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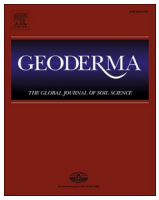

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ABSTRACT

It is well known that biopores are crucial for soil functioning. However, their dynamics is rarely studied and their origin with regards to the soil organisms involved is still hard to determine. In this study we investigated the diversity of biopores and their regeneration rates *in situ* in various pedoclimatic conditions. Our approach involved field incubation of repacked soil cores with lateral openings across nine study sites in five countries (France, Vietnam, India, Laos and Thailand). After 12 months, biopores were characterized by X-ray computed tomography and grouped according to their diameter, length and sphericity index using principal component analysis followed by K-means clustering. The regeneration dynamics of biopores was assessed by comparing those created after one year of incubation to the biopores determined in soil cores taken from the surrounding soils (assuming the latter are in a steady-state). Additionally, we examined the relationships between newly formed biopores and soil macrofauna taxa. Our results evidenced significant variability in biopore diameter (0.90 to 15.84 mm), length (1 to 1600 mm) and sphericity index (0.03 to 0.93). We propose 10 biopore groups allowing to distinguish most of the study sites. Complete regeneration of biopores after 12 months was achieved in seven out of nine sites. Three groups of biopores showed a positive relation with earthworm abundance (r values ranged from 0.69 to 0.90), whereas the other groups of biopores showed no association with any macrofauna taxa. We conclude that biopore formation can be assessed under field conditions with repacked soil cores, regardless the pedoclimatic conditions. However, the involvement of macrofauna other than earthworms in biopore formation still remains to be unraveled. To capture their contribution to biopore formation, improvements of the repacked soil core approach and complementary laboratory experiments were suggested.

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1. Introduction

Soil macrofauna, accounting for a significant proportion of soil faunal biomass (Gongalsky, 2021), comprise diverse taxa with a variety of diets and habitats. Certain members of this group are recognized as ecosystem engineers (Jones et al., 1997; Jouquet et al., 2016) due to their ability to modify the soil habitat in a way that will benefit other organisms through soil translocation and modification of its organization (Bottinelli et al., 2015). This phenomenon, known as bioturbation, has been extensively studied with earthworms, ants, and termites, although some species of millipedes, beetles, bees, fly larvae are also capable of bioturbation (Lavelle et al., 2006; Tschanz et al., 2023).

Cavities created by soil engineers are biopores. It has been shown that their presence affects soil porosity and thus modifies the transfer of water, air and nutrients through the soil profile (Kautz, 2015). Consequently, the accurate quantification of biopores, including their origin, volume and morphology is essential for understanding and modelling the functioning of soil systems (Flores et al., 2021). Biopores can take the form of tubular structures with varying degrees of branching, including chambers and galleries (García Ibarra et al., 2024; Pham et al., 2023), or they may be irregular in shape, such as galleries filled up with soil (Bottinelli et al., 2010). The shape and dimensions of biopores, and the characteristics of gallery walls are influenced by the size and burrowing behaviour of the organisms involved in their formation (Mele et al., 2021; Capowiez et al., 2024b; Pham et al., 2024). Meanwhile, the intensity of bioturbation is affected by climatic conditions (García Ibarra et al., 2024) and soil properties (Capowiez et al., 2024a). In natural settings, biopores arise from the collective actions of a diverse array of species interacting over prolonged periods. The complexities introduced by the varying ages and interactions among these species pose a significant challenge in isolating the individual contributions of each macrofauna taxon.

Since the early 90 s, X-ray computed tomography (CT) has been a growing application in quantifying the 3D structure of biopores (Taina et al., 2008). This technique has proven to be useful in distinguishing biopores created by various taxa (Cheik et al., 2019; Mele et al., 2021) or different species (Capowiez et al., 2024b) in both controlled and *in situ* conditions. However, no attempt has been made to use X-ray CT technology to differentiate the contributions of various macrofaunal groups inhabiting the same volume of soil to biopore formation.

To unravel the formation of natural biopore complexity by various macrofaunal groups, we installed repacked soil cores with lateral openings at 9 field sites, added litter to attract macrofauna and studied biopore formation during 12 months by comparing the volume and shape of newly formed biopores to an undisturbed core from the same site. We conducted the experiment over a large longitudinal gradient to capture the variability in macrofauna communities and pedoclimatic conditions. The purpose of this study was to demonstrate the suitability (proof-of-concept) of using open soil repacked cores and X-ray CT technology to differentiate the contributions of various macrofaunal groups to biopore formation. We hypothesized that the diversity and volume of regenerated biopores would depend on macrofauna composition and climatic conditions at the sites of exposure.

2. Materials and method

2.1. Study sites

Nine study sites were chosen to represent various environments presenting different climates, soil properties and vegetations (Table 1). The study sites were located in India, Thailand, Laos, Vietnam and France. Four sites were located in France: with continental climate in Bondy (France_B) and Chambord (France_C) as well as in Mediterranean climate in Roujan (France_R) and Avignon (France_A). One site was located in Hanoï, Vietnam under subtropical humid climate. Three sites were located in a tropical savanna climate in Luang Prabang, Laos and two sites in Khon-Kaen, Thailand (Thailand_K1 and K2). Finally, a one site was located in Athirampuzha, India under tropical monsoon climate.

