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Luana Caiafa

Magnitude and drivers of greenhouse gas emission from ant nests

Juiz de Fora
2021

Luana Caiafa

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Dissertação apresentada ao Programa de Pós-Graduação em Biodiversidade e Conservação da Natureza, Instituto de Ciências Biológicas da Universidade Federal de Juiz de Fora como requisito parcial para a obtenção do grau de Mestre.

Orientadora: Profa. Dra. Juliane Floriano Lopes Santos
Coorientador: Prof. Dr. Nathan Oliveira Barros

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“Magnitude and drivers of greenhouse gas emission from ant nests”

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Dissertação apresentada ao Instituto de Ciências Biológicas, da Universidade Federal de Juiz de Fora, como parte dos requisitos para obtenção do Título de mestra em Biodiversidade e Conservação da Natureza.

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Profa. Dra. Juliane Floriano Lopes Santos
Universidade Federal de Juiz de Fora – UFJF

Nathan O Barros

Prof. Dr. Nathan Oliveira Barros
Universidade Federal de Juiz de Fora – UFJF

Simone Jaqueline Cardoso

Profa. Dra. Simone Jaqueline Cardoso
Universidade Federal de Juiz de Fora - UFJF

Prof. Dr. Flavio Roces
Universidade de Würzburg

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“*Not only is it important to ask*
questions and find the answers, as a
scientist I felt obligated to communicate
with the world what we were learning”.
(Stephen Hawking)

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Abstract

Greenhouse gas (GHG) emission is a worldwide concern, making the identification of emission sources and their magnitude an urgent issue. Ants release large amounts of carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) from their nests. Beyond the already known anthropogenic sources of CO_2 emissions, such as fossil fuel combustion in the power generation, industrial, residential, and transport sectors, the evaluation of GHG emissions from ant nests is a current research target. However, there are still gaps regarding the real impact of ant nests on GHG emission, primarily because most surveys were conducted excluding anthropogenic areas. In the present study, we aimed to fill part of these gaps by evaluating the magnitude and drivers of GHG emission from ant nests. Our results were organized over three chapters. First, chapter I discussed the current scenario and highlighted the methodological issues of the studies on GHG emissions from ant nests using a systematic review as methodology. We present a scientometry of available publications, which revealed the lack of investigations of ant nests emission in anthropogenic areas and on countries that are higher sources of anthropogenic GHG. Second, in Chapter II, we've made a methodology test related to the closed chamber method, guided by some warnings which stood out in the systematic review. Results revealed a dilution effect of the soil surface over the CO_2 measurements when using chambers whose diameters are significantly larger than the nest hole. Finally, in Chapter III, we've made field measurements of CO_2 emissions from *Acromyrmex subterraneus* nests in urban areas, considering the effect of features of the nest. This investigation revealed an association of CO_2 flux with the function of the nest hole, a component of ant nests not yet investigated. Obtained results also showed that ant nests are on a par with recognized anthropogenic CO_2 sources, adding one more point to consider in the context of climate change, considering the facility of installation of some ant species in urban and disturbed areas. We hope that these results contribute to expanding knowledge regarding the role of ant nests in CO_2 emissions and could even guide the next steps for future investigations.

Keywords: GHG flux, soil biogeochemistry, closed chamber method, ant nest features.

