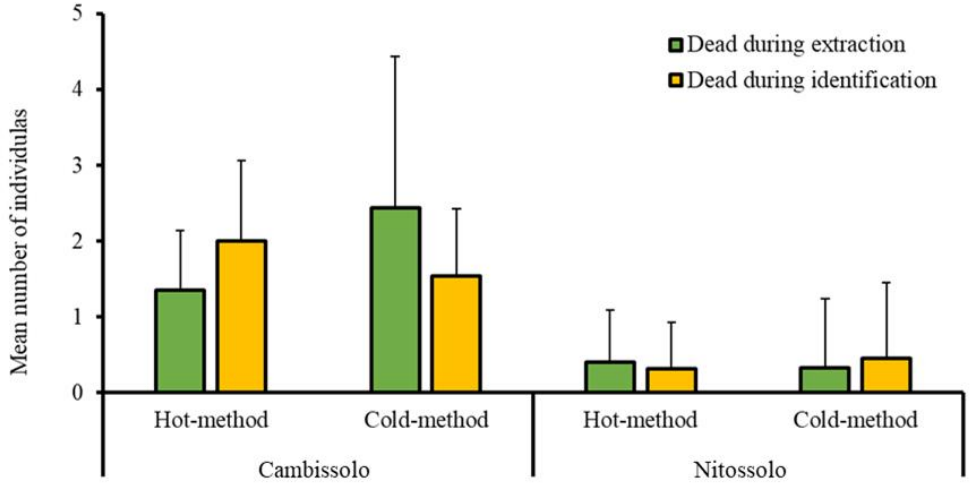
Figure 10 – Principal coordinates Analysis (PCoA) from community obtained by Hot and Cold extraction methods in a) Cambissolo and b) Nitossolo,



Prepared by the author, 2023.

In addition to the effects on the diversity accessed by the extraction methods, other aspects of their performances were evaluated: the number of dead individuals during the process of extraction (Figure 11) in addiction the number of days required for complete extraction in the cold method (Figure 12) were also observed.

Figure 11 - Number of individuals dead during extraction and identification at in cold and hot extraction on Nitssolo (Campo Belo do Sul) and Cambissolo (Urupema).

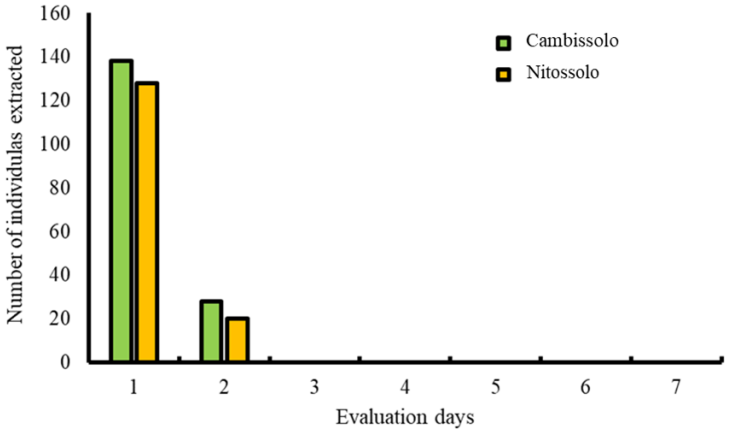


Prepared by the author, 2023.

When comparing the methods in each site, no difference was observed in the mean number of dead individuals during the processes of extraction and identification. When observing the soil type, Nitssolo showed a slightly higher number of dead individuals than Cambissolo. Those differences where possibly due differences in the initial status of the sites (e.g., soil moisture, disturbances…).

To understand the time required to ensure and efficient cold extraction, the number of individuals extracted each day, during sever days were evaluated (Figure 11). After the second day no individuals were extracted from any sample.

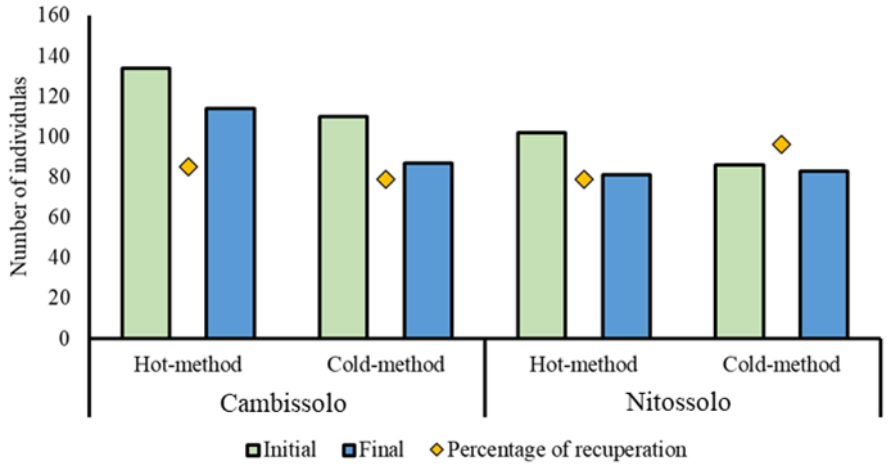
Figure 12 - Number of individuals extracted by cold method for 7 days.



Prepared by the author, 2023.

Ather extraction and identification procedures, the individuals were submitted to an post-extraction survival test, after 14 days the individuals where counted, in figure 13 were showed the number of individuals initially added and the number of individuals observed after 14 days, additionally is presented a percentage of recuperation in relation to the number of individuals initially added to the experimental units.

Figure 13 - Number of individuals at the beginning and end of test, and percentage of recuperation by hot and cold extraction method in Cambissolo and Nitossolo.



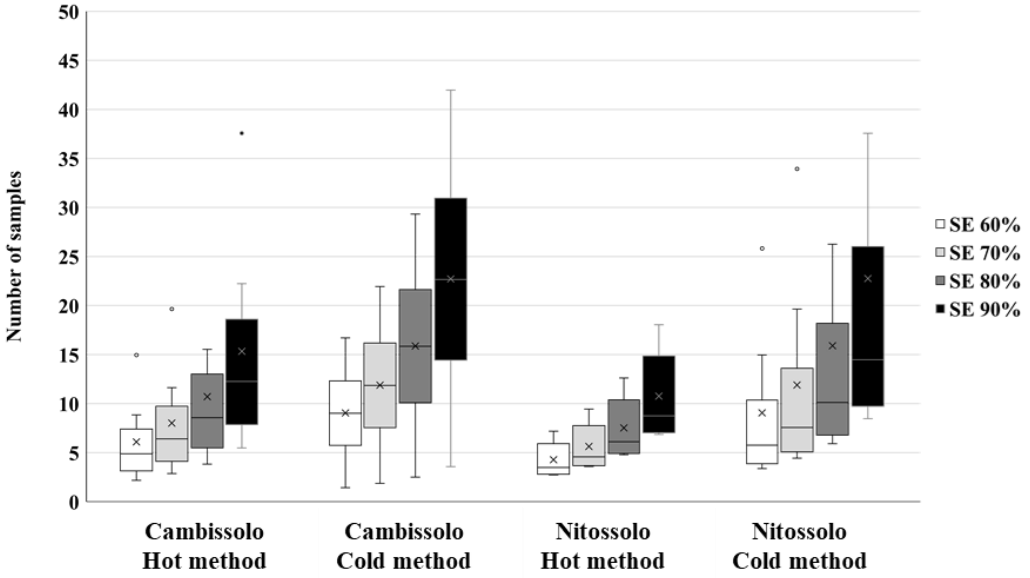
Prepared by the author, 2023.

In all method and sites, a decrease in the number of individuals were observed (Figure 13), being in Nitossolo with the Cold method the highest percentage of recuperation of individuals (96%), followed by Hot-method on Cambissolo (85%) and Cold-method in Cambissolo and Hot method in Nitossolo (79% booth).

The asymptote of the models were 6 points on Hot method in both sites, and by cold method were 8 and 10 in Cambissolo e Nitossolo, respectively. The average sampling effort per extraction method varied significantly (Figure 14), being the col-method the one requiring more samples to obtain a given fraction of species richness, and the method with higher number of samples required to reach the asymptote.

The applied negative exponential model allowed the estimation of the number of samples required to estimate 60, 70, 80 and 90% of the sites richness being, respectively, 6, 8, 11 and 15 in Nitossolo by Hot extraction method; 9, 12, 16 and 23 in Urupema by Cold extraction method, 5, 7, 9 and 13 in Cambissolo by Hot extraction method; 9, 12, 16 and 23 in Cambissolo by Cold extraction method.

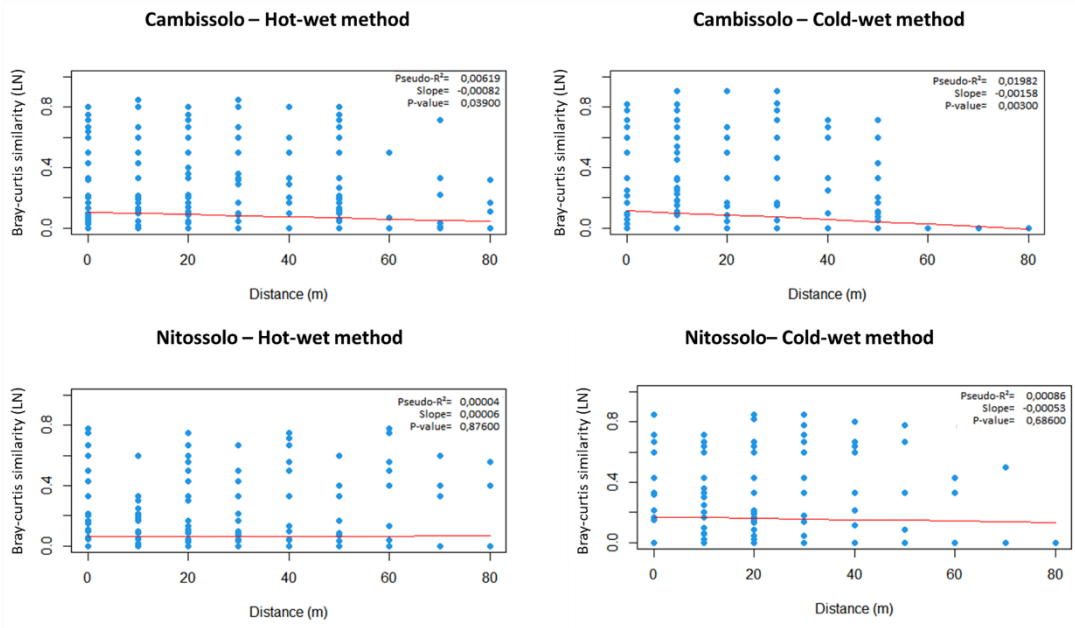
Figure 14 - Boxplot of the median number of samples required to estimate 60, 70, 80 and 80 % of the total richness per extraction method and site.



(CBS) Campo Belo do Sul; (URU) Urupema; (HOT) Hot extraction method; (COLD) Cold extraction method and (SE) Sampling Effort. Prepared by the author, 2023.

To evaluate the spatial scale dependence of enchytraeids community diversities, through distance–decay models of beta pairwise Bray–Curtis dissimilarities (Figure 15)

Figure 15 – Distance-decay patterns among pairs of enchytraeids communities, within samples from 0 to 90 m, for each site and extraction method.



The x-axis represents the distance between pairwise enchytraeids communities and the y-axis represents the Bray-Curtis similarity for each pair of sampling point (blue circles). The red lines represent the fitted

GLM model. Slope = decay rate; Pseudo-R^2= coefficient of determination; p = χ^2-test probability. Prepared by the author, 2023.

Decay rates between enchytraeid pairwise similarities were only significant ($p < 0.05$) in Cambissolo for both extraction methods; with Cold-method (d=-0.00152) and hot-method (d=-0.0082) presenting the larges decay slopes and the highest coefficients of determination (R^2 = 0.00619 and 0.01892 in hot and cold-method, respectively).

Nitossolo does not show a distance-decay pattern, which means that the similarity between sampling points does not decrease with a growth in distance.

3.4 DISCUSSION

The results obtained in this study provide insights into the performance of the two extraction methods in relation to the diversity accessed in the field. The analysis of the genus diversity accessed between the extraction methods in both sites showed no significant differences, indicating that the extraction method did not influence the genus diversity of enchytraeids. This finding supports the hypothesis (i) that the extraction method does not influence the diversity accessed in the field.

When considering morphospecies richness and abundance, the results revealed that both extraction methods sampled similar morphospecies richness in both sites. Indicating that the extraction method did not affect the morphospecies richness accessed. These results support the notion that the extraction method does not impact the community structure accessed in terms of morphospecies composition, providing further evidence in favor of hypothesis (i).

So far two studies aimed to compare the extraction methods Pachenko (2005) collected twelve samples in a pine forest in Sweden, of which six were hot-extracted and six cold-extracted, the author found a greater abundance in the cold-extracted samples, while Niva *et al.* (2015) evaluated a fragment of Araucária Forest (FOM) in the state of Paraná in southern Brazil (n=8), found a better performance of the hot extraction method, given the speed of extraction and a smaller number of damaged individuals.

Furthermore, the abundance of enchytraeids accessed by each extraction method in both sites was not statistically different. This suggests that both extraction methods were equally effective in sampling enchytraeids, regardless of the site. Therefore, the

sampling effort required to obtain a similar abundance of enchytraeids was comparable for both methods, supporting hypothesis (ii) that the sampling effort is positively related to the genus and morphospecies richness.

In terms of number of dead organisms during the processes of extraction and identification none of the methods stood out. The extraction method does not affect the survival of organisms after extraction, the percentage of recuperation of organisms after 14 days were high in both methods. Indicating a similar level of stress caused in the organisms during the extraction in both methods. Nonetheless, the overall similarity in the number of dead individuals suggests that both extraction methods had similar effects on the survival of enchytraeids, thus confirming hypothesis (iii).

Those studies evaluated the methods concerning their performance in taxonomic and ecological studies. To develop a methodology to assess the effects of pollutants on natural communities of enchytraeids, some aspects must be considered – as time and resources necessary for its execution.

The time required for efficient cold extraction was also investigated. The results indicated that after the second day, no individuals were extracted in any of the sites, suggesting that the majority of enchytraeids were extracted within the initial days of the extraction process. This finding implies that a longer extraction period may not significantly increase the number of individuals obtained. Therefore, a short-duration extraction protocol may be sufficient for extracting enchytraeids effectively.

The communities accessed by both methods were similar, soon the method chosen will depend on the available resources, the hot method requires the construction of an extractor, and the number of samples to be extracted will depend on the size of the extractor, however, each batch of extraction takes a maximum of 3 hours. The cold method depends on a simpler structure, although the extraction takes up to three days.

The evaluation of sampling effort revealed that the cold extraction method required more samples to obtain a given fraction of species richness compared to the hot extraction method. Additionally, the cold extraction method showed a higher number of samples required to reach the asymptote. These findings suggest that the cold extraction method may be less efficient in terms of sampling effort and may require a larger number of samples to achieve a desired level of species richness. Conversely, the hot extraction method demonstrated an increase in the number of sampled individuals, indicating that it may be a more efficient approach.

To date, no work has determined the sampling effort required to sample enchytraeids. The guideline ISO 23611-3 (2007) does not establish a sampling scheme, or a minimum number of samples per area. The first proposal of a sampling plan for tropical regions was made by Römbke (2007) in which 10 samplings were carried out in an area of 20 x 50 m, in two parallel lines, the author indicates a minimum of 10 samplings per 1000 m², number also recommended by Niva *et al.* (2010), the authors also address that the samples usually present a high variability, in addition tropical and subtropical regions have low abundance of enchytraeids, so care must be taken in determining the number of samples to ensure a significant number of individuals collected.

The distance-decay analysis of beta pairwise Bray-Curtis dissimilarities provided insights into the spatial scale dependence of enchytraeids community diversities. The results showed significant distance-decay patterns in Urupema for both extraction methods, with the cold extraction method exhibiting a steeper decay slope and higher coefficient of determination compared to the hot extraction method. This suggests that the similarity between sampling points in Urupema decreases rapidly with increasing distance when using the cold extraction method. In contrast, Campo Belo do Sul did not exhibit a distance-decay pattern, indicating that the similarity between sampling points in this site does not decrease with distance.

3.5 CONCLUSION

In conclusion, the results of this study support the hypotheses put forward. The extraction method did not influence the diversity accessed in the field, as evidenced by the comparison of genus and morphospecies richness between the extraction methods in both sites. The sampling effort was positively related to the genus and morphospecies richness, with the cold extraction method requiring more samples to achieve a given fraction of species richness. Additionally, both extraction methods had similar effects on the survival of enchytraeids after extraction. These findings provide valuable insights for the development of a methodology to assess the effects of pollutants on enchytraeids communities and contribute to the optimization of extraction methods and sampling effort in future studies.