2.2. Experimental design

Cores consisted of polyvinyl chloride cylinders (15 cm in height and 15 cm in diameter) with 96 holes (1 cm diameter and spaced by 2 cm). Cores were two-thirds filled (10 cm) with soil sampled at the 0–20 cm depth and homogenized by sieving soil at 2 mm. The soil was manually repacked to obtain a dry bulk density similar to that found in the field. To reduce variations in soil bulk density between the top and bottom of the cores, the soil was compacted stepwise in two layers until it reached a height of 10 cm. To attract macrofauna, organic matter was applied to the soil surface. Its quality varied depending on the study sites and is detailed in Table 1. The bottom and top of the soil cores were sealed with a nylon mesh of 250 µm. The goal of the top cover was to prevent bioturbation from the top to restrict our study on soil inhabiting macrofauna. The soil cores were arranged randomly in the field, and spaced with a minimal distance of 1 m between them and within an area covering 50 m x 50 m. A total of 36 soil cores were installed (i.e., 9 study sites x 4 replicates) for 12 months at 0–10 cm depth. In each site, four undisturbed soil columns (10 cm in height and 15 cm in diameter) were sampled at the end of the incubation and used as control to quantify the volume of biopores existing in each study field.

2.3. Soil macrofauna

Soil organisms visible to the naked eye (generally > 2 mm) were hand-sorted from 25 cm $\times$ 25 cm samples of the litter layer and soil down to a depth of 25 cm ($n = 4$) during the peak activity period of macrofauna (humid seasons in Asia and spring or autumn in France). Individuals were preserved in 70 % alcohol until they were counted in the laboratory. Individuals were classified into earthworms, ants, termites, Coleoptera, Myriapoda and others.

2.4. Quantification of biopores

Repacked and undisturbed control soil cores were scanned with medical X-ray CT at hospitals located near each study site. The specifications for image acquisition and segmentation are detailed in Table S1.

Table 1
Description of the nine study sites (soil, vegetation and climate) and type of organic matter added at the surface of repacked soil cores.

City	Acronyme	Latitude	Longitude	Rainfall (mm)	Air temp. (°C)	Vegetation	OC (%)	Texture	Organic matter added
Athirampuzha	India	9.66	76.54	3600	28	Rubber trees	3.6	Sandy clay	Cow dung
Khon Kaen	Thailand_K1	16.47	102.84	1250	27	Rubber trees	≈2-3	Silt loam	Leave litter
Khon Kaen	Thailand_K2	16.76	103.1	1110	27	Rubber trees	≈2-3	Sandy loam	Leave litter
Luang Prabang	Laos	19.85	102.17	1900	24	Teak trees	0,84	Clay	Leave litter
Hanoï	Vietnam	20.95	105.48	1800	24	Mixed tress	2.6	Clay	Buffalo dung
Roujan	France_R	43.3	3.19	650	14.1	Shrub trees	0,76	Clay loam	Horse manure
Avignon	France_A	43.55	4.48	752	14.9	Pear trees	2.7	Silty clay loam	Horse manure
Chambord	France_C	47.63	1.55	700	11	Oak trees	6.9	Clay loam	Oak litter
Bondy	France_B	48.9	2.48	723	11.6	Grass	4.5	Sandy loam	Horse manure

The softwares Avizo3D (Thermo Fisher Scientific) and Fiji/ImageJ (Schindelin et al., 2012) were used to extract biopores and characterize them. Images were transformed into 8-bits and rendered isotropic with a voxel size ranged between 0.35 to 0.43 mm. A 3D median filter with a 2-voxel radius was applied to reduce noise and an unsharp mask filter with a standard deviation of one voxel and a filter weight of 0.6 was applied to enhance the sharpness of object edges. To exclude the PVC, a cylindrical region of interest with a circular diameter of 125 mm was used. For each dataset, different global thresholding methods were tested to separate the imaged pores from the soil matrix, with careful attention to exclude roots from the pores. The segmentation method selected for each dataset is indicated in Table S1. We applied the workflow described in Fig. S1 to separate imaged pores having a tubular or ball shape from the rest having a plate shape. The ImageJ plugin "BoneJ – particle analyser" (Doube et al., 2010) was used to determine the volume, diameter (mean, maximum and standard deviation), number and length of skeletons. The plugin "morpholibJ" (Legland et al., 2016) was used to determine the sphericity, the orientation and size of the equivalent ellipsoid of biopores. The volume of biopores was transformed into percentage (volume of biopores / volume of region of interest x 100). The sphericity is defined as the ratio of the squared volume over the cube of the surface area, normalized such that the value for a ball equals one.