Resumo

A emissão de gases de efeito estufa (GEE) é uma preocupação mundial, tornando urgente a identificação das fontes de emissão e sua magnitude. As formigas liberam grandes quantidades de dióxido de carbono (CO_2), metano (CH_4) e óxido nitroso (N_2O) de seus ninhos. Além das já conhecidas fontes antropogênicas de emissões de CO_2, como a queima de combustíveis fósseis nos setores de geração de energia, industrial, residencial e de transporte, a avaliação das emissões de GEE por ninhos de formigas é um alvo atual de pesquisa. No entanto, ainda existem lacunas em relação ao real impacto dos ninhos de formigas na emissão de GEE, principalmente porque a maioria dos estudos foram feitos em áreas naturais, não incluindo as áreas antrópicas. No presente estudo, nosso objetivo foi preencher parte dessas lacunas avaliando a magnitude e os *drivers* da emissão de GEE por ninhos de formigas. Nossos resultados foram organizados em três capítulos. Primeiramente, o capítulo I, através de uma revisão sistemática como metodologia, discutiu o cenário atual e destacou as questões metodológicas dos estudos sobre emissões de GEE por ninhos de formigas. Apresentamos uma cienciometria das publicações disponíveis, que revelou a falta de investigações sobre a emissão de formigueiros em áreas antropogênicas e em países que são maiores fontes de GEE antrópicos. Em segundo lugar, no Capítulo II, norteados pela lacuna encontrada na revisão sistemática, fizemos um teste de metodologia relacionado ao método de câmara fechada. Os resultados revelaram um efeito de diluição da superfície do solo sobre as medições de CO_2 ao usar câmaras cujos diâmetros são significativamente maiores do que o buraco do ninho. Por fim, no Capítulo III, fizemos medições em campo das emissões de CO_2 de ninhos de *Acromyrmex subterraneus* em áreas urbanas, considerando o efeito das características do ninho. Essa investigação revelou uma associação do fluxo de CO_2 com a função do olheiro, um componente dos ninhos de formigas ainda não investigado nesse contexto. Os resultados obtidos também mostraram que os ninhos de formigas se equiparam a importantes fontes antrópicas de CO_2, acrescentando mais um ponto a se considerar no contexto das mudanças climáticas, levando em conta a facilidade de instalação de algumas espécies de formigas em áreas urbanas e perturbadas. Esperamos que esses resultados contribuam para ampliar o conhecimento sobre o papel dos formigueiros nas emissões de CO_2 e possam orientar os próximos passos para futuras investigações.

Palavras-chave: fluxo de GEE, biogeoquímica do solo, método de câmara fechada, características dos olheiros.

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Summary

1 | Thesis Description

The carbon dioxide (CO_2) emission from the soil results from a combination of physical and biological features, which include water content, temperature, and nutrient availability on the soil (Davidson et al., 2006). These features associated with the presence of the fauna and microorganisms are important drivers of the CO_2 emissions and increase the spatial and temporal heterogeneity of carbon in the soil (Ohashi et al., 2005).

The edaphic fauna, especially ants, stands out to induce variations of CO_2 emissions and other greenhouse gases (GHG), such as methane (CH_4) and nitrous oxide (N_2O) (Bender and Wood, 2003; Wu et al., 2013; Jílková et al., 2015; Majeed et al., 2018; Soper et al., 2019), due to the bioturbation effect of their nests on the soil properties (Kilpeläinen et al., 2007; Wu et al., 2013). Although the CO_2 emissions from ant nests are part of the natural carbon cycle, human intervention in natural areas facilitates the installation and development of more nests, which could put the ant nests as villains in the context of GHG emissions to the atmosphere.

It is still unclear how the ant nests contribute to GHG emissions to the atmosphere, which opens the way for some questions: (i) what gaps still exist in studies already carried out about this topic? (ii) what nest characteristics should be evaluated that allow measuring ants' influence in the greenhouse gas emissions context? (iii) what is the real magnitude of GHG emissions from the ant nests and how much it is comparable to other recognized GHG emission sources? Herein, I aimed to answer these and other questions, presenting the obtained results in three chapters.

In the first chapter, I present a systematic review aiming to provide a current overview of GHG emissions from ant nests exploring questions about the methodologies used, highlighting the gaps in the studies included in the scientometry, such as the lack of investigations of GHG emissions from ant nests in anthropogenic areas. In Chapter II, I tested two parameters of the closed chamber methodology to check if the diameter and insertion depth of the respirometric chamber in the soil is a source of bias in CO_2 flux measurements in the field. Finally, in Chapter III, I carried out fieldwork using a species model *Acromyrmex subterraneus*, a leaf-cutting ant species common in urban environments, to evaluate how physical features of the nest are associated with the magnitude of the CO_2 emission. Also, we compared the obtained results with published data of other ant species and recognized anthropogenic CO_2 emission sources.

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2 | Chapter I

Greenhouse gas emissions from ant nests: A systematic review

Luana Caiafa[1,3], Nathan Oliveira Barros[1,2], Juliane Floriano Santos Lopes[1,3]

[1] MirmecoLab, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora 36036–900, Juiz de Fora, Minas Gerais, Brazil

[2] Laboratório de Ecologia Aquática, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora 36036–900, Juiz de Fora, Minas Gerais, Brazil

[3] Programa de Pós-Graduação em Biodiversidade e Conservação da Natureza, Universidade Federal de Juiz de Fora, 36036–900, Brazil

Corresponding author: caiafaluana96@gmail.com

Highlights

- Ant nests are ecologically important emission sources of greenhouse gases.
- Regarding GHG emissions, the most investigated ant species were the cold climate specialists.
- Standardization of GHG measurement methods is needed to allow comparison of the impact of ant nests.
- Investigation of GHG fluxes from ant nests in anthropogenically disturbed areas is critical.
- Collaborative and global studies must be encouraged to close knowledge gaps.