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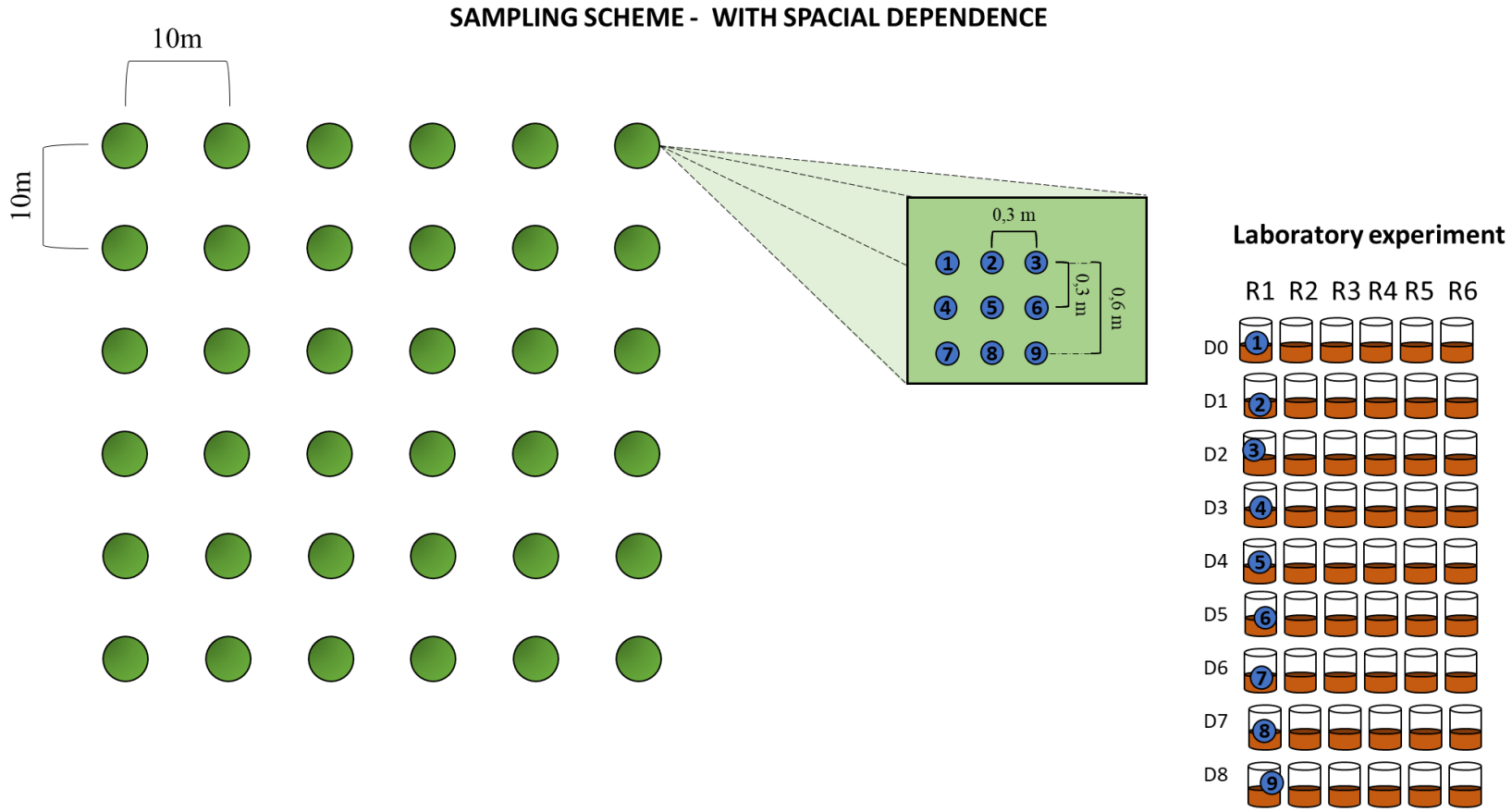
APPENDIX A - Morphospecies found in the study, with their morphological characterization.

Morphospecies codification	Genus	Chaetae	Chaetae format	Lateral Chaetae (n)	Ventral Chaetae (n)	Intestinal diverticulum	Location of Intestinal diverticulum	Oesophaegeal appendage	Full-developed clitellum	Nephridium insertion	Anucleate bodies	Epidermal glands	Epidermal glands format
GUA1	*Guaranidrilus*	Present	Straight	2	2	Present	VI	Absent	Absent	Postseptale	Absent	Absent	-
GUA2	*Guaranidrilus*	Present	Straight	2	2	Present	VI	Absent	Present	Anteseptale	Absent	Present	Transversely elongate
GUA3	*Guaranidrilus*	Present	Straight	2	2	Present	VI	Absent	Present	Postseptale	Absent	Absent	-
GUA4	*Guaranidrilus*	Present	Bent	2	2	Present	n.o.	Absent	Absent	-	Absent	Present	Transversely elongate
ENC1	*Enchytraeus*	Present	Straight	2	3	Absent	-	Absent	Present	Postseptale	Absent	Present	Irregular
HEM1	*Hemienchytraeus*	Present	Straight	2	2	Absent	-	Present	Absent	-	Absent	Absent	-
GUA5	*Guaranidrilus*	Present	Bent	2	2	Present	-	Absent	Present	Postseptale	Absent	Absent	-
ENC2	*Enchytraeus*	Present	Bent	2	3	Absent	-	Absent	Present	Anteseptale	Absent	Absent	-
GUA6	*Guaranidrilus*	Present	Straight	2	2	Present	n.o.	Present	Absent	-	Absent	Absent	-
HEM2	*Hemienchytraeus*	Present	Bent	2	2	Absent	-	Present	Present	n.o.	Presents	Present	Transversely elongate
GUA7	*Guaranidrilus*	Present	Bent	2	2	Present	n.o	Absent	Present	n.o.	Absent	Present	Irregular
GUA8	*Guaranidrilus*	Present	Bent	2	2	Present	n.o.	Absent	Absent	-	Absent	Present	Transversely elongate
GUA9	*Guaranidrilus*	Present	Bent	2	2	Present	n.o.	Absent	Present	n.o	Absent	Present	Transversely elongate
HEM3	*Hemienchytraeus*	Present	Bent	2	2	Absent	-	Present	Absent	-	Absent	Absent	-
HEM4	*Hemienchytraeus*	Present	Bent	2	2	Absent	-	Present	Absent	-	Absent	Present	Transversely elongate
HEM5	*Hemienchytraeus*	Present	Bent	2	2	Absent	-	Present	Present	-	Absent	Absent	-
ENC3	*Enchytraeus*	Present	Bent	2	2	Absent	-	Absent	Absent	-	Absent	Absent	-
GUA9	*Guaranidrilus*	Present	Bent	2	2	Present	n.o.	Absent	Absent	-	Absent	Present	Irregular
FRI1	*Fridericia*	Present	Fan-shaped	4	2	Absent	-	Present	Absent	-	Absent	Present	Transversely elongate
ACH1	*Achaeta*	Absent	-	0	0	Absent	-	Absent	Absent	-	Absent	Present	Irregular
ENC4	*Enchytraeus*	Present	Bent	2	3	Absent	-	Present	Absent	-	Absent	Present	Irregular

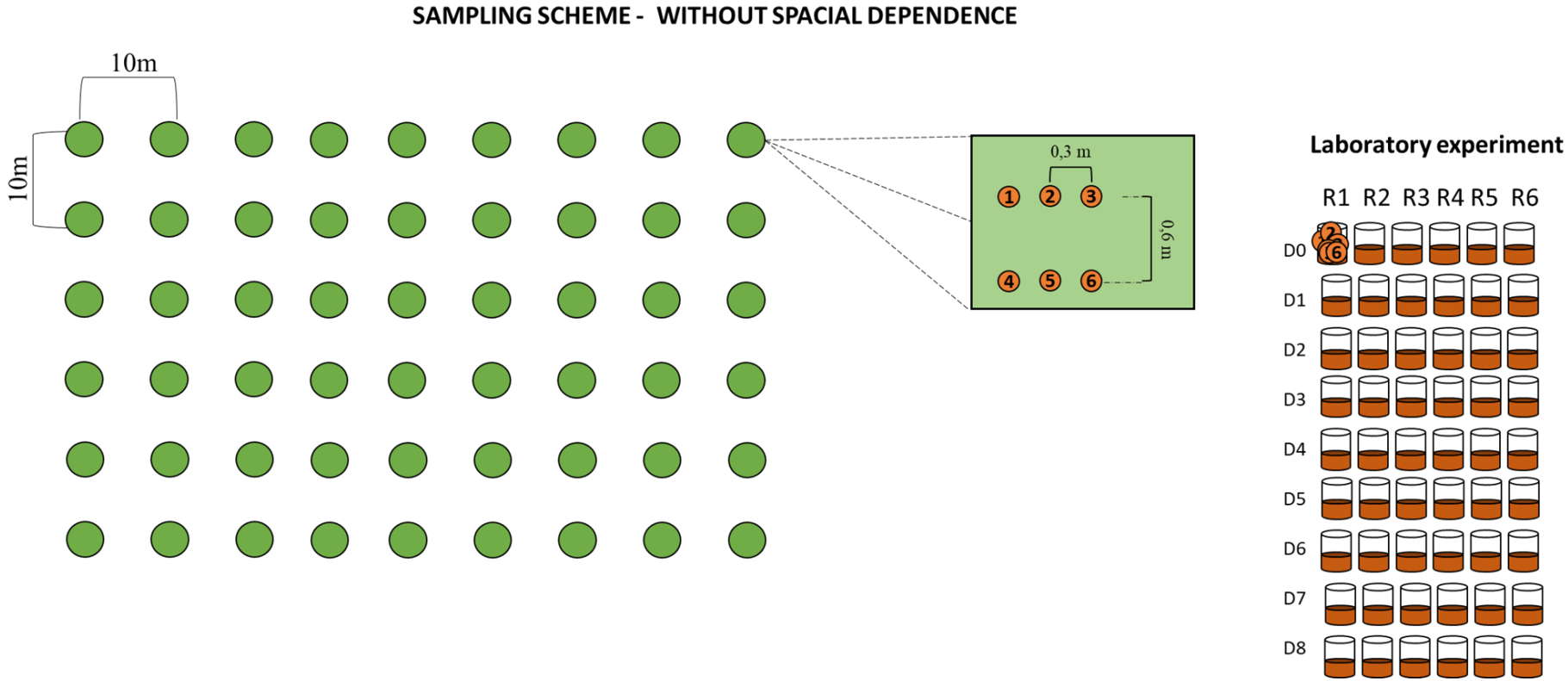
Prepared by the author, 2023.

APPENDIX B – Sampling scheme in sites with spatial dependence.



In sites with spatial dependence, we need to guarantee that the samples collected in the field are well distributed in the treatments. Therefore, at each point a core corresponding to each treatment must be collected.

APPENDIX C – Sampling scheme in sites without spatial dependence.



In sites without spatial dependence, at each sampling point the number of colors needed to form an experimental unit should be collected. Therefore, each point in the field corresponds to a sample unit in the laboratory.

4 CHAPTER 3: RESPONSE OF NATIVE ENCHYTRAEIDS COMMUNITIES UNDER LABORATORY CONDITIONS

4.1 INTRODUCTION

Developing a methodology to evaluate the effects of pesticides on native enchytraeid communities is essential for effective environmental risk assessment. By considering a broader ecological context and incorporating community-level responses, rather than focusing only on individual species, the methodology can provide a more realistic and ecologically relevant understanding of the potential direct and indirect impacts of pesticides (EFSA, 2017). Understanding how enchytraeid communities respond to pesticide exposure, including changes in abundance, diversity, and survival, provides valuable information for conservation efforts and the development of more sustainable pesticide use practices.

Understanding the behavior of natural enchytraeid communities under laboratory conditions is of utmost importance when developing a methodology to assess the effects of pesticides on these communities. The main goal of this research is to assess the effects that temperature and exposure time (days of evaluation) may have on native enchytraeid communities, including potential impacts on community dynamics, including shifts in morphospecies composition and population sizes.

We hypothesized that factors such as temperature and duration of pesticide exposure will influence the response of enchytraeids, including their life cycle and survival, in community tests.

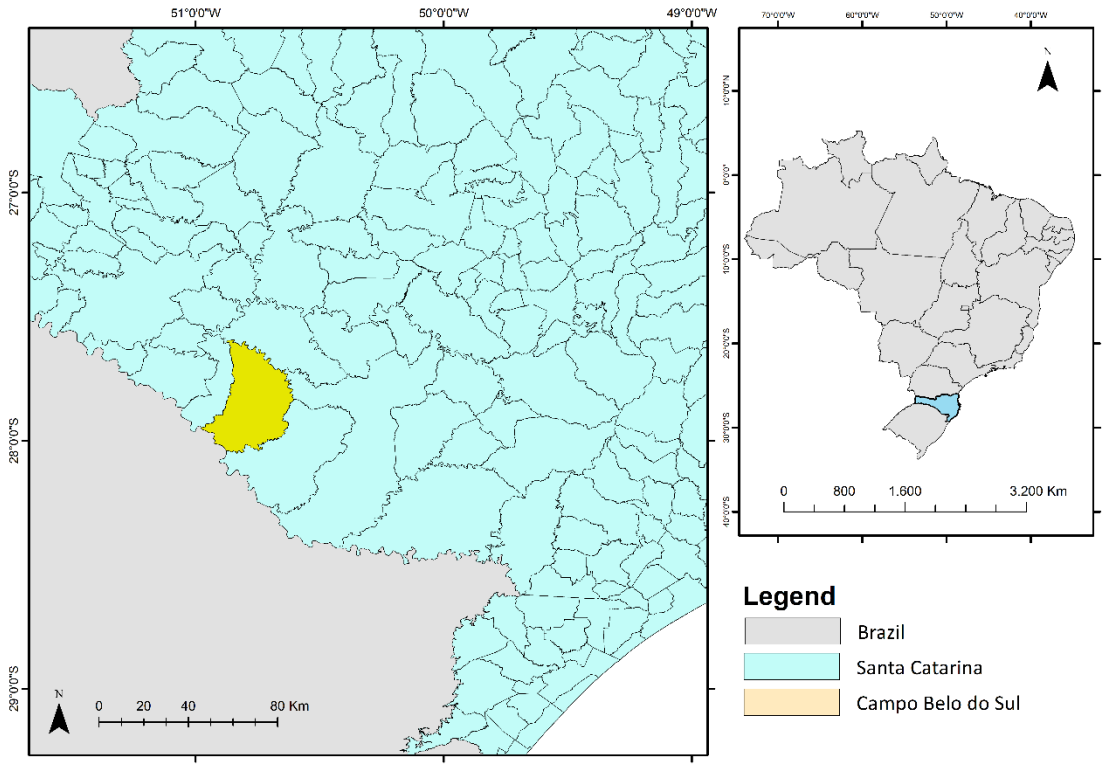
Those evaluations serve as a crucial bridge between controlled experiments and on-field conditions. Hence laboratory experiments allow the manipulation of key variables and precise measurements, enabling a more comprehensive understanding of how enchytraeid communities respond.

4.2 MATERIAL AND METHODS

4.2.1 Characterization of study area

To conduct the study, a natural pastures area with predominance of pastures of the genera *Plantago* and *Axonopus,* with Nitossolo Bruno with no historic of pesticide application were selected in the municipality of Campo Belo do Sul (27°53'42.5" S 50°40'18.4" W) in the plateau of the Santa Catarina State in Brazil (Figure 16). The climate in Campo Belo do Sul is subtropical type Cfb according to the Köppen classification, rainy and with mild winters and summers, and altitude of 923 m asl.

Figure 16 - Location of the study site.

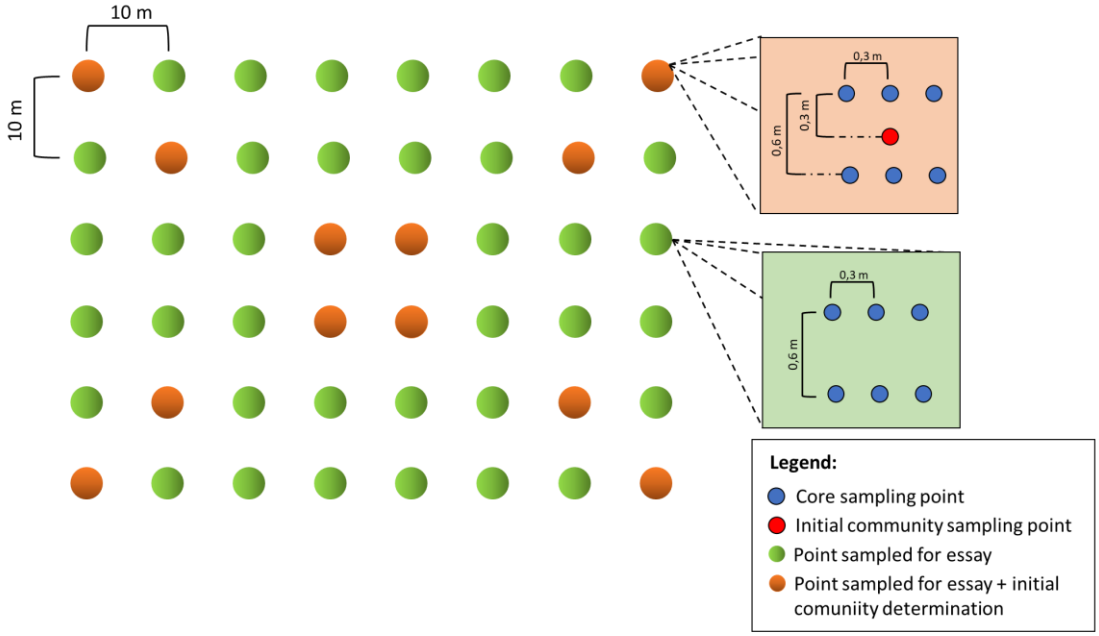


Prepared by the author, 2023.

4.2.2 Sampling and extraction

A grid of 6 x 8 points (n = 48) was established in the field, spaced 10 m apart, at each point 6 cores were collected, in a 2 x 3 grid spaced 0.3 m apart (Figure 17). In addition, on 12 random sampling points one extra core was collected to determinate de initial community (IC) of the area, of the site, and serve as a control parameter.

Figure 17 – Enchytraeids sampling scheme.



Prepared by the author, 2023.