2.5. Statistical analysis

Statistical analyses were performed in R version 4.4.1 (R Core Team, 2024) and figures were produced with the package "ggplot2" (Wickham et al., 2016). Principal components analysis (PCA) was performed with the package "ade4" (Dray and Dufour, 2007) to ordinate biopores, described by the 11 log-transformed parameters. Then, highly correlated parameters and those poorly related to the first two PCA axes were removed. Ultimately, only three parameters were selected: sphericity index, branch length and maximum diameter. To determine groups of biopores, we applied a K-means clustering method to the values on the two first axes of the PCA. The optimal number of groups was assessed visually. We determined that an acceptable choice for the number of groups could be set to 10 (thereafter named B1 to B10). Between-class analysis was carried out with the package "ade4" to differentiate the nine study sites according to their biopore properties. Co-inertia analysis from the package "ade4" was applied between matrices of log-transformed abundance of macrofauna and biopore properties, allowing us to examine the relationship between the two datasets. The level of association between these two datasets was measured by the Monte Carlo test (RV score) with 1000 permutations. T-tests were used to compare biopore volume in repacked soil cores and control soils in each

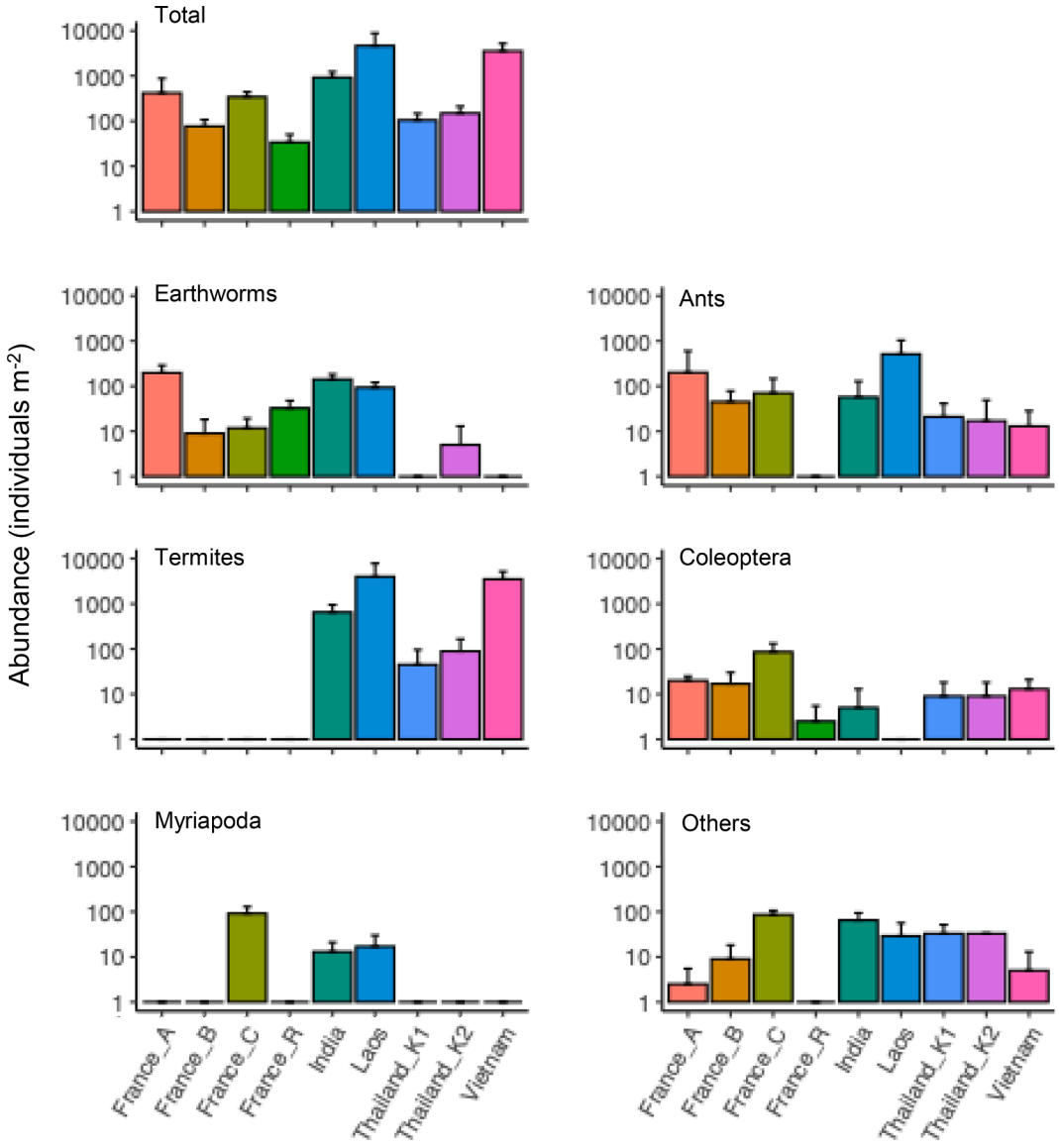

Fig. 1. Means + standard deviations of soil macrofauna abundance sampled across nine study sites in France and Asia.

study site. Before using *t*-test, parameters were tested for normality and homogeneity of variances using the Shapiro–Wilk test and Levene's test. Finally, Pearson correlations were used to study the relationships between biopore groups and macrofauna taxa (n = 9). Differences among treatments were declared significant at the 0.05 probability level.