Abstract: Greenhouse gas (GHG) emission is a worldwide health and environmental concern and identifying emission sources and their magnitude an urgent matter. Ant nests are important emission sources or sinks of carbon dioxide, methane, and nitrous oxide. Therefore, a current outlook regarding advances on GHG flux from ant nests, especially methods, equipment, and similarity between investigated ant species, could provide a more comprehensive grasp of their role in this context. Here, we evaluated these worldwide advances based on a systematic review. We registered that CO_2 was the most frequently examined gas and that the nest's characteristics were the factors most authors used authors to justify their choice of investigated ant species. The ant species were in nine different functional groups, with cold climate specialists as the most frequent. Concerning the measurement methods, a closed chamber system associated with an infrared gas analyzer was the most used, containing several variations related to the chamber's size and material. The authors' collaboration network revealed some interconnected clusters of research teams, which belonged to European countries. Consequently, most studies were on this continent and in journals inserted in the soil science knowledge area. Natural environments without anthropogenic influence made up most of the studied habitats. Among the GHG flux drivers from ant nests, the most important ones are related to the activity of ants and microbes and the properties of the soil. Our study points out the need to inventory GHG flux from ant nests, especially in anthropogenic areas. Furthermore, it is necessary to standardize the measurement method. These advances will only be possible with collaborative studies, increasing the interaction network between researchers through projects on a continental or global scale.

Keywords: GHG flux, CO_2 efflux, CO_2 production, biogeochemistry, nutrient cycling.

1. Introduction

Greenhouse gases (GHG) emissions have risen substantially since 1990, with evidence supporting that this growth is accelerated by anthropogenic activities. Therefore, the global concerns about the effects of GHG concentration on the atmosphere, and its indirect effects on environmental and human health, have increased, as did the acknowledgment of how natural sources are also linked to GHG emission and sink.

Existing data suggest that edaphic fauna, especially ants, are responsible for releasing large natural amounts of GHG from the soil (Ohashi et al., 2007; Ohashi et al., 2017; Bender and Wood, 2003; Berberich et al., 2018a; Soper et al., 2019). This high importance attributed to ants in raising GHG release is related to their nest building activity, which modifies the nutrient cycling in the soil by altering its structure and biogeochemical processes (Jones et al., 1994; Frouz and Jílková, 2008; Whitford and Eldridge, 2013; Farji-Brener and Werenkraut, 2017). Additionally, ants have a worldwide distribution and represent a large proportion of animal biomass (Hölldobler and Wilson, 1990; Agosti et al., 2000). Therefore, their participation as GHG emission agents is of global interest and should be included when discussing atmospheric GHG increases and their associated effects.

Carbon dioxide (CO_2), the most significant GHG produced by ant nests, originates mainly from the ants' respiration, and is strongly related to the size and activities of the colony population (Risch et al., 2005a, Jílková and Frouz, 2014). Additionally, as ant nests cause alterations in the soils' chemical properties, they also interfere in the microorganisms' activity, and hence in the soil respiration (Kilpeläinen et al., 2007; Wu et al., 2010; Sousa-Souto et al., 2012; Jílková and Frouz, 2014).

On the other hand, ant nests can represent either a source or sink of methane (CH_4). Their conflicting functions concerning the CH_4 cycle is related to the material used to build the nests (Jílková et al., 2015b) and the alterations this may cause in the soil's properties, for instance, methane oxidation depends on factors such as temperature (Adamsen and King, 1993; Borken and Beese, 2006), soil porosity (Ball et al., 1997), and aeration (Vor et al., 2003). Often, CH_4 production is verified in the upper part of nests, mainly from microbial processes (Bender and Wood, 2003; Jílková et al., 2015b; Berberich et al., 2018a), while the consumption of CH_4 by soil methanotrophic bacteria occurs deep in the nest (Jílková et al., 2016).

Regarding nitrous oxide (N_2O), which originates from microbial nitrification and denitrification processes (Wrage et al., 2001; Bateman and Baggs, 2005; Fangueiro et al., 2010), emissions are higher in sites with ant nests than in surrounding soil surfaces (Bender and Wood, 2003; Wu et al., 2013; Wu et al., 2015). It has been identified that the external refuse dumps of a leaf-cutting ant species are N_2O hotspots (Soper et al., 2019).