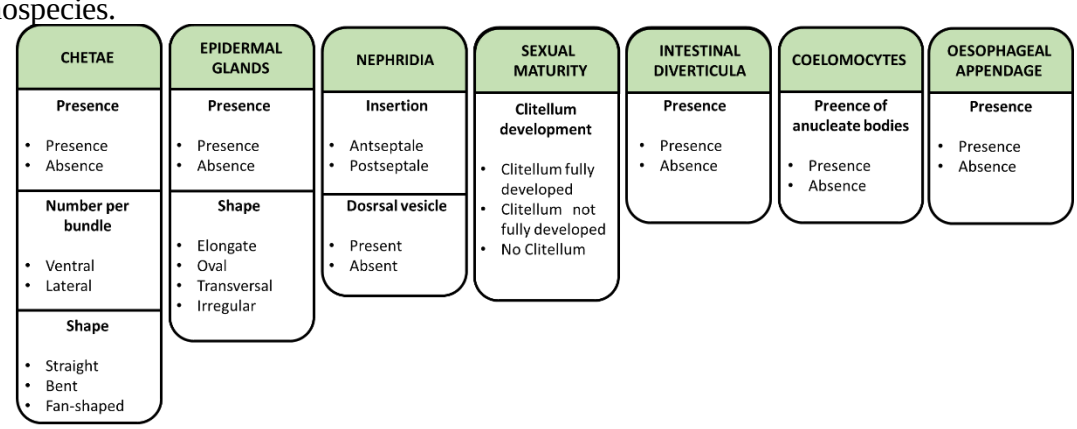
The enchytraeids were collected following the ISO 23611-3 (2007) which consists of collecting soils samples, with a metallic ring measuring 5 x 5 cm. The samples were transported to the laboratory and kept in a temperature-controlled room (16 ± 2 °C) until further processing.

Individuals were extracted by the hot- wet extraction method (O'CONNOR, 1955) with adaptation from Niva *et al.* (2010, 2015). Each samples was placed in plastic sieves (15 cm diameter), lined with a porous flannel in a plastic funnel (19 cm diameter) with a hose and a valve attached to its end, the set being filled with water. The sample was heated by a lamp so that the surface temperature of the water reached between 40-50 °C. The heat gradient produced by the lamp causes the enchytraeids to move downwards from the soil to the water, falling through the connected valve in the lower end of the funnel. After 2.5 hours of heating, the valve was opened and the water and enchytraeids were collected in containers with a capacity of 1.5 liters. After allowing the sample to decant for about 10 minutes, the excess water was carefully discarded, avoiding the loss of any sediment. The decanted material was transferred to petri dishes and visualized under a stereoscopic microscope to count the enchytraeids.

4.2.3 Diversity assessment

Live individuals with preserved morphological integrity were identified to the genus level according to Schmelz and Collado (2010) and classified in morphospecies according to seven morphological traits. The identification was carried out *in vivo* through the observation of internal and external morphological characteristics of the enchytraeids under an optical microscope.

Figure 18 – Morphological traits of enchytraeids used in the attribution of morphospecies.



Prepared by the author, 2023.

4.2.4 Test soils

Two substrates were used, a subtropical soil sampled in native pasture areas without historic of pesticides application, in southern Brazil, in the municipality of Campo Belo do Sul. Soil samples were collected at a depth of 0.00 – 0.10 m, the soil is classified as a Nitossolo (Table 8) the samples were air dried, sieved (2 mm mesh), and then stored in laboratory until further use. Physicochemical characteristics of the soils are shown in table 7. Additionally, a modified version of the Tropical Artificial Soil (TAS) is a proposed by Garcia (2004) and is composed of 75% fine sand (washed and dried), 20% kaolin clay, and 5% coconut coir dust. The pH was adjusted to 6.0 ± 0.5 by the addition of $CaCO_3$.

Table 8 - Physicochemical characteristics of the study soil.

Soil attribute	Nitossolo Bruno
Clay (%)[a]	41
pH in water[a]	4.4
P (mg/dm³) [a]	3.7
K (mg/dm³) [a]	120
Organic matter content (%)[a]	5.5
Al ($cmol_c/dm^3$) [a]	4.7
Ca ($cmol_c/dm^3$) [a]	1.7
Mg ($cmol_c/dm^3$) [a]	1.6

[a] Determined according to Tedesco et al. (1995). Prepared by the author, 2023.

4.2.5 Experiment procedure

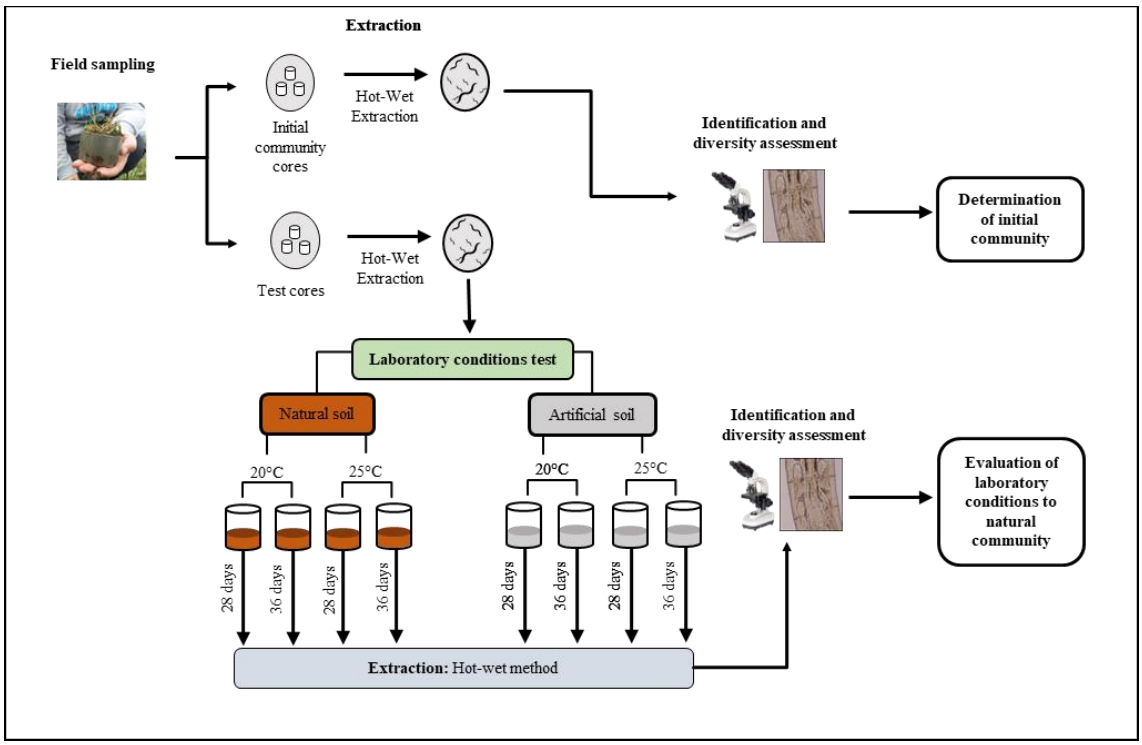
To evaluate the effect of laboratory conditions on native enchytraeids communities, treatments in this study (Figure 19) consisted of three factors with two levels each: substrate type (TAS and Nitossolo), temperature (20 ± 2 °C [ISO, 2014] and 25 ± 2 °C [Niva *et al.*, 2016], and exposure period (28 and 36 days). For each treatment combination, there were six replicates (n=48).

Each unit of the experiment consisted of a plastic container containing 200 g of we substrate. The substrate was moistened with distilled water to reach 50% of its water-holding capacity. The natural community of enchytraeid extracted from a set of 6 cores were introduced into each experimental unit.

Weekly the experimental units were opened the moisture was adjusted (by weight difference), restocked, additionally, 1 g of rolled oats was added to each experimental unit as a food source for the enchytraeids. At the end of each exposure period, the individuals were extracted from soil with the hot-wet methodology, then counted and identified until morphospecies level.

In addiction six cores were collected and identified to determinate the initial community (IC) of the site and serve as a control parameter.

Figure 19 – Experimental procedure



Prepared by the author, 2023.

4.2.6 Data analysis

The number of individuals added to each unit experiment in the beginning of the text were compared to the number of individuals obtained at the end of the test by a Wilcoxon Matched Pairs Test in the software STATISTICA 7.0.

The abundance and richness at the end of the test were compared with the control by a Dunnet test in the software STATISTICA 7.0

To determinate the effect of the factors (substrate type, temperature and expose period) a three-way ANOVA for the abundance and richness matrix were performed with R 3.3.0 (R CORE TEAM, 2018)

To understand the effects of the factors evaluated (substrate type, temperature and duration of test) in the dissimilarity of the enchytraeids communities a PERMANOVA based on the Bray-curtis for abundance matrixes were performed in PAST 4 (2001).

4.3 RESULTS

In the initial community, 107 individuals were found, distributed in 3 genera (*Guaranidrilus*, *Enchytraeus* and *Hemienchytraeus*) and 10 morphospecies.

Regarding to abundance of enchytraeids, the highest number of individuals were observed in the treatments with a duration of 28 days, regardless temperature, and substrate. Specifically, the treatments TAS 20–28, NITO 25–28, and NITO 20–28 showed the highest total abundances (Table 9) indicating that the enchytraeid populations thrived better under these conditions.

Table 9 - Total abundance, morphospecies and genus richness of enchytraeids.

Morphospecies	**IC**	**Treatments**							
		TAS 20-28	**NITO 20-28**	**TAS 20-36**	**NITO 20-36**	**TAS 25-28**	**NITO 25-28**	**TAS 25-36**	**NITO 25-36**
GUA1	39	32	43	21	14	26	28	25	14
GUA2	15	11	2	0	2	2	3	0	33
GUA3	10	16	6	17	9	13	20	7	3
GUA4	9	2	7	0	7	1	1	7	2
GUA5	2	2	0	0	6	8	0	2	1
GUA7	3	0	1	7	2	7	0	0	0
ENC1	14	5	8	3	8	14	2	6	2
ENC2	8	5	6	0	0	1	4	0	4
HEM1	2	8	0	2	5	0	16	3	1
HEM2	5	3	0	0	1	1	0	0	7
Total abundance	**107**	**84**	**73**	**50**	**54**	**73**	**74**	**50**	**67**
Richness	10	9	7	5	9	9	7	6	10
Genus richness	3	3	2	3	3	3	3	3	3

IC (Initial community); TAS (Tropical Aritifical Soil); NITO (Nitossolo); 20 – 28 (20 °C and 28 days); 20 – 36 (20 °C and 36 days); 25 – 28 (25 °C and 28 days); 25 – 36 (25 °C and 28 days); GUA (*Guaranidilus*); ENC (*Enchytraeus*); HEM (*Hemienchytraeus*). Prepared by the author, 2023.

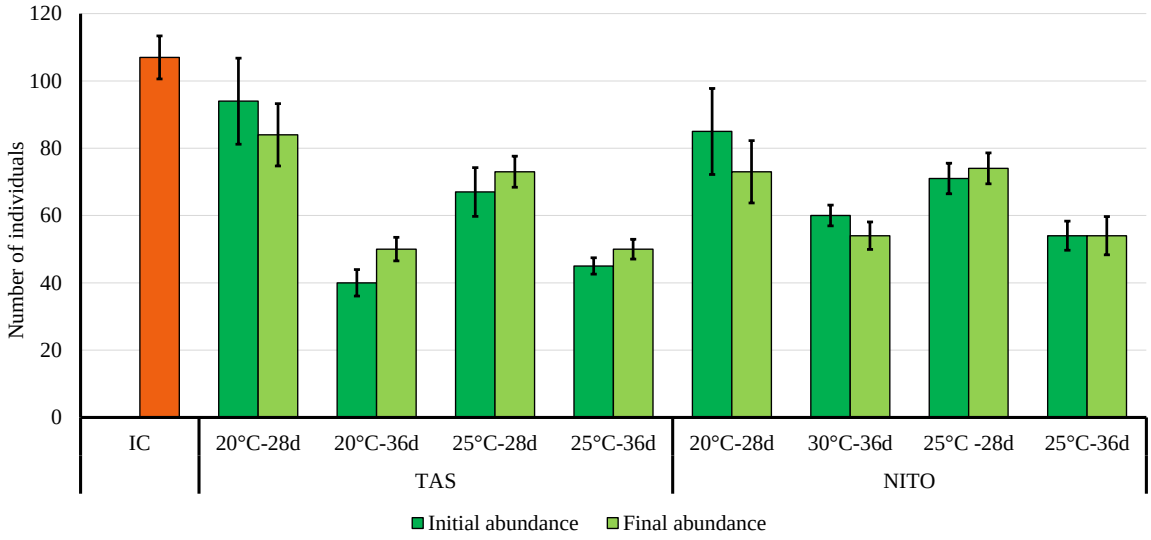
However, it is important to note that the overall abundance did not significantly differ from the control group (IC), indicating that the observed differences were within the natural variability of the system.

Furthermore, the morphospecies richness showed a more complex pattern. The treatment with the highest morphospecies richness was NITO 25–36 (10 morphospecies), suggesting that this specific combination of temperature and duration of exposure might

have a positive effect on enchytraeid diversity. On the other hand, treatments TAS 25–36 and TAS 20–36 exhibited the lowest morphospecies richness (with 6 and 5 morphospecies, respectively).

No differences were found when comparing the number of individuals added initially and the number observed at the end of the tests (Table 10; Figure 20) This indicates that enchytraeid survival was consistent across all treatments, regardless of the initial number of individuals.

Figure 20 - Initial and final average abundance of native enchytraeids in laboratory conditions.



TAS (Tropical Artificial Soil); Nito (Nitossolo); IC (Initial community). Prepared by the author, 2023.

Table 10 - Wilcoxon Matched pairs Test between initial and final abundance of enchytraeids under laboratory conditions.

Treatment	T	Z	p-level
TAS 20 – 28	4.500	0.182	0.855^{ns}
TAS 20 – 36	1.000	1.753	0.797 ^{ns}
NITO 20 – 28	3.000	1.121	0.225 ^{ns}
NITO 20 – 36	7.500	0.629	0.530 ^{ns}
TAS 25 – 28	5.000	0.674	0.500 ^{ns}
TAS 25 – 36	2.000	1.148	0.138 ^{ns}
NITO 25 – 28	7.000	0.135	0.893 ^{ns}
NITO 25 – 36	4.009	0.365	0.715 ^{ns}

$^{(*)}$ significant at p<.0500; (ns) non significative difference between initial and final abundance.; IC (Initial community); TAS (Tropical Artificial Soil); NITO (Nitossolo); 20 – 28 (20° C and 28 days); 20 – 36 (20° C and 36 days); 25 – 28 (25° C and 28 days); 25 – 36 (25° C and 28 days).

To further understand the effects of temperature, substrate type, and duration of exposure, statistical analyses were performed. The three-way ANOVA (Table 11) indicated a significant effect of duration of exposure on abundance (p = 0.0142) This suggests that the length of exposure plays a crucial role in influencing the abundance of enchytraeids in the laboratory setting. However, no other significant effects were observed for the other factors (temperature and substrate type) or their interactions, suggesting that the combined effect of these factors did not significantly impact the abundance and richness of enchytraeids in this study.

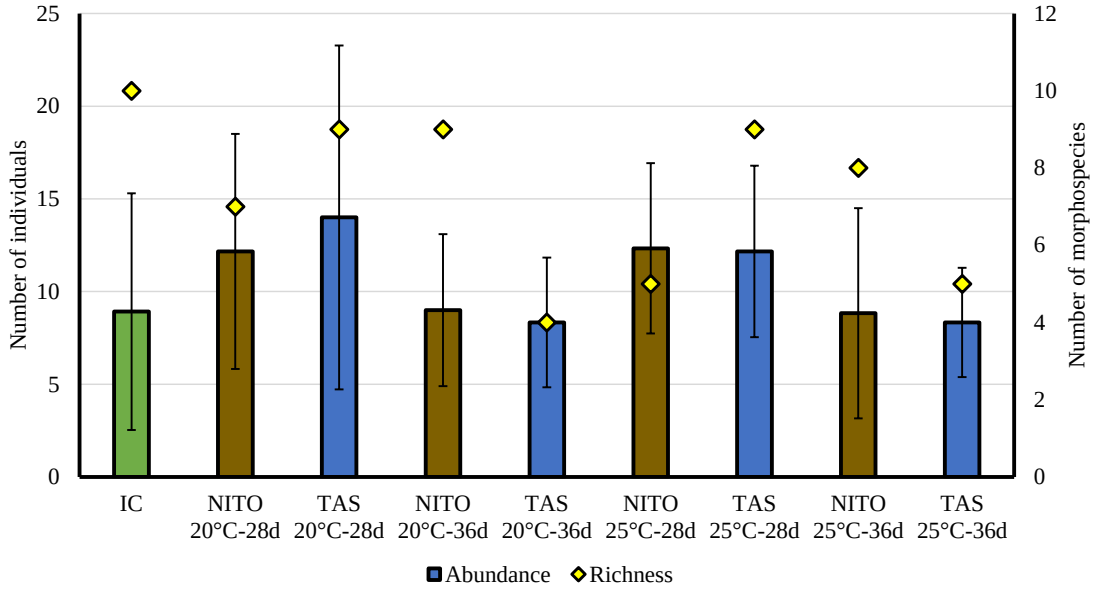
Table 11 - Three-way ANOVA for Abundance and Richness for the factors Temperature, Substrate and Duration of test.