3. Results

3.1. Macrofauna communities

The mean total densities of macrofauna varied between 33 individuals m^{-2} in France_R to 6,056 individuals m^{-2} in Laos (Fig. 1). The main macrofaunal taxa in terms of abundance were earthworms (on average 14 %), termites (on average 18 %) and ants (on average 15 %) while the other taxa each represented less than 10 %. Earthworms were found in all sites, except in Vietnam and Thailand_K1, with largest abundance recorded in France_A (197 individuals m^{-2}). Ants were found in all sites, except in France_R, with largest abundance recorded in Laos (512 ind m^{-2}). Termites were only found in Asia and reached their largest abundance in Laos with 4,012 ind m^{-2}. Coleoptera and Myriapoda were mainly found in France_C with 85 and 90 ind m^{-2}, respectively. Other taxa were much less represented, with a maximum of 85 ind m^{-2} in France_C and 64 ind m^{-2} in India.

3.2. Bioporosity in repacked and control soils

The mean bioporosity (percentage of biopores volume as referred to the total volume of soil) including control soils and repacked soil varied from 0.2 to 3.7 % (Fig. 2). Largest values were observed for France_A and Thailand_K2, whereas lowest values were found for France_C and France_R. Bioporosity in repacked soil cores differed from that in controls at two sites, *i.e.*, France_C and France_R where it was found to be significantly lower. At all other study sites, no differences were observed.

Representative biopore systems in control and repacked soils for each site are displayed in Fig. 2. Biopores exhibited significant variation in both length and diameter. While it remained impossible to visually determine the origin of the majority of biopores, some could, based on our expertise, be clearly attributed to earthworm activity (e.g., the large U-shaped gallery in France_B or the thin gallery likely filled up by dejections in France_A), ants (e.g., large chambers in France_B), termites (e.g., the small, flat chambers in Vietnam).

3.3. Classification of biopores according to their shape

A total of 4,864 biopores was found in the 36 repacked soil cores. The two first axes of the final PCA represented 63 and 36 % of the variability, respectively (Fig. 3a). The first axis opposed the sphericity index and length of biopores whereas the second axis was correlated to the diameter of biopores. The 10 group types extracted by K-means clustering gathered between 132 and 856 biopores in each group. Three groups were characterized by a higher length (B2, B4 and B8), one groups by a higher sphericity (B3) and two groups by a higher diameter (B4 and B7). On the other hand, groups B9 and B10 were characterized by a low diameter. Finally, B1 was neither specifically characterized by length, diameter or sphericity.

The distribution of the sphericity index, diameter and length of biopores is shown in Fig. 3b. Sphericity index varied between 0.03 and 0.93, with a mode value of 0.25, indicating that most of the biopores were neither simple linear tubes nor ball shape. The diameter varied between 0.90 to 15.84 mm with a mode value of 2.39 mm. The total length varied between 1 to 1599.4 mm with a mode value of 13.8 mm.

Between class analysis carried out on the volume of the 10 groups of biopores (Fig. 4) separated most of the study sites, although overlap occurred for some sites: (i) France_R, F, C and Thailande_K1 and (ii) Laos, India and Thailand_K2. The first axis, explaining 66 % of the variability, separated France_A, Laos, India and Thailand_K2 from the other sites. This axis was negatively correlated to the volume of all biopores except biopores having large diameter and medium to low sphericity (biopore groups B4 and B7). The second axis (explaining 17 % of the variability) opposed France_B and Thailand_K2 to the most of the other sites. This axis was positively correlated to the volume of biopore for groups B4 and B7.

3.4. Relationships between macrofauna and biopores

Co-inertia analysis only showed a small and non-significant relationship between the abundance of different macrofauna taxa and the volume of the different biopore groups (RV = 0.31; $p > 0.05$). Pearson correlations (Fig. 5) showed that earthworm abundance was positively correlated to biopores having intermediate diameter, greater length and lower sphericity, specifically B8 (r = 0.74), B5 (r = 0.69), B1 (r = 0.82) and B2 (r = 0.90) whereas the other macrofauna taxa were not related to any biopore group.

4. Discussion

4.1. Regeneration of the bioporosity

Biopores in repacked soil cores from seven out of nine sites reached a similar level as the control cores and were thus considered as fully regenerated indicating that biopores regenerated in less than one year. This duration is in agreement with studies carried out in France (Capowiez et al., 2012; Pelosi et al., 2017) and in Brazil (Barros et al., 2001). For instance, Barros et al., (2001) using soil blocks exchanged between a pasture and a forest found that one year was enough to transform the compact structure of the pasture soil into a highly porous framework with interconnected galleries characteristic for forest. Similarly, Capowiez et al. (2012) found that the recovery of burrow systems takes between 12 and 24 months after being degraded by soil compaction. Finally, Pelosi et al., (2017) found that 60 % of the volume of the biopores made by earthworms recovered within five months after being degraded by tillage. In our experiment, bioporosity was however not fully regenerated at two of the four French sites (R and C). This indicates that, in temperate conditions, bioporosity regeneration is not directly linked to climate, as both mediterranean and continental sites were concerned. Differences in macrofauna density and the type of organic matter used to attract macrofauna could have influenced the regeneration duration considering that several macrofauna organisms can show feeding preferences (Caner et al., 2004). For instance, horse manure, used in three sites, may be more attractive than leaf litter used at France_C (Curry and Schmidt, 2007). Additionally, France_R had the lowest macrofauna abundance characterized by the absence of ants and Myriapoda.