The most comprehensive review on the impact of ant nests on soil demonstrates that the CO_2 efflux from ant mounds is higher than that of the surrounding soil in European forests (Jurgensen et al., 2008). Another review corroborates that ant nests, especially from the *Formica rufa* group, are hotspots of CO_2 production (Frouz and Jílková, 2008). Both studies associate the increase of CO_2 emission with microbial activity, which may be higher inside the nest mainly because of nutrient availability. However, these reviews do not focus on the emission of CH_4 and N_2O from ant nests, and after their publication other authors have quantified GHG flux from ant nests in different species or habitats and applied distinct methodologies and equipment (Golichenkov et al., 2009; Sousa-Souto et al., 2012; Wu et al., 2013, 2015; Wang et al., 2018), which opens the way for a new synthesis.

Therefore, this systematic review aims to evaluate the advances regarding the flux assessment of the three greenhouse effect gases (CO_2, CH_4, and N_2O) from ant nests considering the methods, equipment, similarity between investigated ant species, and the factors used as selection criteria of species for the study. Furthermore, we discuss the need to standardize the sampling methods of GHG emission from ant nests and suggest the broadening of the investigation by including neotropical ant species, both in natural and anthropogenic areas, where some ant species have more ease in their establishment.

2. Methods

We collected data following the steps proposed by the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) methodology (Moher et al., 2009, 2015). This methodological approach includes the development of selection criteria, definition of search strategies, and assessment of study quality.

To identify global-scale records about the topic "Greenhouse gas (CO_2, CH_4, and N_2O) emissions from ant nests", we performed a search in the Scopus (Elsevier) and Web of Science (Thomson Reuters) databases, which only contained original peer-reviewed scientific articles, published until April 2020. To access all registers, we used the following search code: ((("carbon dioxide emission *" OR "CO_2 emission *" OR "methane emission *" OR "CH_4 emission *" OR "nitrous oxide emission *" OR "N_2O emission") OR ("carbon dioxide" OR CO_2 OR "methane" OR CH_4 OR "nitrous oxide"

OR N_2O) OR (“carbon dioxide flux *” OR “CO_2 flux *” OR “methane flux *” OR “CH_4 flux *” OR “nitrous oxide flux *” OR “N_2O flux *”) OR (“carbon dioxide efflux *” OR “CO_2 efflux *” OR “methane efflux *” OR “CH_4 efflux *” OR “nitrous oxide efflux *” OR “N_2O efflux *”) OR (“carbon dioxide product *” OR “CO_2 product *” OR “methane product *” OR “CH_4 product *” OR “nitrous oxide product *” OR “N_2O product *”) OR ("respiration rate") OR ("greenhouse gas *" OR "greenhouse gas * emission *" OR GHG * OR "GHG * emission *")) AND (“ant nest *” OR “ant mound *” OR anthill * OR ant *)).

We made the inclusion and exclusion of publications at two levels, (i) screening of title and abstract, followed by (ii) the screening of the full text. The eligibility criteria in both screenings were: studies approaching GHG emissions from ant nests.

We retrieved the following information from the studies: (i) year of publication; (ii) journal and respective impact factor; (iii) country of the study area; (iv) author and their collaboration network; (v) investigated ant species and reason for the author's choice; (vi) drivers of GHG flux; (vii) habitat of study; (viii) used method for GHG emission measurement (closed chamber, air probe, among others); (ix) type of equipment for measuring flux of gases. We also categorized the studied ant species by their functional group (Andersen, 1997; Hung, 2006; Brandão et al., 2012; Berman et al., 2013) to verify shared characteristics among them.

We adapted the Prisma flowchart from PRISMA (Moher et al., 2009) and built the graphics using the following packages “ggplot2” (Wickham, 2016), “ggpubr” (Kassambara, 2019), “rworldmap” (South, 2011), “RColorBrewer” (Neuwirth, 2014), “maps” (Minka and Deckmyn, 2018), “wordcloud” (Fellows, 2018), “wordcloud2” (Lang and Chien, 2018), “ggwordcloud” (Le Pennec and Slowikowski, 2019), “gridExtra” (Auguie, 2017), “cowplot” (Wilke, 2019), “treemapify” (Wilkins, 2019), “treemap” (Tennekes, 2017), and “dplyr” (Wickham et al., 2020), through R Statistical Software version 3.6.0 (R Core Team, 2019).