Font	Df	Sum sq	Mean Sq	F value	p
Abundance					
Duration	1	196.0	196.02	6.575	0.0142*
Substrate	1	0.2	0.19	0.006	0.9372^{ns}
Temperature	1	2.5	2.52	0.085	0.7272^{ns}
Duration:Substrate	1	6.0	6.02	0.202	0.6556^{ns}
Duration:Temperature	1	1.7	1.69	0.527	0.8132^{ns}
Substrato:Temprature	1	2.5	2.52	0.85	0.7272^{ns}
Duration:Substrate:Temperature	1	3.5	3.52	0.118	0.7329^{ns}
Residuals	40	1192.5	29.81		
Richness					
Duration	1	5.33	5.333	2.623	0.113^{ns}
Substrate	1	1.33	1.333	0.656	0.423^{ns}
Temperature	1	0.08	0.083	0.041	0.841^{ns}
Duration:Substrate	1	1.33	0.750	0.566	0.423^{ns}
Duration:Temperature	1	0.75	0.750	0.369	0.547^{ns}
Substrato:Temperature	1	0.75	0.750	0.369	0.547^{ns}
Duration:Substrate:Temperature	1	0.75	0.750	0.369	0.547^{ns}
Residuals	40	81.33	2.033		

(n=48; α=5%). Prepared by the author, 2023.

A Dunnet test comparing the abundance and richness values of each treatment with the control group (IC) revealed no significant differences (Figure 21).

Figure 21 – Enchytraeids abundance and morphospecies richness in different substrates, temperatures as exposure days.



IC (Initial community); TAS (Tropical Artificial Soil); NITO (Nitossolo). Prepared by the author, 2023.

Similarly, the PERMANOVA analysis indicated that none of the factors significantly affected the dissimilarity of enchytraeid morphospecies (Table 12). This suggests that the variations in temperature, substrate type, and exposure duration tested in this study did not lead to noticeable shifts in enchytraeid morphospecies composition.

Table 12 - PERMANOVA of enchytraeids communities based on Bray-Curtis coefficient for abundance matrixes.

Source	**Sum of squares**	**df**	**Mean square**	**pseudo-F**	**p**
Substrate	0.0834254	1	0.083425	0.78052	0.5681
Temperature	0.0442538	1	0.044254	0.41403	0.9016
Interaction	0.123603	1	0.1236	11.564	0.3081
Residual	470.294	44	0.10688		
Total	49.542	47			
Substrate	0.0834254	1	0.083425	0.79121	0.5623
Duration	0.166929	1	0.16693	15.832	0.1361
Interaction	0.064503	1	0.064503	0.61175	0.7331
Residual	463.936	44	0.10544		
Total	49.542	47			
Temperature	0.0442538	1	0.044254	0.42243	0.8925
Duration	0.166929	1	0.16693	15.934	0.1411
Interaction	0.1336	1	0.1336	12.753	0.2496
Residual	460.944	44	0.10476		

Total	49.542	47

Prepared by the author, 2023.

4.4 DISCUSSION

Of all the factors evaluated only the exposure time played a significant role in the enchytraeids community, in all the subtracts and temperatures the abundance of enchytraeids tends to be lower in treatments with duration of 36 days compared with the ones with 28 days.

When dealing with natural communities of enchytraeids several aspects of the bioecology and demographic dynamics of these populations are unknown, such as the life cycle, life spawn, reproduction strategies (k or r strategists), reproduction modes (fragmentation, sexual reproduction, parthenogenesis, or self-fertilization), and rates of migration.

Other factors not considered in this study, such as microhabitat preferences or interspecific interactions, could have a stronger influence in the enchytraeid community. Those aspects could be influencing in enchytraeids response under laboratory conditions. In previous experiences in cultivating native enchytraeids on laboratory we observed that enchytraeids survive but don't thrive, we believe that this is due to close interactions between enchytraeids and soil microorganisms or even with their gut microbiome.

Niva *et al* (2010b) tried to cultivate enchytraeids extracted from soil samples in Parana state and observed similar behavior in specimens form genus *Fridericia*, they kept alive and apparently healthy for many months, but did not reproduce, however, they were successful in the cultivation of species of the genus *Enchytraeus*.

Another aspect influencing those dynamics could be the competition for resources as food, or even space. For instance, the species *E. bigeminus* shows different reproduction forms in response to density when cultivate in laboratory, showing sexual reproduction at low densities (less than 300), but suppressing sexual reproduction and favoring fragmentation-regeneration at higher densities (above 400) (CRISTENSEN, 1979).

Furthermore, substrate type was found to influence the reproduction of *E. bigeminus* in laboratory settings. According to Niva *et al.* (2010b) no differences were observed between natural soil (514 individuals) and TAS (455 individuals) but in Agar

1% the reproduction was about 13 times lower than in the other substrates (34 individuals).

It is worth mentioning that in the current experiment no contaminants were added to the soil. Nevertheless, other studies have shown that increasing temperatures can impact the toxicity of pesticides towards soil invertebrates. For example, Hennig *et al* (2022a) observed that the toxicity of Fipronil to *Eisenia andrei* increased at 27 ºC (39.89 $mg.kg^{-1}$) when compared with 25 (312.87 $mg.kg^{-1}$) and 20 °C (277.57 $mg.kg^{-1}$) when exposed in a Oxisol. Likewise, Hennig *et al.* (2022b) observed that the pesticide Fipronil was more toxic to *Foslomia candida* in higher (27 °C) than at lower temperatures (25 and 20°C) when exposed in a TAS and Oxisol.

4.5 CONCLUSION

These findings contribute to our understanding of enchytraeid community dynamics under controlled laboratory conditions. However, it is essential to consider that other untested factors and their interactions might play crucial roles in shaping enchytraeid communities. Further research is needed to investigate additional environmental variables and their potential effects on native enchytraeid communities, providing a more comprehensive understanding of their ecology and responses to environmental changes.

The results of this study demonstrate that temperature, substrate type, and duration of exposure did not significantly influence enchytraeid community dissimilarity. However, the duration of exposure did have a significant effect on the abundance of enchytraeids, with shorter exposure periods leading to higher abundances.

Our findings can contribute to the development and optimization of a methodology to assess the effects of pesticides towards those communities.

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APENDIX D- Morphospecies found in the study, with their morphological characterization.

Morphospecies codification	Genus	Chaetae	Chaetae format	Lateral Chaetae (n)	Ventral Chaetae (n)	Intestinal diverticulum	Location of Intestinal diverticulum	Oesophaegeal appendage	Full-developed clitellum	Nephridium insertion	Anucleate bodies	Epidermal glands	Epidermal glands format
GUA1	*Guaranidrilus*	Present	Straight	2	2	Present	VI	Absent	Absent	Postseptale	Absent	Absent	-
GUA2	*Guaranidrilus*	Present	Straight	2	2	Present	VI	Absent	Present	Anteseptale	Absent	Present	Transversely elongate
GUA3	*Guaranidrilus*	Present	Straight	2	2	Present	VI	Absent	Present	Postseptale	Absent	Absent	-
GUA4	*Guaranidrilus*	Present	Bent	2	2	Present	n.o.	Absent	Absent	-	Absent	Present	Transversely elongate
ENC1	*Enchytraeus*	Present	Straight	2	3	Absent	-	Absent	Present	Postseptale	Absent	Present	Irregular
HEM1	*Hemienchytraeus*	Present	Straight	2	2	Absent	-	Present	Absent	-	Absent	Absent	-
GUA5	*Guaranidrilus*	Present	Bent	2	2	Present	-	Absent	Present	Postseptale	Absent	Absent	-
ENC2	*Enchytraeus*	Present	Bent	2	3	Absent	-	Absent	Present	Anteseptale	Absent	Absent	-
GUA6	*Guaranidrilus*	Present	Straight	2	2	Present	n.o.	Present	Absent	-	Absent	Absent	-
HEM2	*Hemienchytraeus*	Present	Bent	2	2	Absent	-	Present	Present	n.o.	Presents	Present	Transversely elongate
GUA7	*Guaranidrilus*	Present	Bent	2	2	Present	n.o	Absent	Present	n.o.	Absent	Present	Irregular
GUA8	*Guaranidrilus*	Present	Bent	2	2	Present	n.o.	Absent	Absent	-	Absent	Present	Transversely elongate
GUA9	*Guaranidrilus*	Present	Bent	2	2	Present	n.o.	Absent	Present	n.o	Absent	Present	Transversely elongate
HEM3	*Hemienchytraeus*	Present	Bent	2	2	Absent	-	Present	Absent	-	Absent	Absent	-
HEM4	*Hemienchytraeus*	Present	Bent	2	2	Absent	-	Present	Absent	-	Absent	Present	Transversely elongate
HEM5	*Hemienchytraeus*	Present	Bent	2	2	Absent	-	Present	Present	-	Absent	Absent	-
ENC3	*Enchytraeus*	Present	Bent	2	2	Absent	-	Absent	Absent	-	Absent	Absent	-
GUA9	*Guaranidrilus*	Present	Bent	2	2	Present	n.o.	Absent	Absent	-	Absent	Present	Irregular
FRI1	*Fridericia*	Present	Fan-shaped	4	2	Absent	-	Present	Absent	-	Absent	Present	Transversely elongate
ACH1	*Achaeta*	Absent	-	0	0	Absent	-	Absent	Absent	-	Absent	Present	Irregular
ENC4	*Enchytraeus*	Present	Bent	2	3	Absent	-	Present	Absent	-	Absent	Present	Irregular

5 CHAPTER 4: ECOTOXICOLOGICAL TESTS WITH NATIVE ENCHYTRAEIDS COMMUNITES IN SUBTROPICAL SOILS

5.1 INTRODUCTION

Pesticides, while designed to target specific pests, can also impact a wide range of beneficial organisms in the soil ecosystem (LI *et al.*, 2019). Soil-dwelling organisms, such as enchytraeids play crucial roles in nutrient cycling, soil structure maintenance, and overall soil health (ALBRECHTOVÁ *et al.*, 2012). Exposure to pesticides can pose direct effects by impacting their gene expression, disrupting their populations, altering their behaviors, reproduction rates, and even causing mortality. This disruption can also have indirect effects throughout the entire soil food web modifying interactions between individuals and populations, leading to imbalances and potential long-term ecological consequences.

Ecotoxicological tests play a crucial role in assessing the effects of chemicals on soil organisms in their natural environment (BÁNSZEGI *et al.*, 2014). These tests help provide valuable information on the potential risks and impacts of chemicals on the soil ecosystem (MÓNOK *et al.*, 2020). Laboratory single-species tests do not allow to address properly these complex effects of chemical exposure, since they only focus on individual species under highly standardized conditions, which does not accurately represent the intricate interactions and dynamics within a real community setting.

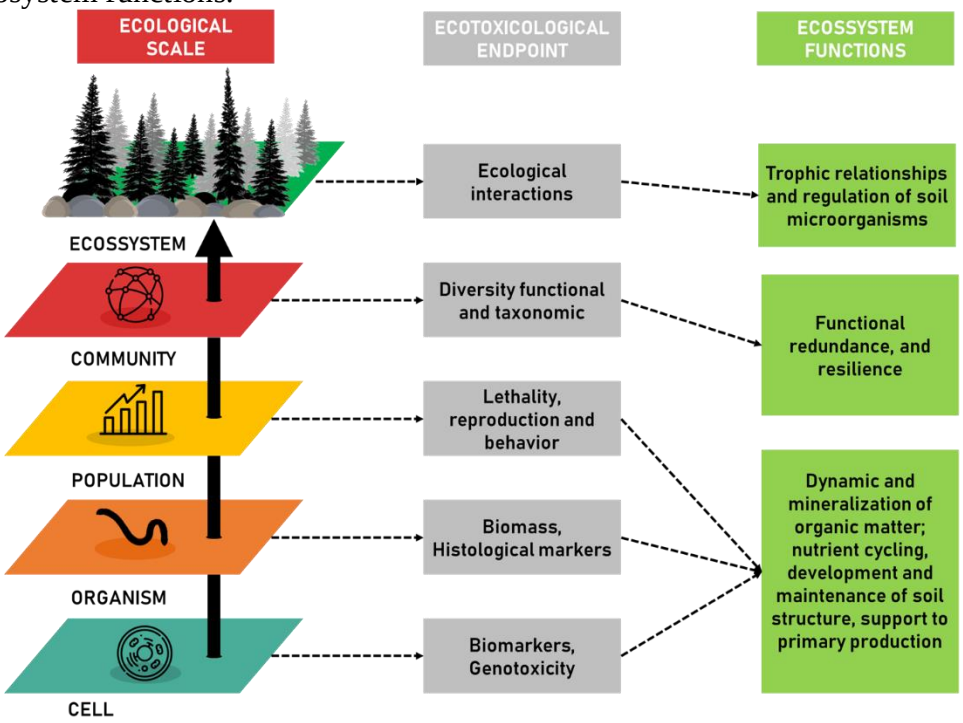
Several standardized ecotoxicological tests are commonly performed with enchytraeids to assess the effects of chemicals on their populations. These tests include acute and chronic tests, which measure the immediate and long-term effects of chemicals, respectively (ISO, 2014.; OCDE, 2004).To gain a comprehensive understanding of the effects of chemical exposure, it is essential to examine not only individual-level responses but also the cascading impacts at higher levels of biological organization. Adopting approaches that allow for a more comprehensive assessment of higher hierarchical levels, including populations, communities, and ecosystems (Figure 21)

The European Commission (2009) emphasizes the importance of considering these multiple levels in the evaluation and management of ecosystem services. Understanding the relationship between changes in community structure and the overall level and stability of ecosystem services is crucial for successful ecosystem management.

This knowledge helps us grasp how the presence of enchytraeids and their interactions with other organisms influence the functioning and resilience of ecosystems over space and time.

The main goal of this study is to validate an ecotoxicological protocol to access the effect of the fungicide Mancozeb towards a natural enchytraeids communities in natural soil. We hypothesize that natural enchytraeids communities are sensitive and will show changes in their structure (abundance, diversity, and composition of genera/morphospecies).

Figure 22 - interrelations between ecological scale, ecotoxicological endpoint evaluated and ecosystem functions.



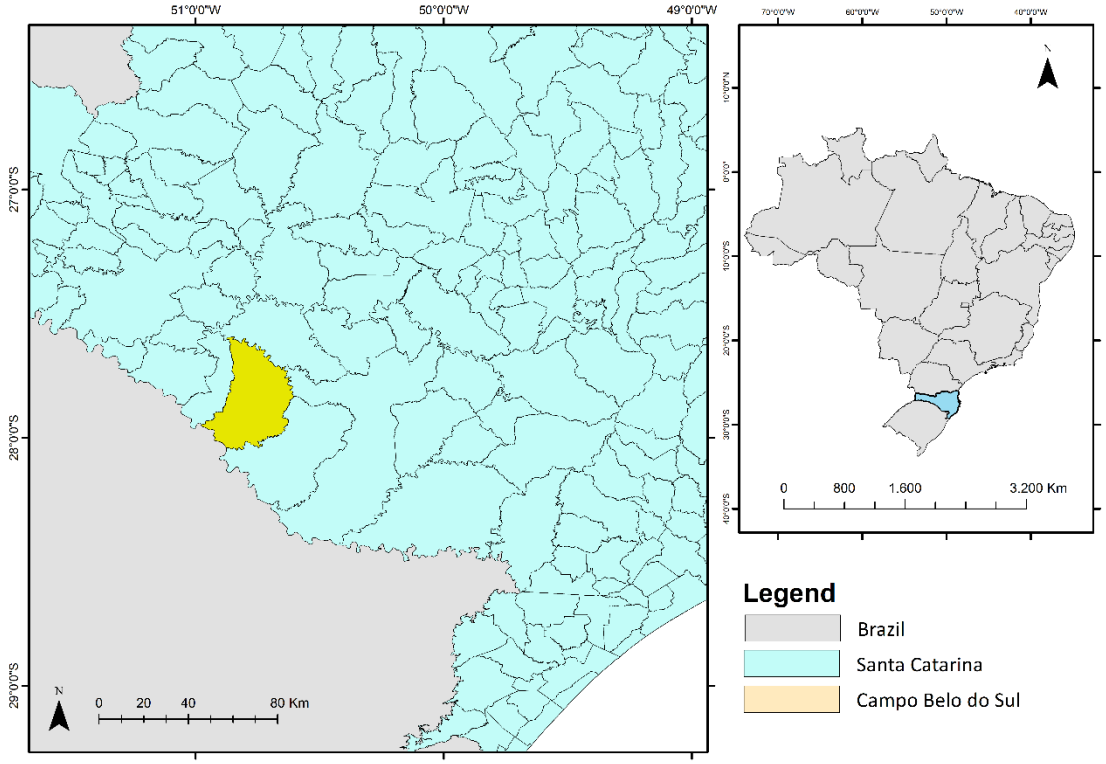
Prepared by the author, 2023.

5.2 MATERIAL AND METHODS

5.2.1 Characterization of study area

To conduct the study, a natural pastures area with predominance of pastures of the genera *Plantago* and *Axonopus*, with Nitossolo Bruno with no historic of pesticide application were selected in the municipality of Campo Belo do Sul (27°53'42.5" S 50°40'18.4" W) in the plateau of the Santa Catarina State in Brazil (Figure 23). The climate in Campo Belo do Sul is subtropical type Cfb according to the Köppen classification, rainy and with mild winters and summers, and altitude of 923 m above sea level.

Figure 23 – Location of the study site.