4.2. Origin of biopores

Studies reporting the origin of biopores are often limited to laboratory experiments (Capowiez et al., 2024b; Jégou et al., 2001; Mele et al., 2021; Nahmani et al., 2005) or field experiments, with one dominant species (Cheik et al., 2019; Tschanz et al., 2023). With one exception (Pérès et al., 1998), where earthworm galleries on soil thin sections were assigned to different earthworm ecological categories, the contribution of different taxa to the volume of soil biopores has never been addressed in field conditions. Here, we showed that biopores made by macrofauna are variables in both shape and size. Their diameter, length, and sphericity index allowed for differentiation between most of study sites, indicating that biopores can be good indicators of the local macrofaunal diversity and activity. However, our hypothesis that macrofauna produce different groups of biopores was not totally confirmed. We found that earthworms were associated with biopores which were characterized by intermediate diameters, great lengths and low sphericity index.

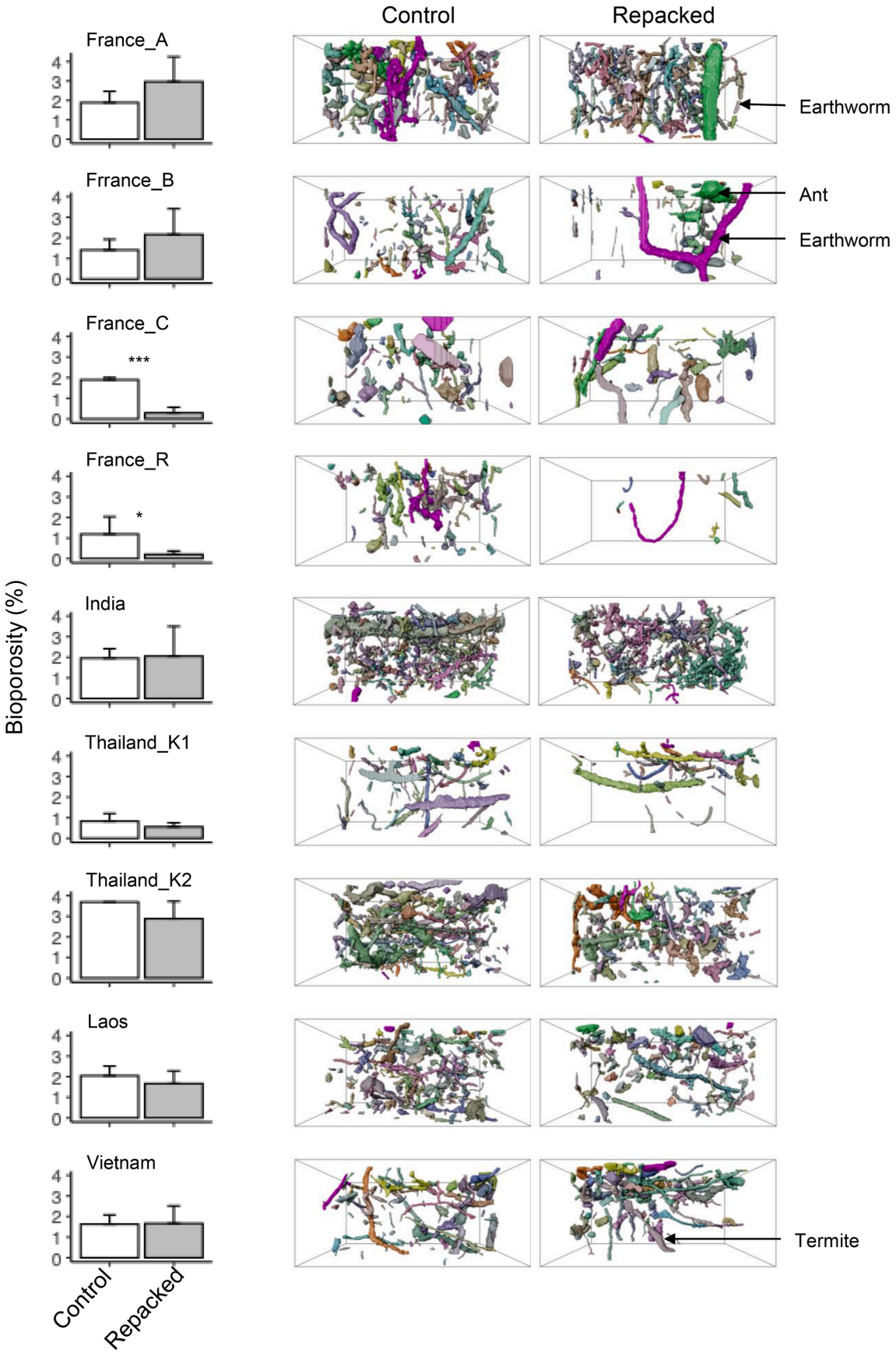

Fig. 2. Mean + standard deviation of the volume percentage of biopores (bioporosity) observed in repacked soil cores and surrounding control soils across nine study sites in France and Asia. Examples of biopore systems for each treatment are illustrated in the accompanying images. Different pore colors indicate unconnected individual pores regardless of their shape.