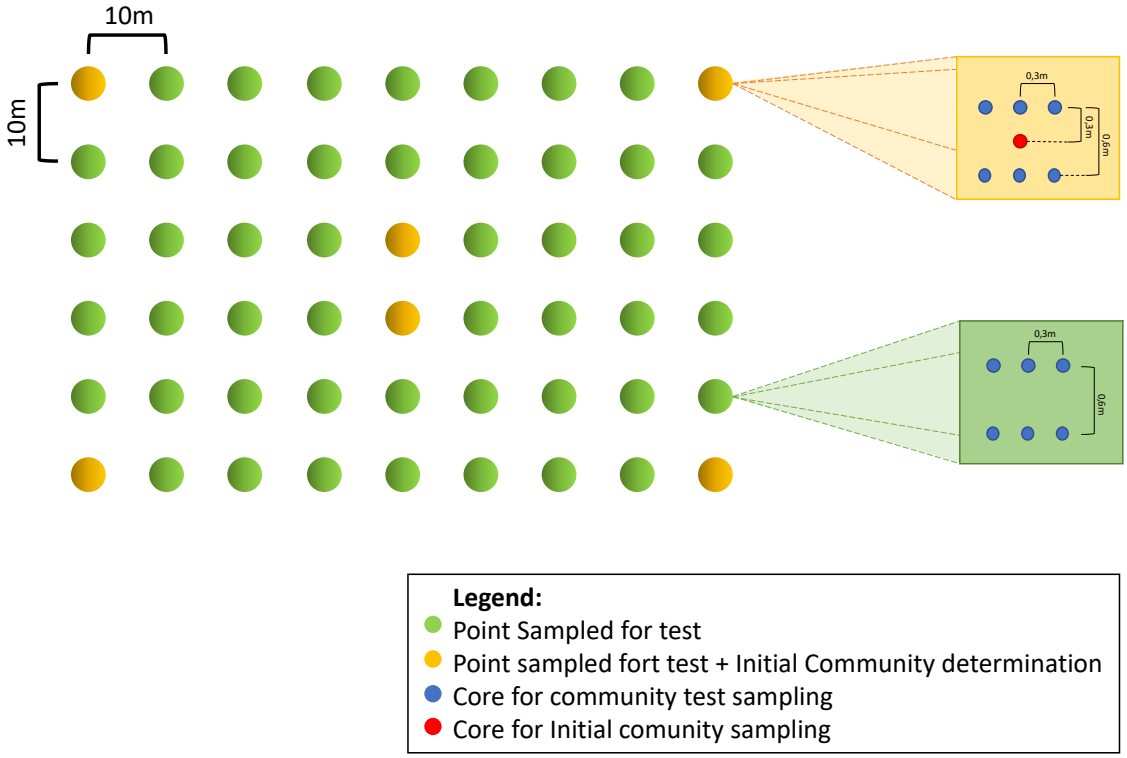


Prepared by the author, 2023.

5.2.2 Sampling and extraction

The enchytraeids were collected following the ISO 23611-3 (2007) which consists of collecting soils samples, with a metallic ring measuring 5 x 5 cm. The samples were transported to the laboratory and kept in a temperature-controlled room (16 ± 2 °C) until further processing (Figure 24).

Figure 24 – Enchytraeids sampling scheme.



Prepared by the author, 2023.

Individuals were extracted by the hot- wet extraction method (O'CONNOR, 1955) with adaptation from Niva *et al.* (2010, 2015). In which the samples were placed in plastic sieves (15 cm diameter), lined with a porous flannel in a plastic funnel (19 cm diameter) with a hose and a valve attached to its end, the set being filled with water. The sample was heated by a lamp so that the surface temperature of the water reached between 40-50 °C. The heat gradient produced by the lamp causes the enchytraeids to move downwards from the soil to the water, falling through the connected valve in the lower end of the funnel. After 2.5 hours of heating, the valve was opened and the water and enchytraeids were collected in containers with a capacity of 1.5 liters. After allowing the sample to decant for about 10 minutes, the excess water was carefully discarded, avoiding the loss

of any sediment. The decanted material was transferred to petri dishes and visualized under a stereoscopic microscope to count the enchytraeids.

5.2.3 Test substrate

A subtropical soil was sampled in native pasture areas without historic of pesticides application, in southern Brazil, in the municipality of Campo Belo do Sul. Soil samples were collected at a depth of 0.00 – 0.10 m, the soil is classified as a Nitossolo, the samples were air dried, sieved (2 mm mesh) and then stored in laboratory until further use. Physicochemical characteristics of the soils are shown in table 13.

Table 13 – Physicochemical attributes of the study soil.

Soil attribute	Nitossolo Bruno
Clay (%)[a]	41
pH in water[a]	4.4
P (mg/dm³) [a]	3.7
K (mg/dm³) [a]	120
Organic matter content (%)[a]	5.5
Al ($cmol_c/dm^3$) [a]	4.7
Ca ($cmol_c/dm^3$) [a]	1.7
Mg ($cmol_c/dm^3$) [a]	1.6

[a] Determined according to Tedesco et al. (1995). Prepared by the aurhor, 2023.

5.2.4 Test substance

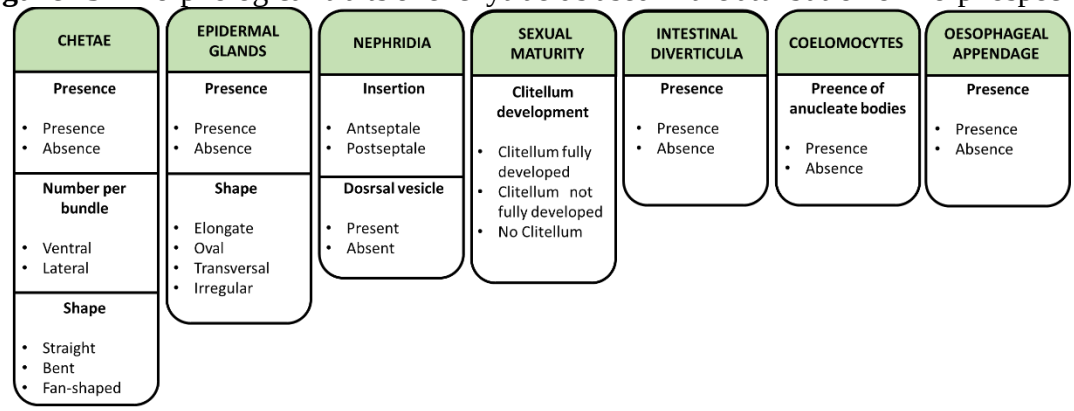
A commercial formulation of the fungicide Mancozeb (Manzate 800®, 800 g a.i. Kg^{-1}) was used for soil contamination. In the concentrations: 0, 0.2, 0.4, 0.8, 1.5, 4.5, 9, 15 and 30 mg a.i kg $soil^{-1}$)

The product characteristics are, Chemical Abstracts Service (CAS): 8018-01-7; Log K_{oW}: 1.33; solubility 6.2 ppm at pH 7.5, 25 °C. Mancozeb has low soil persistence with half-life pointed in literature as less than 2 days in aerobic soils (Xu, 2000) and in European legislation as less than one day (EC, 2009).

5.2.5 Test organisms - diversity assessment

Live individuals with preserved morphological integrity were identified to the genus level according to Schmelz and Collado (2010) and classified in morphospecies according to seven morphological traits (Figure 25). The identification was carried out *in vivo* through the observation of internal and external morphological characteristics of the enchytraeids under an optical microscope.

Figure 25 - Morphological traits of enchytraeids used in the attribution of morphospecies.



Font: prepared by the author, 2023.

5.2.6 Experiment procedure

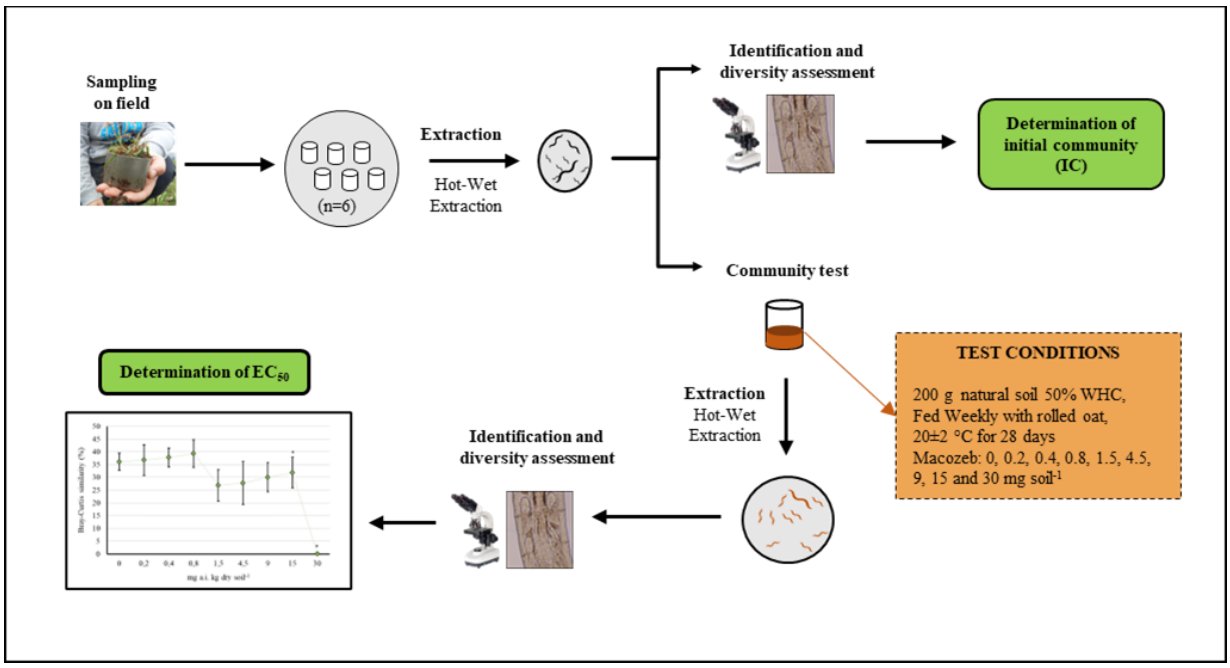
The natural enchytraeids community were extracted from the set of cores sampled in the field (n=6 per experimental unity) and then added to their corresponding treatments (Figure 26).

Each experimental unities consists of a vessel containing 200 g of soil (Contaminated with mancozeb or control, n=6 for each treatment) and the extracted enchytraeid community (from a set of 6 cores per experimental unit).

The vessels were placed in an incubation room (20 +/- 2 °C; 16:8 h of light:dark) for 28 days. During test incubations, weekly: 3 mg of rolled oat was provided to each experimental unit, water content of soil was adjusted with distilled water.

After 28 days, each experimental unit were submitted to extraction, and the organisms were identified to morphospecies levels in the same conditions as the initial (Topics 5.2.2 and 5.2.5).

Figure 26 – Experimental procedures of enchytraeids community test.



Prepared by the author, 2023.

5.2.7 Data analysis

The abundance morphospecies dataset (data transformed to fourth root) was used to calculate Bray-Curtis distance matrices using the software PRIMER 6.0 (CLARKE; GORLEY, 2006). In these matrices, the similarity within control and between control and each concentration were selected to perform dose-response curves. Effective concentrations EC_{50} were estimated using nonlinear regressions using Statistica 7.0 (STAT. SOFT. Inc., 2004).

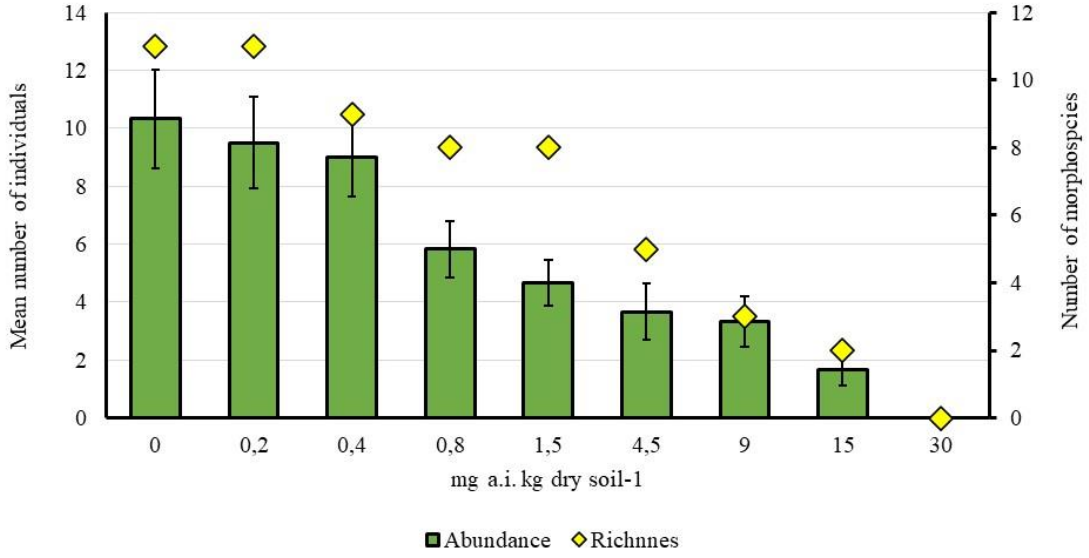
Permutational multivariate analysis of variance (PERMANOVA) was used to verify significant differences ($p < 0.05$) in similarity between control and treatments and allowed to estimate the non-observed effect concentration (NOECs).

In addition, the morphospecies that contributed to significant differences in PERMANOVA test were observed though the similarity of percentages analysis (SIMPER). These analyses were performed using the software PAST 4.0 (2001)

5.3 RESULTS

In figure 27 we can observe the dose-response in abundance and morphospecies richness of enchytraeids from natural communities when exposed to mancozeb in a natural subtropical soil.

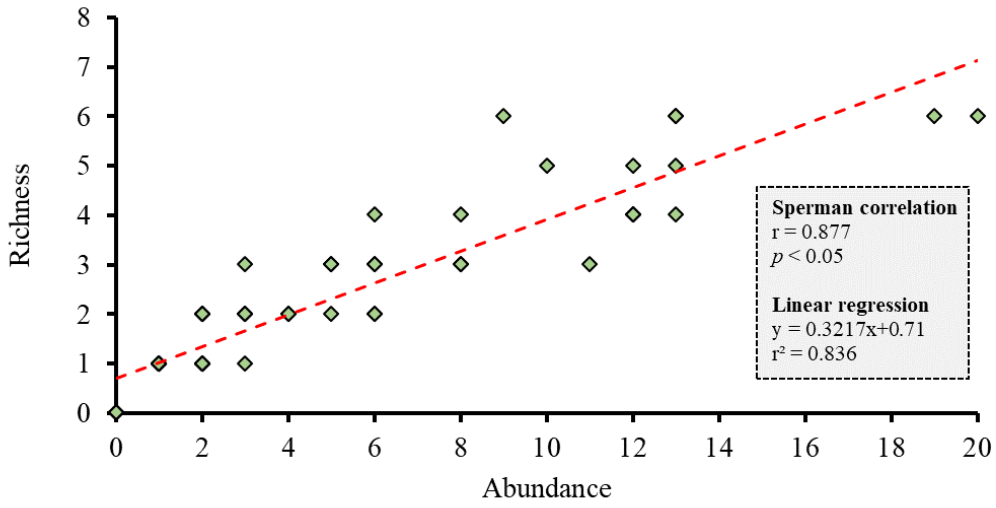
Figure 27 - Natural community enchytraeids mean abundance (±SD) and morphospecies richness in increasing concentrations of Mancozeb in natural subtropical soil.



Prepared by the author, 2023.

To better understand the correlation between the response in abundance and the response in morphospecies richness when exposed to mancozeb in natural soil we performed a liner regression and a spearman's correlations analyses. We found a high positive correlation coefficient (0.877; $p < 0.05$) indicating a positive relation between the responses in enchytraeids abundance and morphospecies richness (Figure 28).

Figure 28 - Correlation between enchytraeids abundance and morphospecies richness on community test in natural subtropical soil spiked with increasing concentrations on Mancozeb.

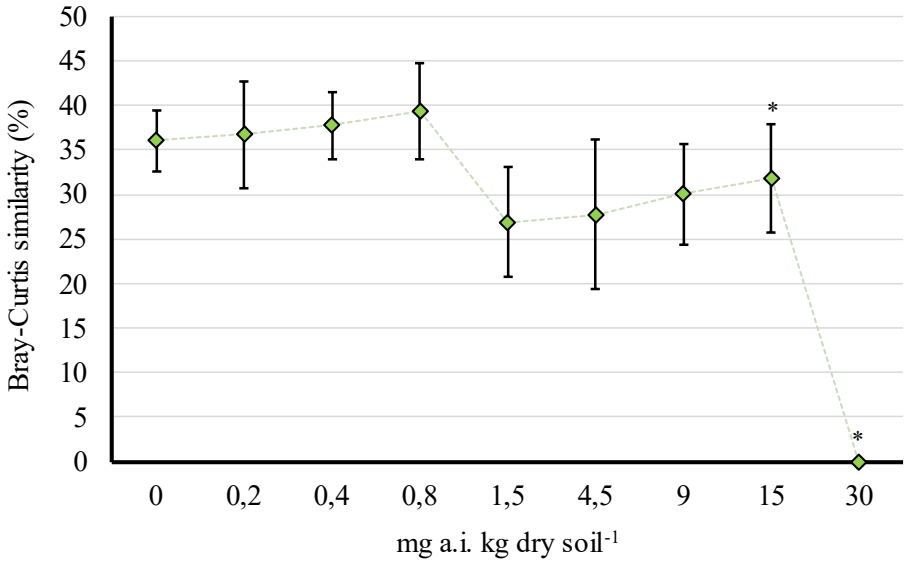


Prepared by the author, 2023.

In figure 29 is shown the similarity between the communities in each treatment and with community in the control. The PERMANOVA indicates that only after the 15 mg soil^{-1} dose the dissimilarity in relation to the control becomes significant. Being the other treatments similar to control.

For the overall enchytraeids community exposed to Mancozeb it was possible to estimate the EC_{50} value (32.753 mg a.i. kg^{-1}) and PERMANOVA analysis pointed to dissimilarities between control from 15.00 mg.kg^{-1} to the highest concentration ($p < 0.05$, NOEC: 9.000 mg a.i. kg^{-1}). It was also possible estimate the EC_{50} value to the enchytraeids abundance (1.310 mg a.i. kg^{-1}) and morphospecies richness (6.294 mg a.i. kg^{-1}), as shown in table 15.