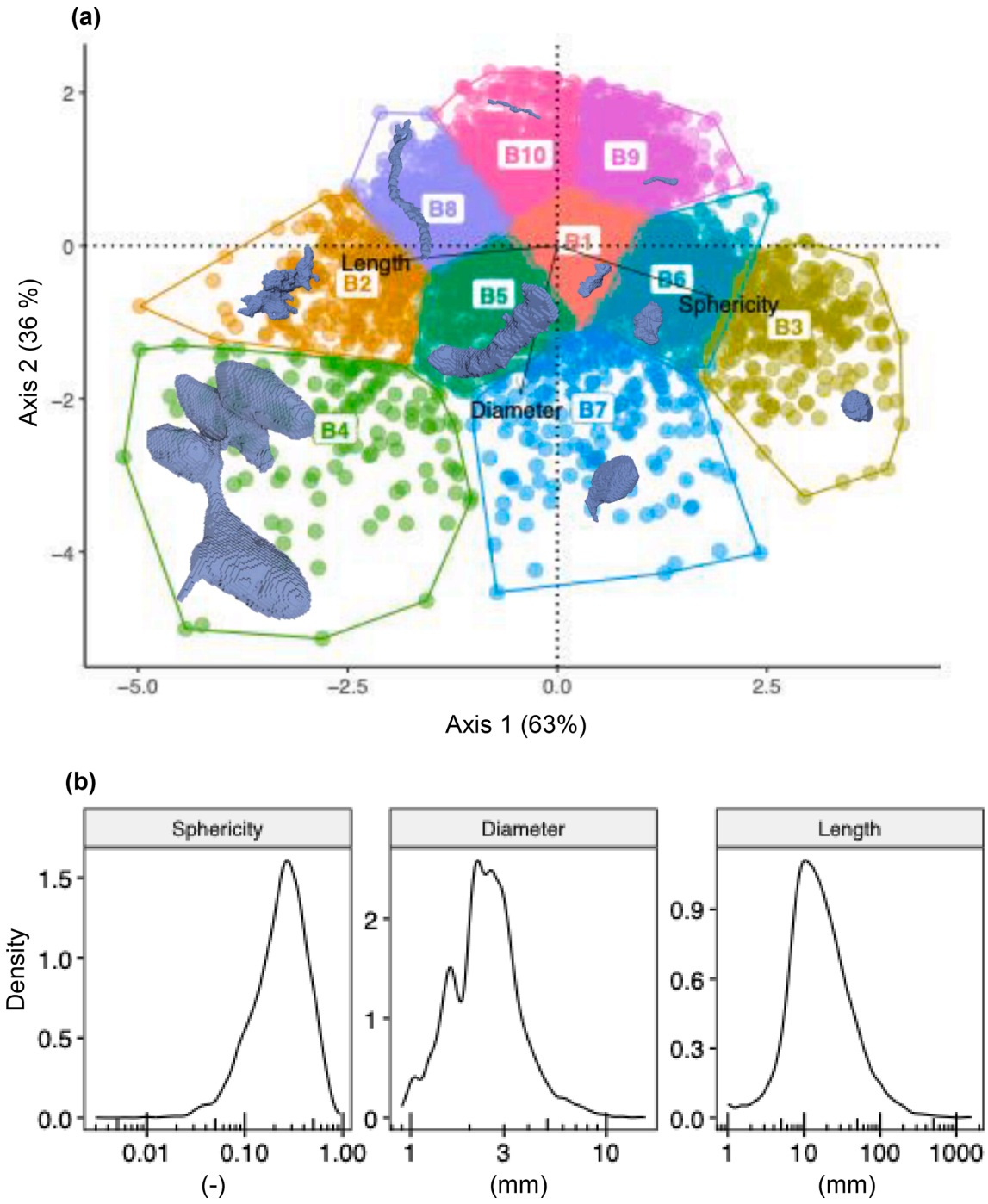

Fig. 3. (a) Results of the K-means clustering method applied to 4864 biopores, projected onto the first two axes of the principal component analysis based on their maximum diameter, skeleton length, and sphericity index; (b) density plots displaying the skeleton length, maximum diameter, and sphericity index of biopores.