Figure 29 - Enchytraeids community in increasing concentrations of Mancozeb spiked in a natural subtropical soil. Points represents the mean values of similarity to control (±SD).



Asterisks (*) indicates differences between each treatment and control (PERMANOVA, $p < 0.05$). Prepared by the author, 2023.

To better understand which morphospecies are contributing to the differentiation between the treatments indicated by PERMANOVA, a SIMPER analyses (Table 14) where performed. Highlighting the great contributions from GUA1 and GUA 2, booth form genus *Guaranidrilus*, in the differentiations between the treatments and controls and to overall dissimilarity.

Table 14 - Percentage dissimilarities (SIMPER) to statistically differentiate Mancozeb treatments from control (PERMANOVA, $p < 0.05$).

Morphospecies	**Dissimilarity contribution (%)**		
	Control x 15 mg.kg^{-1}	Control x 30 mg.kg^{-1}	Overall
GUA1	26.02	37.57	22.67
GUA2	25.47	20.13	20.13
GUA3	11.00	9.52	10.02
GUA5	10.13	9.11	8.16
ENC2	7.95	6.97	5.38
HEM2	7.41	6.44	3.11
HEM1	5.25	4.42	6.29
GUA7	2.10	1.77	4.86
GUA6	1.59	1.39	6.30
GUA4	1.59	1.39	2.25
ENC1	1.48	1.29	10.84

Contribution > 10% are highlighted. (GUA) *Guaranidrilus;* (ENC) *Enchytraeus*; (HEM) *Hemienchytraeus*. Prepared by the author, 2023.

Table 15 - Toxicity values to Mancozeb estimated trough Enchytraeids abundance, morphospecies richness and dissimilarities between control and treatments on community tests in subtropical natural soil.

Parameter	EC_{50}	NOEC	LOEC
	mg a.i kg soil^{-1}		
Abundance	1.310 (0.109 – 2.511)	0.800	1.500
Richness	6.294 (1.384 – 11.240)	9.000	15.000
Dissimilarity	21.753 (3.457 – 39.959)	9.000	15.000

EC_{50} (Effective concentrations for 50% of effect); NOEC (Non-observed effect concentration); LOEC (Lowest observed concentration effect).

5.4 DISCUSSION

Even the results showing an $EC_{50\text{-dissimilairity}}$ of 21.753 mg kg^{-1}, the morphospecies showed different sensitivity to Mancozeb (Appendix E); GUA4, GUA5 and HEM2 weren't observed in treatments higher than 0.8 mg kg^{-1}, while GUA7, ENC2 and HEM1 weren't observed in treatments higher than 1.5 mg kg^{-1}, GUA3 and GUA6 weren't observed in treatments higher than 4.5 mg kg^{-1}. GUA1 and GUA2 were the less sensitive morphospecies, being observed in all treatments, excepted in the higher dose tested (30 mg kg^{-1}). If the $EC_{50\text{-dissimilarity}}$ (21.753 mg kg^{-1}) were used as a threshold value 8 of 11 morphospecies would be affected. The observed shifts in community structure, as well as the elucidation of specific morphospecies driving these changes, provide valuable information for future ecological risk assessments.

The principal morphospecies driving the dissimilarity between treatments and control belongs to the genus *Guaranidrilus*, this genus is native to South America, with the highest species richness (SCHMELZ e*t al.*, 2013).

Kraft *et al.*, (2022) evaluating the diversity of enchytraeids on native forest and no-tillage system cultivated with soybean in southern Brazil, reported occurrence of

Guaranidrilus only at the Native forest system. Similarly, Alexandre *et al.*, (2022) also reported the occurrence of *Guaranidrilus* only at natural systems int the Atlantic forest and Cerrado biomes. This highlights the sensitivity of this genus to anthropic interventions.

The higher correlation between effects in enchytraeids abundance and morphospecies richness could indicate that they are interchangeable, considering the lack of enchytraeids taxonomists and availability of taxonomic keys, also the time demanded to count the number of individuals *versus* the time required do identify the individuals until genus or morphospecies level, the evaluation of enchytraeids abundance could be an efficient alternative on community tests.

Regarding the effects of Mancozeb on enchytraeids community, there is a lack of available information. However, Carniel *et al.* (2019) found effects on lethality and reproduction of *E. crypticus* in Oxisol (LC_{50} = 6.97 mg soil^{-1}; EC_{50} = 3.56 mg soil^{-1}) and Ultisol (LC_{50} = 280.21 mg soil^{-1}; EC_{50} = 29.67 mg soil^{-1}).

5.5 CONCLUSION

The assessment of community similarity revealed that significant dissimilarity from the control was only evident after the application of a 15 mg a.i kg soil^{-1} of Mancozeb.

The estimation of EC_{50} values provided critical insights into the toxicological impact of Mancozeb on the enchytraeid communities. The abundance of enchytraeids is the most sensitive attribute (EC_{50} = 1.310 mg a.i. kg^{-1}) followed by morphospecies richness (EC_{50} = 6.294 mg a.i. kg^{-1}) and overall community dissimilarity (EC_{50} = 31.753 mg a.i. kg^{-1}). These values offer crucial thresholds for evaluating the potential effects of Mancozeb exposure on these community attributes. And supports our initial hypothesis that natural enchytraeid communities are sensitive to Mancozeb exposure.

Notably, we observed a strong positive correlation between enchytraeid responses on abundance and morphospecies richness, implying that shifts in these parameters were closely linked. And the evaluation of enchytraeids abundance is a great endpoint in community tests.

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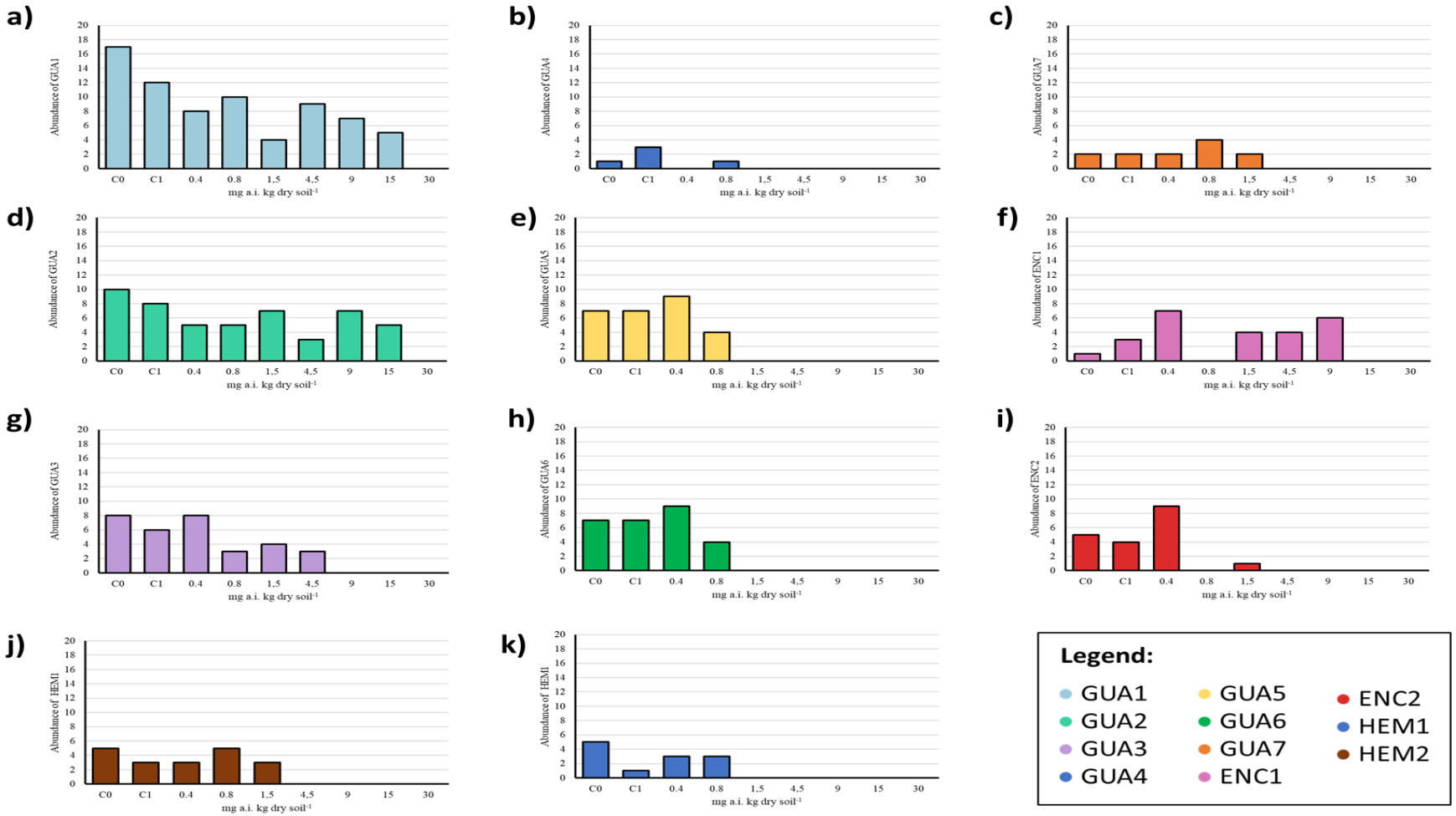
APENDIX D- Morphospecies found in the study, with their morphological characterization.

Morphospecies codification	Genus	Chaetae	Chaetae format	Lateral Chaetae (n)	Ventral Chaetae (n)	Intestinal diverticulum	Location of Intestinal diverticulum	Oesophaegeal appendage	Full-developed clitellum	Nephridium insertion	Anucleate bodies	Epidermal glands	Epidermal glands format
GUA1	*Guaranidrilus*	Present	Straight	2	2	Present	VI	Absent	Absent	Postseptale	Absent	Absent	-
GUA2	*Guaranidrilus*	Present	Straight	2	2	Present	VI	Absent	Present	Anteseptale	Absent	Present	Transversely elongate
GUA3	*Guaranidrilus*	Present	Straight	2	2	Present	VI	Absent	Present	Postseptale	Absent	Absent	-
GUA4	*Guaranidrilus*	Present	Bent	2	2	Present	n.o.	Absent	Absent	-	Absent	Present	Transversely elongate
ENC1	*Enchytraeus*	Present	Straight	2	3	Absent	-	Absent	Present	Postseptale	Absent	Present	Irregular
HEM1	*Hemienchytraeus*	Present	Straight	2	2	Absent	-	Present	Absent	-	Absent	Absent	-
GUA5	*Guaranidrilus*	Present	Bent	2	2	Present	-	Absent	Present	Postseptale	Absent	Absent	-
ENC2	*Enchytraeus*	Present	Bent	2	3	Absent	-	Absent	Present	Anteseptale	Absent	Absent	-
GUA6	*Guaranidrilus*	Present	Straight	2	2	Present	n.o.	Present	Absent	-	Absent	Absent	-
HEM2	*Hemienchytraeus*	Present	Bent	2	2	Absent	-	Present	Present	n.o.	Presents	Present	Transversely elongate
GUA7	*Guaranidrilus*	Present	Bent	2	2	Present	n.o	Absent	Present	n.o.	Absent	Present	Irregular
GUA8	*Guaranidrilus*	Present	Bent	2	2	Present	n.o.	Absent	Absent	-	Absent	Present	Transversely elongate
GUA9	*Guaranidrilus*	Present	Bent	2	2	Present	n.o.	Absent	Present	n.o	Absent	Present	Transversely elongate
HEM3	*Hemienchytraeus*	Present	Bent	2	2	Absent	-	Present	Absent	-	Absent	Absent	-
HEM4	*Hemienchytraeus*	Present	Bent	2	2	Absent	-	Present	Absent	-	Absent	Present	Transversely elongate
HEM5	*Hemienchytraeus*	Present	Bent	2	2	Absent	-	Present	Present	-	Absent	Absent	-
ENC3	*Enchytraeus*	Present	Bent	2	2	Absent	-	Absent	Absent	-	Absent	Absent	-
GUA9	*Guaranidrilus*	Present	Bent	2	2	Present	n.o.	Absent	Absent	-	Absent	Present	Irregular
FRI1	*Fridericia*	Present	Fan-shaped	4	2	Absent	-	Present	Absent	-	Absent	Present	Transversely elongate
ACH1	*Achaeta*	Absent	-	0	0	Absent	-	Absent	Absent	-	Absent	Present	Irregular
ENC4	*Enchytraeus*	Present	Bent	2	3	Absent	-	Present	Absent	-	Absent	Present	Irregular

Prepared by the author, 2023.

APPENDIX E- Enchytraeids morphospecies abundance in communities tests.



Prepared by the author, 2023

6 CHAPTER 5: BORIC ACID AS A SUSBTANCE REFERENCE FOR ECOTOXICOLOGICAL TESTS WITH NON-STANDARDIZED ENCHYTRAEIDS SPECIES IN SUBTROPICAL ARTIFICIAL SOIL

6.1 INTRODUCTION

Standardized test methods are essential for assessing the harmful effects of chemicals (RÖMBKE; AHTIAINEN, 2007). According to Gourmelon and Ahtiainen (2007) the selection of an appropriate reference substance, also known as positive control, is a critical aspect of validating a new test method. Also, regular testing of reference substances, with known toxicity, is necessary to ensure the continuous sensitivity of the test cultivated organisms and to demonstrate the precision and reliability of laboratory data (EC, 2004; OECD 2005).

Initially, various reference substances, primarily pesticides, are used for each of the established or developing test methods, such as Carbendazin for enchytraeids (ISO 11268-2, 2014), a substance no longer available on UE and Brazil. Gourmelon and Ahtiainen (2007) describes criteria recommend in the selection of a reference substance as; (i) the substance should be bioavailable during the test and it affects the respective test species and the chosen endpoint in a reproducible way, (ii) it shouldn't be too difficult to obtain, (iii) should be feasible to handle in the laboratory (manageable health and environmental risks), and (iv) a practical and affordable analytical method is available.

According to OECD and ISO criteria, it has been proposed that effects should occur within a reasonable range, i.e., <1,000 $mg.kg^{-1}$ for reasons of practicality, costs and because higher concentrations are difficult to achieve in practice, but the absolute toxicity shouldn't be significant criterion for selecting a reference substance (RÖMBKE; AHTIAINEN, 2007; BECKER *et al.* 2011).

Boric Acid has been suggested as a substance reference for all soil ecological test guidelines (RÖMBKE; AHTAINEN, 2007; BECKER *et al.*, 2011; AMORIN *et al.*, 2012), since then a broaden set of data was developed for soils organisms, as demonstrated by Princz *et al.* (2017), and has been incorporated into international guidelines (EC, 2004; EC, 2005; ISO, 2008; ISO, 2012).

Most of the data are for OECD soil and temperate regions, lacking information on Tropical Artificial Soil (TAS) and using non-standardized species.

We hypothesize that Boric Acid is a suitable substance reference for ecotoxicological tests with non-standardized enchytraeids species in Artificial Subtropical Soil (TAS).

The aim of this study is to evaluate the suitability of boric acid (BA) as a refence substance for ecotoxicological tests with non-standardized enchytraeids species in subtropical regions.

6.2 MATERIAL AND METHODS

6.2.1 Test substrate

Tropical Artificial Soil (TAS) and is composed of 75% fine sand (washed and dried), 20% kaolin clay, and 5% coconut coir dust (OECD, 2016). The pH was adjusted to 6.0 ± 0.5 by the addition of $CaCO_3$.

6.2.2 Test organisms

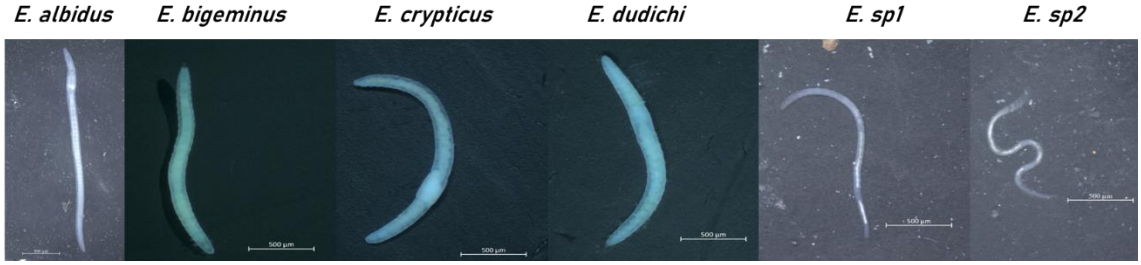
Enchytraeids of six distinct species were used to conduct the reproduction tests (Figure 30):

- The species *Enchytraeus crypticus*, a standardized species, is widely used in ecotoxicological tests (ISO, 2004; KUPERMAN et al., 2004; ZHANG et al., 2019). The species reproduces by self-fertilization, cross-fertilization, and fragmentation (COLLADO et al, 2012; GONÇALVES et al., 2016).
- The species *Enchytraeus albidus* is a standardized species, widely used in ecotoxicological tests (ISO, 2004).
- The species *Enchytraeus bigeminus* reproduces primarily by fragmentation, but at low densities it can reproduce sexually (CRISTENSEN, 1973).
- The species *Enchytraeus dudichi* reproduces by fragmentation and has physiological similarities with *E. bigeminus* (NIVA et al., 2010).
- A species collected (in the state of Santa Catarina, Brazil) and cultivated by researcher Douglas Alexandre (Laboratório de Ecologia do Solo – UDESC/CAV),

belonging to the genus *Enchytraeus*, with pending description, henceforth referred to as *Enchytraeus sp. 1*. The species reproduces sexually.