Conversely, the other macrofauna taxa were not related to any biopore group although galleries made by termites and ants could be clearly identified by eye on X-ray CT images (see Fig. 2). The lack of relationship between macrofauna and biopore groups can be attributed to several methodological limitations: (i) macrofauna within the repacked soil cores were not quantified, resulting in a limited dataset (n = 9) and it remains unknown whether the macrofauna communities present at each study site effectively colonized the soil cores; (ii) the installation of cores likely caused disturbances that may have influenced the activity of macrofauna; (iii) the soil structure within the cores differed significantly from that of the surrounding soil, potentially making it more challenging for certain taxa to burrow; (iv) over the course of one year, biopores may have disappeared (e.g., filled with soil or collapsed) or been altered by macrofauna activity or water flow, leading to changes in their original shape (Le Mer et al., 2021; Tschanz et al., 2023). Hence, because biopore degradation was not monitored, the effect of texture on biopore stability cannot be discussed. Many of these limitations have been discussed in similar experiments, investigating not macrofauna but fine root growth in natural ecosystems (Steingrobe et al., 2001, 2000). Given these limitations, we believe that the use of repacked soil cores can be significantly improved. First, macrofauna should be directly associated with each soil core, which can be achieved by measuring macrofauna within and around soil cores. Second, reducing the incubation time of soil cores to capture only recent biopores or creating lasting cores that could be

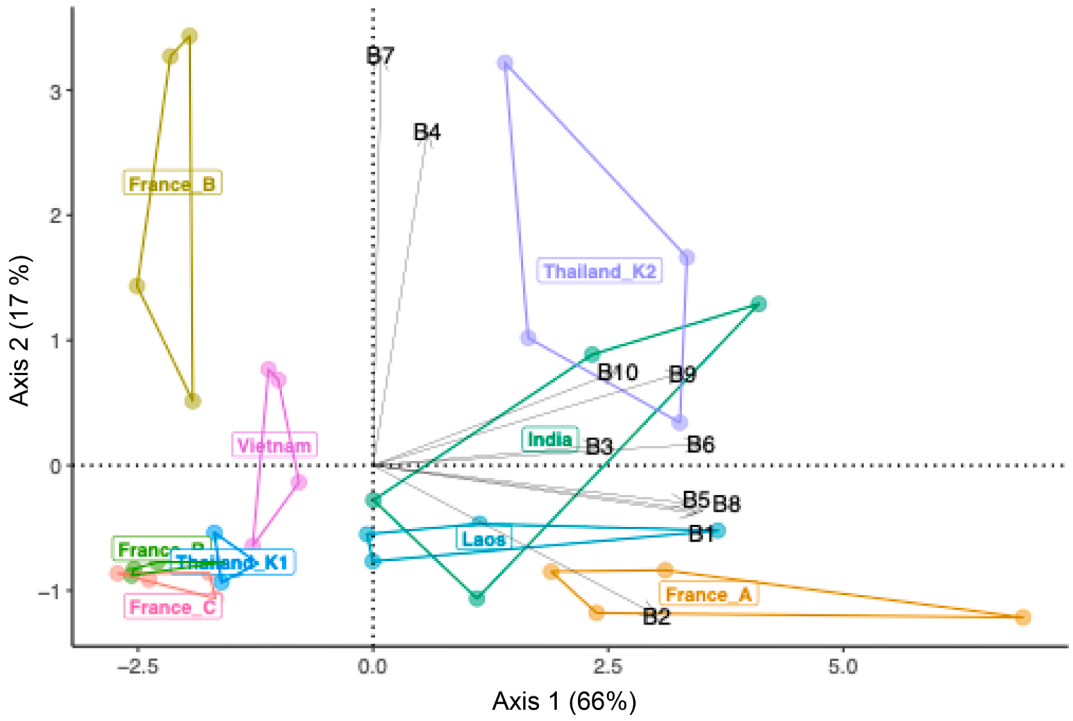

Fig. 4. Biplot showing the between class analysis from variables describing the 10 groups of biopores (B1 to B10) for repacked soil cores incubated in nine study sites in France and Asia.

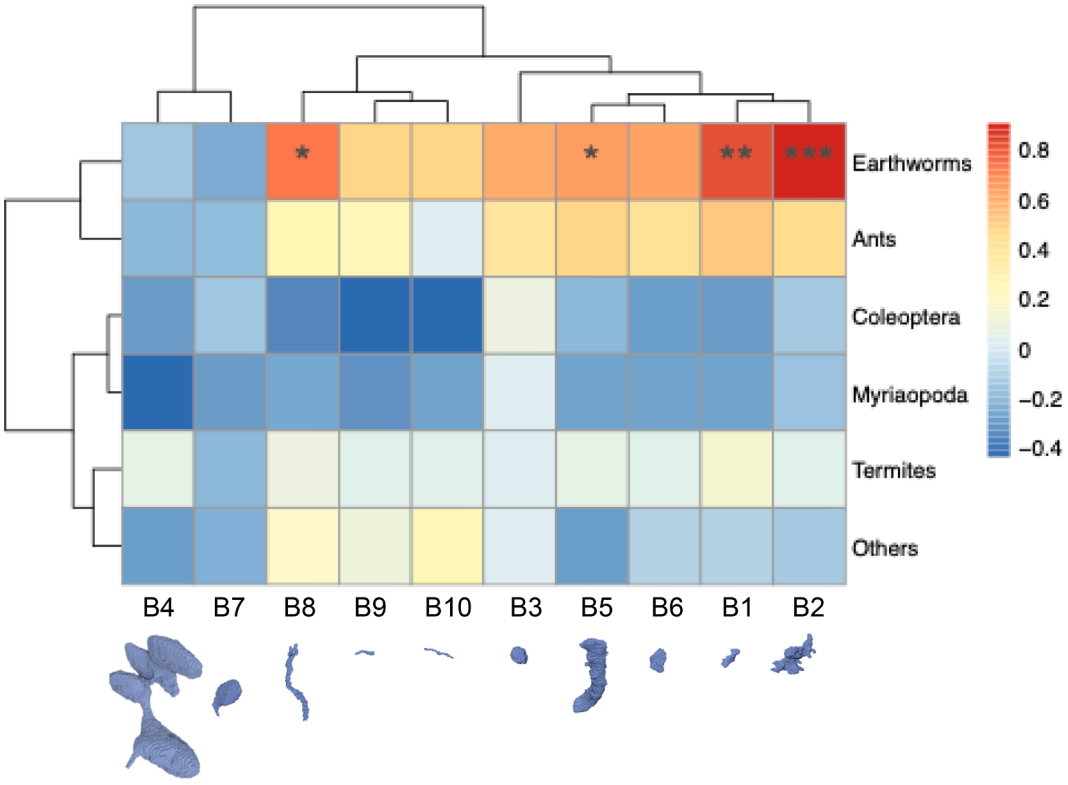

Fig. 5. Heatmap based on the Pearson's correlation matrix of biopores groups measured in repacked soil cores and macrofauna taxa sampled in nine study sites in France and Asia (n = 9). Biopores are sorted according to a cluster analysis. Red colour indicates positive correlations, blue colour indicates negative correlations and the strength of colour indicates the magnitude of correlation coefficient, as shown on the correlation spectrum (inset top right). The number of asterisks for the significance level indicates the p value range (***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

scanned several times might be beneficial to better assess biopore formation dynamics. Incubating soil cores for one month at different times of the year could help identify the peak activity of macrofauna, which may not coincide with their peak of abundance (Schneider et al., 2018) for earthworms. Additionally, the repacked soil core approach should be complemented by laboratory studies where macrofauna taxa are selectively incubated and their biopore systems thoroughly characterized. Such approach has been effectively used to differentiate biopores created by various organisms, including earthworms, millipedes, centipedes and insect larvae (Mele et al., 2021), as well as functional groups of earthworms (Capowiez et al., 2024b). Advances in machine learning and deep learning techniques are poised to play a crucial role in the characterization of soil features (Baveye et al., 2018) and could be useful to identify the origin of biopores.

5. Conclusions

This study improves our understanding of biopore regeneration in soils under field conditions, demonstrating the critical role of soil macrofauna in shaping soil structure. Using repacked soil cores incubated *in situ*, we showed that in most situations, biopores can regenerate within a year, regardless of pedoclimatic conditions. While earthworms were associated with specific biopore shapes, we could not identify and distinguish biopores made by ants, termites, Myriaopoda and Coleopetra. Future research should focus on refining the repacked soil core approach and apply it during shorter periods to be able to better relate macrofauna abundance to biopore formation. Data need to be collected in different climatic conditions and/or at different times throughout the year. Additionally, this work highlights the need of compiling and organizing existing data to develop a comprehensive database on macrofauna-induced biopore formation.

CRediT authorship contribution statement

Charlotte Védère: Formal analysis, Writing – original draft, Writing – review & editing. **Hanane Aroui Boukbida:** Writing – review & editing, Resources, Investigation. **Yvan Capowiez:** Investigation, Resources, Writing – review & editing. **Sougueh Cheik:** Investigation, Resources, Writing – review & editing. **Guillaume Coulouma:** Investigation, Resources, Writing – review & editing. **Rinh Pham Dinh:** Writing – review & editing, Resources, Investigation. **Séraphine Grellier:** Investigation, Resources, Writing – review & editing. **Claude Hammecker:** Investigation, Resources, Writing – review & editing. **Thierry Henry Des Tureaux:** Writing – review & editing, Resources, Investigation. **Ajay Harit:** Investigation, Resources, Writing – review & editing. **Jean Louis Janeau:** Writing – review & editing, Resources, Investigation. **Pascal Jouquet:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing. **Jean Luc Maeght:** Writing – review & editing, Resources, Investigation. **Pascal Podwojewski:** Investigation, Resources, Writing – review & editing. **Cornelia Rumpel:** Writing – review & editing. **Stéphane Sammartino:** Writing – review & editing, Resources, Investigation. **Norbert Silvera:** Investigation, Resources, Writing – review & editing. **Siwaporn Siltecho:** Investigation, Resources, Writing – review & editing. **Lotfi Smaili:** Investigation, Resources, Writing – review & editing. **Bounsamay Soulileuth:** Investigation, Resources, Writing – review & editing. **Nicolas Bottinelli:** Formal analysis, Software, Writing – original draft, Visualization, Conceptualization, Methodology, Writing – review & editing, Investigation, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.geoderma.2025.117177.

Data availability

Data will be made available on request.

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