- A species collected (at Parana State, Brazil) and cultivated by researcher Cintia Carla Niva (Embrapa Cerrados) belonging to genus *Enchytraeus*– with pending description, henceforth called *Enchytraeus sp. 2*. The species reproduces by fragmentation.

Figure 30 – Enchytraeids species used in the experiments.



Prepared by the author, 2023.

The organisms were cultivated in plastic containers containing SAT (Garcia; 2004) adapted for 5% coconut fiber), in a controlled environment (temperature 20 ± 2 °C; photoperiod 18:8h light:dark), the cultures were fed weekly with rolled oats, and had their humidity corrected with mineral water following the recommendations of ISO 16387 (ISO 2014),

6.2.3 Experiment procedure

Reproduction tests were carried out based on the procedures described in ISO standard 16387 (ISO, 2014) and Bandow *et al.* (2013).

For each test, a gradient of laboratory spiked soil with increasing concentrations of Boric Acid (Table 16) was achieved. Each gradient was prepared by a stock solution diluting BA in distilled water. The concentrations of each gradient were selected based on literature data (Niermeyer et al., 2018) and preliminary laboratory tests to assess the full dose-response relationships and to allow the estimation EC_{50} values for each species.

All tests were performed under a photoperiod of 16:8h light: dark and at 20 ºC. Over the experiments, test containers were opened weekly to allow aeration and weight loss of the replicates was reestablished by the addition of distilled water to compensate water losses.

After 21 days, 30 mL of alcohol (95%), and twelve drops of Bengal rose stain (1% in ethanol) were added in the test containers to preserve and color the organisms. After a minimum of 72 h, the organisms were counted using a stereomicroscopic microscope (60× of magnification).

Table 16 - Concentrations of Boric Acid adopted to reproduction tests with *E. albidus, E. bigeminus. E. crypticus, E. dudichi, Enchytraeus* sp. 1 and *Enchytraeus* sp.2 in Subtropical Artificial Soil (TAS).

Species	**Range of concentrations tested (**mg a.i kg^{-1})
E. albidus	0; 12; 25; 50; 100; 200; 400
E. crypticus	0; 25; 50; 100; 200; 400
E. dudichi	0; 2; 4; 8; 16; 32; 64
E. bigeminus	0; 6; 12; 25; 50; 100; 200; 400
Enchytraeus sp. 1	0;25;50;100;200; 400
Enchytraeus sp. 2	0; 25; 50; 100; 200; 400

Prepared by the author, 2023.

6.2.4 Data analysis

In each test, the number of juveniles was compared between contaminated and control soils through analysis of variance (one-way ANOVA), followed by Dunnett's post hoc test. Normality and homogeneity of variances of data were verified by Kolmogorov-Smirnov test and Levene's tests, respectively. Statistical differences found in this analysis allowed to establish NOEC (non-observed effect concentration) and LOEC (low observed effect concentration). Effective concentrations 50% of effect (EC_{50}) were estimated considering measured concentrations and using nonlinear models (ENVIRONMENTAL CANADA, 2007) in Statistica 7.0 software (STATSOFT, Inc., 2004).

6.3 RESULTS

The tests performed were valid according to guideline ISO 16387 (2014) and Bandow *et al.* (2013) (number of juveniles >25 and CV <50%.). Results of BA exposure to Enchytraeids can be observed in Fig. 30 in terms of effects on reproduction. All EC_{50}, LOEC and NOEC values are presented in Table 17.

Table 17 - Reproduction EC_{50}, NOEC and LOEC for enchytraeids species exposed to Tropical Artificial Soil spiked with increasing concentration of Boric Acid.

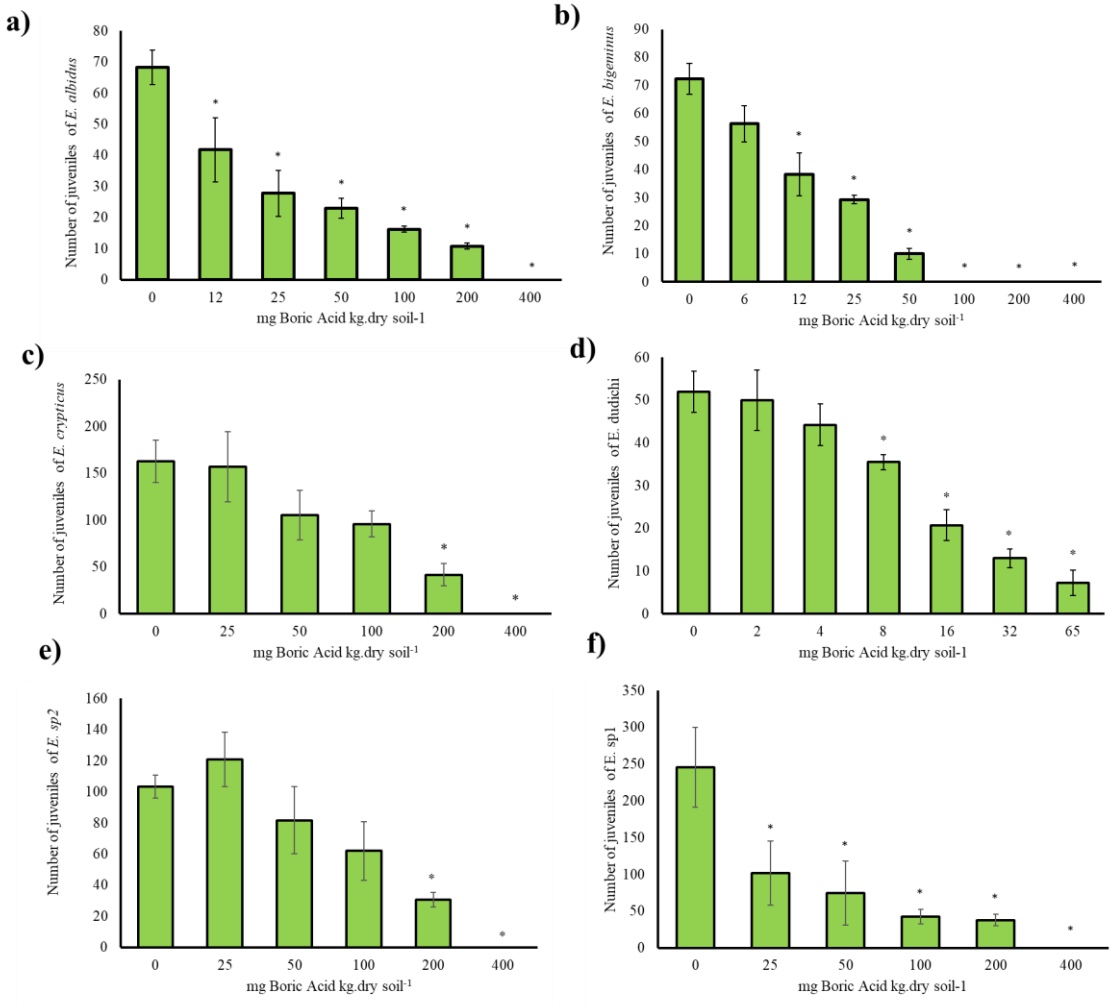
Test species	Endpoint		
	EC_{50} (mg a.i. kg^{-1})	**NOEC** (mg a.i. kg^{-1})	**LOEC** (mg a.i. kg^{-1})
E. albidus	19.593 (11.738 – 27.448)	n.o.	12.000
E. crypticus	100.861 (40.627 – 161.095)	100.000	200.000
E. dudichi	12.671 (10.182 – 15.160)	4.000	8.000
E. bigeminus	14.847 (9.226 – 20.424)	6.000	12.000
Enchytraeus sp1	22.385 (6.899 – 37.870)	n.o.	25.000
Enchytraeus sp2	122.969 (65.037 – 180.902)	100.000	200.000

; Effective concentration for 50% of effect; NOEC: No Observed Effect Concentration; LOEC: Lowest Observed Effect Concentration; n.o.: Parameter not observed. Prepared by the author, 2023.

The results ranged from 12.671 to 122.969 mg a.i. kg^{-1}, with *E. dudichi* being the most sensitive species, followed by *E. bigeminus* (14.847 mg a.i. kg^{-1}), *E. albidus* (19.593 mg a.i. kg^{-1}), *Enchytraeus* sp. 1 (22.385 mg a.i. kg^{-1}), and *E. crypticus* (100.861 mg a.i. kg^{-1}) being *Enchytraeus.* sp. 2 the least sensitive species.

Although *E. dudichi* being the most sensitive, in E. *bigeminus* and *E. albidus* significant effects on reproduction began to be observed at lower doses (12 mg a.i. kg^{-1}) [Figure 31].

Figure 31 – Dose-responses curves of a) *Enchytraeus abidus*; b) *Enchytraeus bigeminus*; c) *Enchytraeus crypticus;* d) *Enchytraeus dudichi;* f) *Enchytraeus sp2* and g) *Enchytraeus sp1* when exposed to Mancozeb in Subtropical Artificial Soil.



Prepared by the author, 2023.

6.4 DISCUSSION

Regarding standardized species our results to *E. crypticus* 100.861 (40.627 – 161.095) mg kg^{-1} were similar with Niemeyer et al (2018) who found EC_{50} values for reproduction of *E. Crypticus* in TAS of 165.2 and 164.8 mg kg-^{1} in an interlaboratory test. But lower than the values reported by Becker et al (2010) for reproduction EC_{50} 220 mg kg^{-1} ^{in} OCDE soil., the authors also evaluated the sensitive of the species *E. luxuriosus* to BA in OCDE soil and reported a reproduction EC_{50} of 228 mg kg^{-1}.

Amorin et al (2012) reported a reproduction EC_{50} of 104 mg kg^{-1} for *E. albidus* in LUFA 2.2, a value higher than our results (19.593 mg kg^{-1}).

Clay and organic matter contents impact boron availability in soil, affecting its bioavailability to organisms. The lower EC_{50} values in TAS when compared to OECD soil can be attributed to their contrasting organic matter content (5% and 10%, respectively), which increases boron availability to organisms. When comparing TAS EC_{50} values to LUFA 2.2 soil results, TAS values tend to be higher, likely due to the clay content in LUFA (17%), which binds boron, reducing its availability to soil organisms (GOLDBERG, 1997; AMORIN *et al.*, 2012; NIEMEYER *et al.*, 2018).

6.5 CONCLUSION

Boric acid is a suitable substance reference for ecotoxicological tests with non-standardized enchytraeids species in Artifical subtropical soil, the results are consistent among the species. Additional tests should be performed in different laboratories to determine if the results are reproducible.

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7 CHAPTER 6: EFFECTS OF MACOZEB ON ECNHYTRAEIDS – SPECIES SENSIBILITY DISTRIBUTION

7.1 INTRODUCTION

Assessing the potential effects of pesticides on non-target organisms is crucial for environmental risk assessment and the development of sustainable agricultural practices (FISHEL, 2005; POSTHUMA *et al.*, 2002; TOOKER & PEARSON, 2021).

Enchytraeids are soil-dwelling organisms that play a significant role in soil ecosystems (JÄNSCH *et al.*, 2005). They are often used as bioindicators for evaluating the ecological risks associated with pesticide exposure (CHELINHO *et al.*, 2012; BANDOW *et al.*, 2013; CARNIEL *et al.*, 2019).

The use of species sensitivity distribution (SSD) curves, derived from toxicity data, can provide valuable insights into the potential effects of pesticides on enchytraeids. SSD curves are statistical tools that allow the interpretation of toxicity data across multiple species or taxa (POSTHUMA *et al.*, 2019).

SSD curves show the distribution of sensitivity within a population or community by showing the concentration or dose-response data of different species against the corresponding proportion of affected organisms (POSTUMA *et al,* 2019; RENAUD *et al.*, 2021)

Since enchytraeids are a diverse group of organisms, their species sensitivity to pesticides varies. The range of pesticide exposures that may have negative effects on these organisms can be predicted by combining data from various enchytraeid species into SSD curves. This information can be further utilized in ecological risk assessments to estimate the potential impacts of pesticide applications on enchytraeid populations in specific environments (Moreover, such information is crucial for setting regulatory guidelines, determining safe exposure levels, and implementing effective risk managements strategies (CARNIEL, 2019).

We hypothesize that, by increasing the number test-species, and using native enchytraeids species will help to determine whether the standard species are reliable and protective surrogates of the sensitivity to pesticides of the enchytraeids group.

The main objective of this work is to elaborate an SSD curve for enchytraeids species to Mancozeb exposed in a natural subtropical soil.

7.2 MATERIAL AND METHODS

7.2.1 Test substance

A commercial formulation of the fungicide Mancozeb (Manzate 800®, 800 g a.i. Kg^{-1}) was used for soil contamination. The product characteristics are, Chemical Abstracts Service (CAS): 8018-01-7; Log K_{oW}: 1.33; solubility 6.2 ppm at pH 7.5, 25 °C. Mancozeb has low soil persistence with half-life pointed in literature as less than 2 days in aerobic soils (Xu, 2000) and in European legislation as less than one day (EC, 2009b).

7.2.2 Test soil

A subtropical soil was sampled in native pasture area without historic of pesticides application, in southern Brazil, in the municipality of Campo Belo do Sul. Soil samples were collected at a depth of 0.00 – 0.10 m, the soil was classified as a Nitossolo Bruno, the samples were air dried, sieved (2 mm mesh) and then stored in laboratory until further use. Physicochemical characteristics of the soil are shown in table 18.

Table 18 – Psychochemical attributes of test-soil.

Soil attribute	Nitossolo Bruno
Clay (%)[a]	41
pH in water[a]	4.4
P (mg/dm^3) [a]	3.7
K (mg/dm^3) [a]	120
Organic matter content (%)[a]	5.5
Al ($cmol_c/dm^3$) [a]	4.7
Ca ($cmol_c/dm^3$) [a]	1.7
Mg ($cmol_c/dm^3$) [a]	1.6

[a] Determined according to Tedesco et al. (1995).Prepared by the author, 2023.

Artificial soil was also used in the laboratory experiments. This soil is Tropical Artificial Soil (TAS) a modified version of the artificial soil proposed by Garcia (2004)

and is composed by 75% of fine sand (washed and dried), 20% of kaolin clay and 5% of coconut coir dust. The pH was adjusted to 6.0 ± 0.5 by the addition of $CaCO_3$.

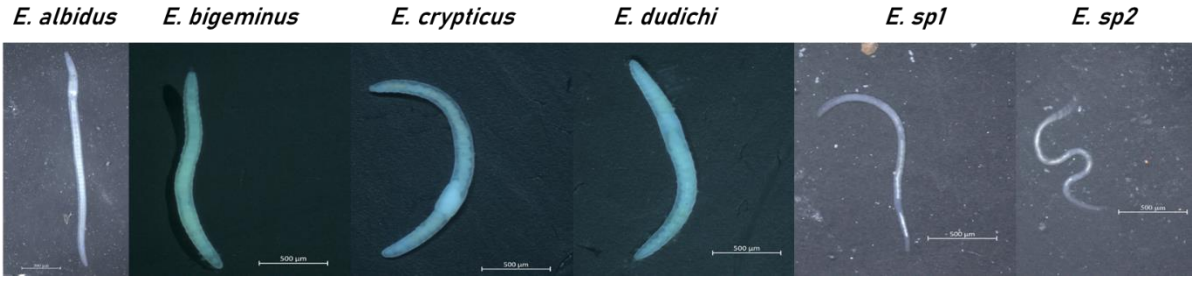
7.2.3 Test Organisms

Enchytraeids of six different species were used to conduct the reproduction tests: (Figure 32).

- The species *Enchytraeus crypticus*, a standardized species, is widely used in ecotoxicological tests (ISO, 2004; KUPERMAN et al., 2004; ZHANG et al., 2019). The species reproduces by self-fertilization and cross-fertilization and fragmentation (COLLADO et al, 2012; GONÇALVES et al., 2016).
- The species *Enchytraeus albidus* is a standardized species, widely used in ecotoxicological tests (ISO, 2004).
- The species *Enchytraeus bigeminus* reproduces primarily by fragmentation, but at low densities it can reproduce sexually (CRISTENSEN, 1973).
- The species *Enchytraeus dudichi* reproduces by fragmentation and has physiological similarities with *E. bigeminus* (NIVA et al., 2010).
- A species collected (at Parana State, Brazil) and cultivated by researcher Cintia Carla Niva (Embrapa Cerrados) belonging to genus *Enchytraeus*– with pending description, henceforth called *Enchytraeus sp. 2*. The species reproduces by fragmentation.
- A species collected (in the state of Santa Catarina, Brazil) and cultivated by researcher Douglas Alexandre (Laboratório de Ecologia do Solo – UDESC/CAV), belonging to the genus *Enchytraeus*, with pending description, henceforth referred to as *Enchytraeus sp. 1*. The species reproduces sexually.

The organisms were cultivated in plastic containers containing SAT (Garcia, [2004], adapted for 5% coconut fiber), in a controlled environment (temperature 20 ± 2 °C; photoperiod 18:8h light:dark), the cultures were fed weekly with rolled oats, and had their humidity corrected with mineral water.

Figure 32 – Enchytraeids test species.



Prepared by the author, 2023.

7.2.4 Experiment procedure

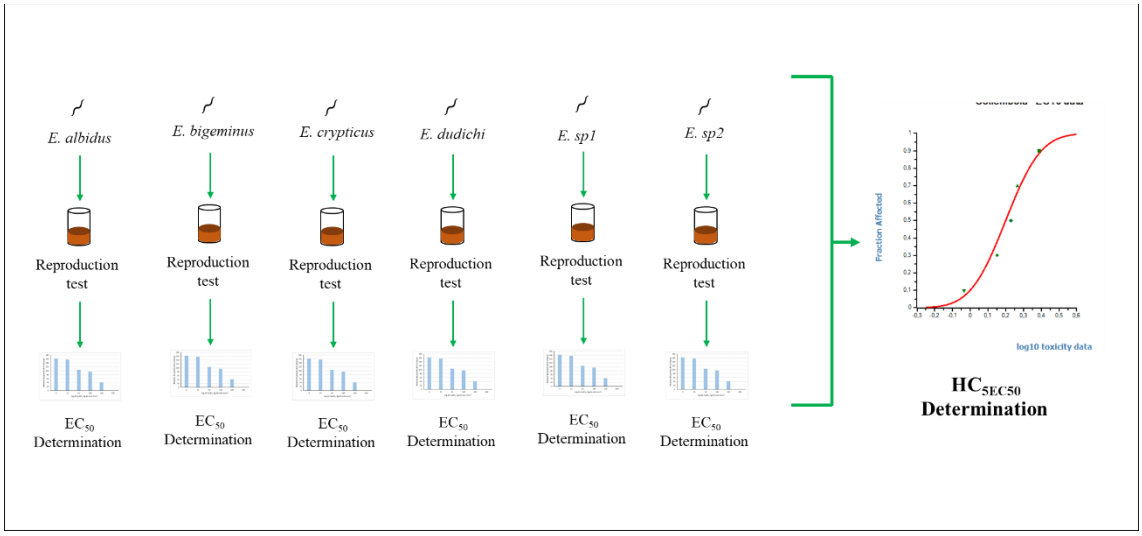
Laboratory reproduction tests were carried out based on the procedures described in ISO standard 16387 (ISO, 2014) and Bandow *et al.* (2013) (Figure 33).

For each test, a gradient of laboratory spiked soil with increasing concentrations of Mancozeb (0, 0.1, 0.2, 0.4, 0.8, 1.5, 3, 6, 10, 20, 40, 80 and 160 mg a.i. kg^{-1} dry soil) was achieved. Each gradient was prepared by a stock solution diluting Manzat 800® in distilled water.

All tests were performed under a photoperiod of 16:8h light: dark and at 20ºC, Over the experiments, test containers were opened weekly to allow aeration and weight loss of the replicates was reestablished by the addition of distilled water to compensate water losses.

After 21 days , 30 mL of alcohol (95%), and twelve drops of Bengal rose stain (1% in ethanol) were added in the test containers to preserve and color the organisms. After a minimum of 72 h, the organisms were counted using a stereomicroscopic microscope (60× of magnification).

Figure 33 – Experiment procedure.



Prepared by the author, 2023.

7.2.5 Data analysis

In each test, the number of juveniles was compared between contaminated and control soils through analysis of variance (one-way ANOVA), followed by Dunnett's post hoc test. Normality and homogeneity of variances of data were verified by Kolmogorov-Smirnov test and Levene's tests, respectively. Statistical differences found in this analysis allowed to establish NOEC (non-observed effect concentration) and LOEC (low observed effect concentration). Effective concentrations for 10% and 50% of effect (EC_{10}, and EC_{50}) were estimated considering measured concentrations and using nonlinear models (ENVIRONMENTAL CANADA, 2007) in Statistica 7.0 software (STATSOFT, Inc., 2004).

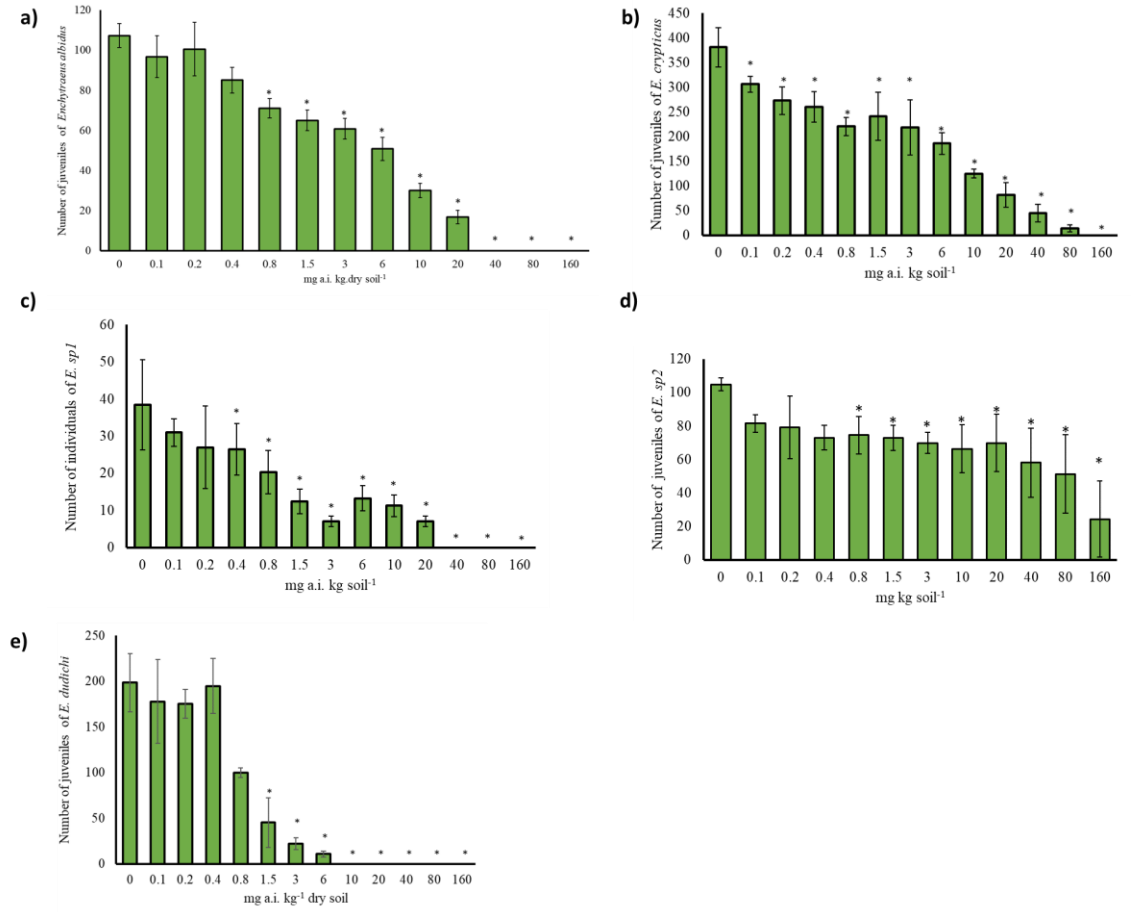
EC_{50} values were used to generate SSD curves through ETX 2.2 software (VAN VLAARDINGEN *et al.*, 2004) and to calculate hazardous concentration for a protection level of 95% and 50% based on EC_{50} values ($HC5_{EC50}$ and $HC50_{EC50}$, respectively).

7.3 RESULTS

The reproduction tests (Figure 34) fulfilled the validity criteria defined in the ISO 16387 (2014), number of juveniles >25 and coefficient of variation <50% in control

vessels. However, the number of adults could only be determined in tests with *E. crypticus, E. albidus e E. sp1*, hence *E. sp2* and *E. dudichi* reproduce by fragmentation.

Figure 34 – Graphics of dose response for enchytraeids reproduction a) *E. albidus*; b) *E. crypticus*; c) *Enchytraues sp1*; d) *Enchytraeus sp2*; e) *E. dudichi* when exposed to mancozeb in nitossolo.



Prepared by the author, 2023.

Toxic values estimated to each species for Mancozeb exposure are presented in table 19.

Table 19 - Reproduction EC_{50} (and corresponding 95% confidence intervals) LOEC and NOEC values estimated for enchytraeids species when exposed to Nitossolo with increasing concentrations of the Mancozeb.

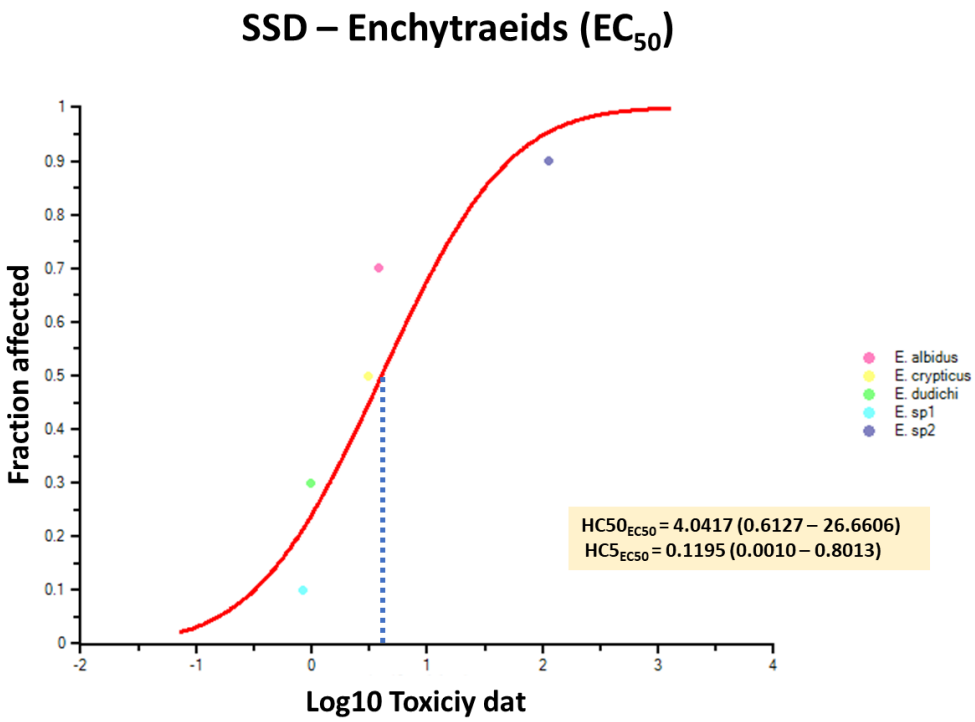
Species	EC_{50}	NOEC	LOEC
	mg a.i kg $solo^{-1}$		
Enchytraeus albidus	3.580 (2.557 - 4.603)	0,2	0,8
Enchytraeus crypticus	3.078 (2.502 - 3.653)	n.o	n.o
Enchytraeus dudichi	0.937 (0.684 - 1.190)	0,8	1,5
Enchytraeus sp1	0.832 (0.289 - 1.375)	0,2	0,4
Enchytraeus sp2	112.417 (82.929 - 141.906)	0,4	0,8

Effective concentration for 50% of effect; NOEC: No Observed Effect Concentration; LOEC: Lowest Observed Effect Concentration; n.o.: Parameter not observed. Prepared by the author, 2023.

The sensibility of the species ranged from 0.832 to 112.417 mg kg. $soil^{-1}$. Being *E. sp1* the most sensitive species and *E. sp2* the least sensitive species. The standardized species *E. albidus* and *E. crypticus* showed similar responses (3.850 and 2.078 mg kg. $soil^{-1}$, respectively).

Using EC50 data the SSDs were performed and the hazardous concentrations (HC) to protect 95% (HC5) and 50% (HC50) of the organisms were estimated of 0.1195 and 4.0416 mg kg. $soil^{-1}$, respectively (Figure 35).

Figure 35 - SSD curves using EC_{50} values of *Enchytraeus spp*. to Mancozeb exposed in Nitossolo.



(SSD) Species Sensitivity Distribution; (EC_{50}) Effective concentration for 50% of effect; ($HC5_{EC50}$) Hazardous Concentration for a protection level of 95% based on EC_{50}; ($HC50_{EC50}$) Hazardous Concentration for a protection level of 50% based on EC_{50}. Prepared by the author, 2023.

7.4 DISCUSSION

It was not possible determinate the number of adults individuals in tests with species with *E. dudichi* and *E. sp2*, those species reproduce by fragmentations. Carniel (2019) highlight the need of establishing validation criteria specific for fragmentary species.

The toxicity of Mancozeb towards enchytraeids presented a high variation between the species (0.832 to 112.417 mg kg. $soil^{-1}$), both species, the most and least sensitive, are native. Which indicates (1) a possible high variability in species sensitivity in the field and (2) the need to use a species with representative sensitivity for ecotoxicological tests, covering this variation.

Carniel *et al.* (2019) found effects on lethality and reproduction of *E. crypticus* in Oxisol (LC_{50} = 6.97 mg $soil^{-1}$; EC_{50} = 3.56 mg $soil^{-1}$) and Ultisol (LC_{50} = 280.21 mg

soil^{-1}; EC_{50} = 29.67 mg soil^{-1}). The result in Oxisol was similar to our finds to *E. crypticus* (3.078 mg kg. soil^{-1}) However, the high variation in toxicity for the same species between soil types highlights the need for attention when comparing data.

To the best of our knowledge, to date, no SSD was elaborated using only enchytraeids species, Carniel (2019) included three enchytraeids species (*E. bigeminus*, *E. dudichi* and *E. crypticus*) in curves containing only oligochaetes (Enchytraeids and earthworms) and also combined with arthropods (Collembola) for the insecticide Chlorpyrifos nevertheless the author sates that the SSD-curves should be for generated for each group individually (arthropods or oligochaetes) to avoid inter-species sensitivity variation.

There are a overlap in the threshold values form *E. crypticus* - EC_{50} and $HC50_{EC50,}$ indicating that this specie is representative and protective for this group.

7.5 CONCLUSION

Enchytraeids species presents a high sensitivity variation when exposded to the fungicide Mancozeb. The native species *E. sp1* were the most sensitive, and *E. sp2* the least sensitive.

The standardized species *Enchytraeus crypticus* are a reliable and protective surrogate of the sensitivity to pesticides of the enchytraeids group.

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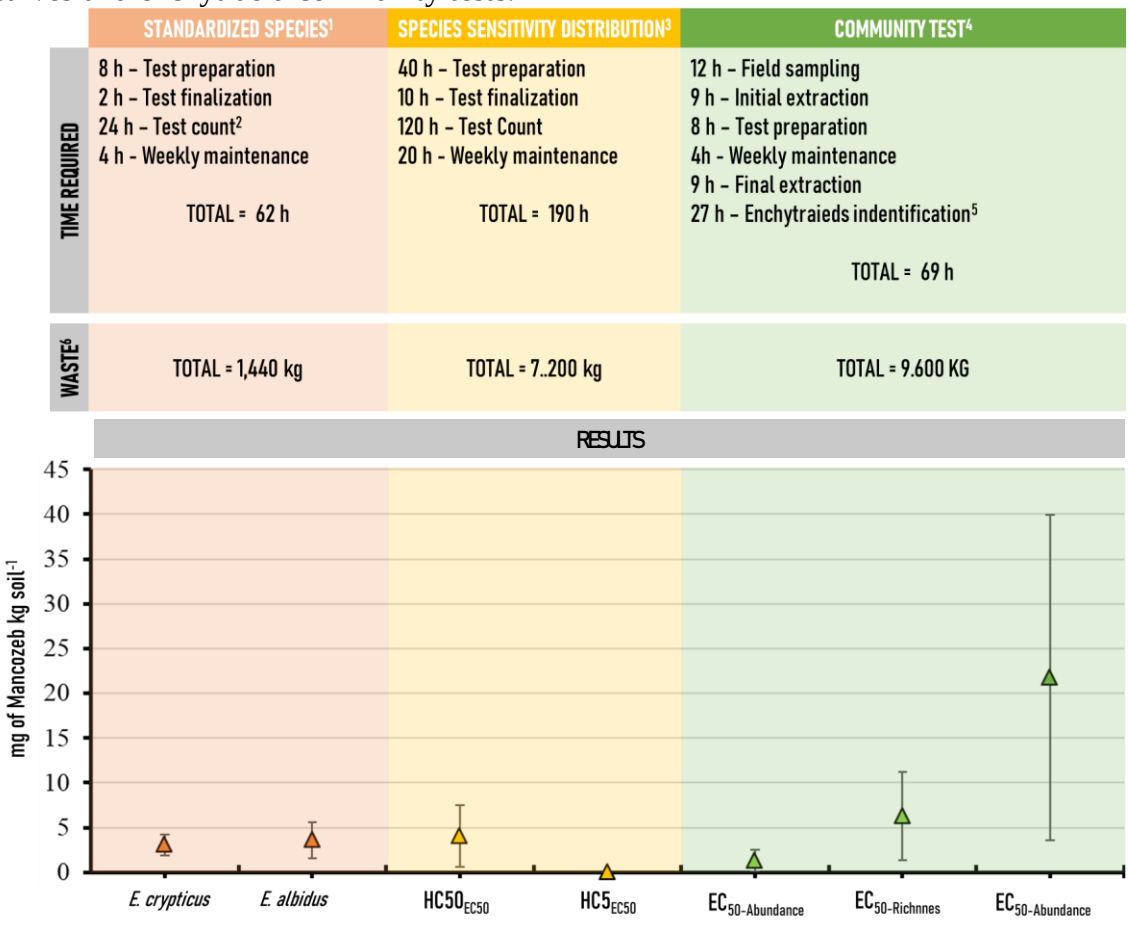
8 FINAL CONSIDERATIONS

Although the SSD approach incorporates the difference in sensitivity between enchytraeids species, and community tests provide valuable information about community dynamics when exposed to a contaminant.

Conducting ecotoxicological tests with standardized species seems to be a good parameter for evaluation and threshold value for enchytraeids protection, in addition to be the approach with less time required and less amount of contaminated waste produced (Figure 36).

Figure 36 - Comparation between the use of Standardized enchytraeids species, SSD curves and enchytraeid community tests.



(1) Each test – with 11 treatments + Control, 4 replicates (n=48); (2) 2 hour/treatment (n=4); (3) 5 Species; Each test – with 11 treatments + Control, 4 replicates (n=48); (4) Test with 8 treatments + control, 6 replicates (n=54); (5) Approximately 0.5 h per replicate; (6) Amount of soil contaminated. Prepared by the author, 2023.