To check if there was any distribution trend in the frequency of publications by year, we adjusted a theoretical distribution curve, using the "descdist" function of the package "fitdistrplus" (Delignette-Muller and Dutang, 2015). We also used the “bibliometrix” R package (Aria and Cuccurullo, 2017) to extract and make a descriptive analysis of the data and the VOSviewer software (version 1.6.15) to build the author's collaboration network (Van Eck and Waltman, 2010, 2014). VOSviewer employs a modularity-based clustering technique by grouping similar objects in the graph (Van Eck and Waltman, 2007), allowing us to understand the stratification and grouping characteristics in the author's collaboration network.

3. Results

3.1 Systematic review and scientific production

Our systematic search resulted in 752 scientific publications. We excluded duplicates (n = 88) and read the title and abstract of each remaining publication (n = 664), selecting only those that included the keywords "CO_2 or CH_4 or N_2O or GHG emissions/flux/efflux from ant nests". We considered the ant nest as the physical component where ants live and the ant colony as the nest and its inhabitants. Thus, the selected studies measured the GHG emissions from the nest's interior in external mounds, nest holes, and dumps. We excluded articles related to the ants' energy metabolism and respiratory physiology, internal nest ventilation, and the soil's nutrient mineralization process. After applying these filters, we were left with 29 publications (Figure 1).

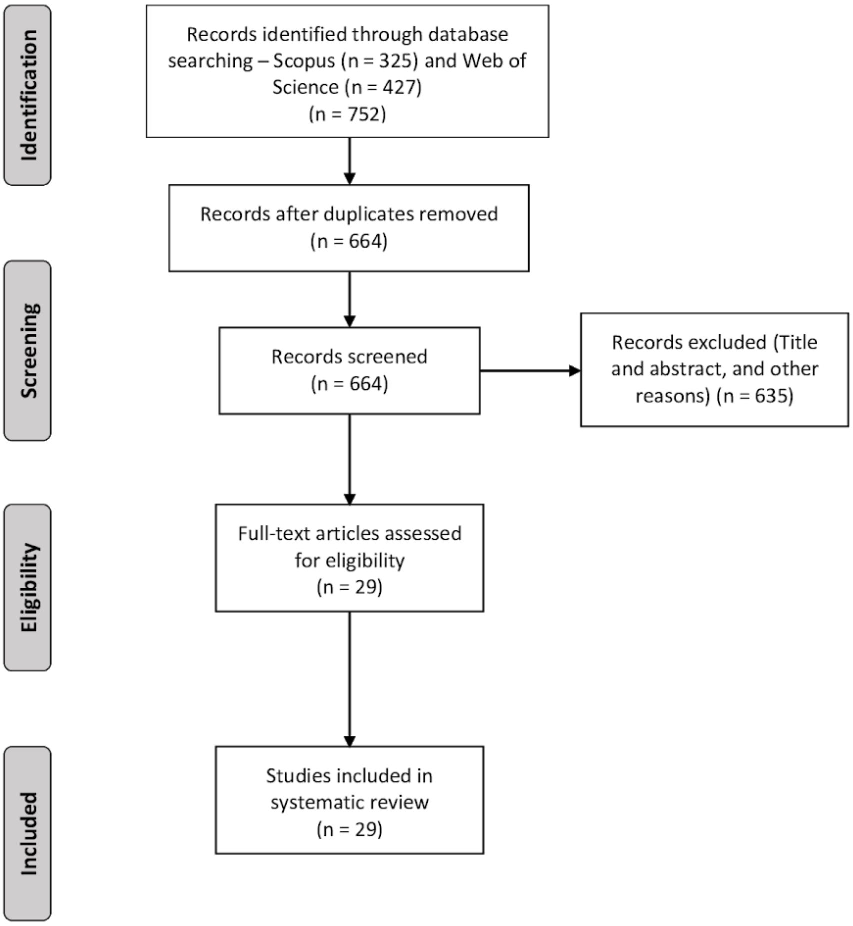

Figure 1. Prisma flowchart shows the selection process for studies on ant nests associated with GHG emissions. Figure adapted from PRISMA (Moher et al., 2009).

In our database, CO_2 flux was the most frequently reported flux from ant nests (93.1%), followed by CH_4 (27.6%) and N_2O (24.1%). Although the first publication occurred in 1987, the last 20 years comprised 96.5% of the publications, with a highlight to 2018 (Figure 2). The frequency distribution of publications along the years fit within the negative binomial theoretical distribution, revealing an exponential distribution of studies over time. This theoretical distribution presents a longer tail than the average (Ross and Preece, 1985), meaning that data is over-dispersed, with periods containing more studies (1987, 2000 - 2009, and 2012 - 2020) and periods of scarcity (until 1987, 1988 - 1999).

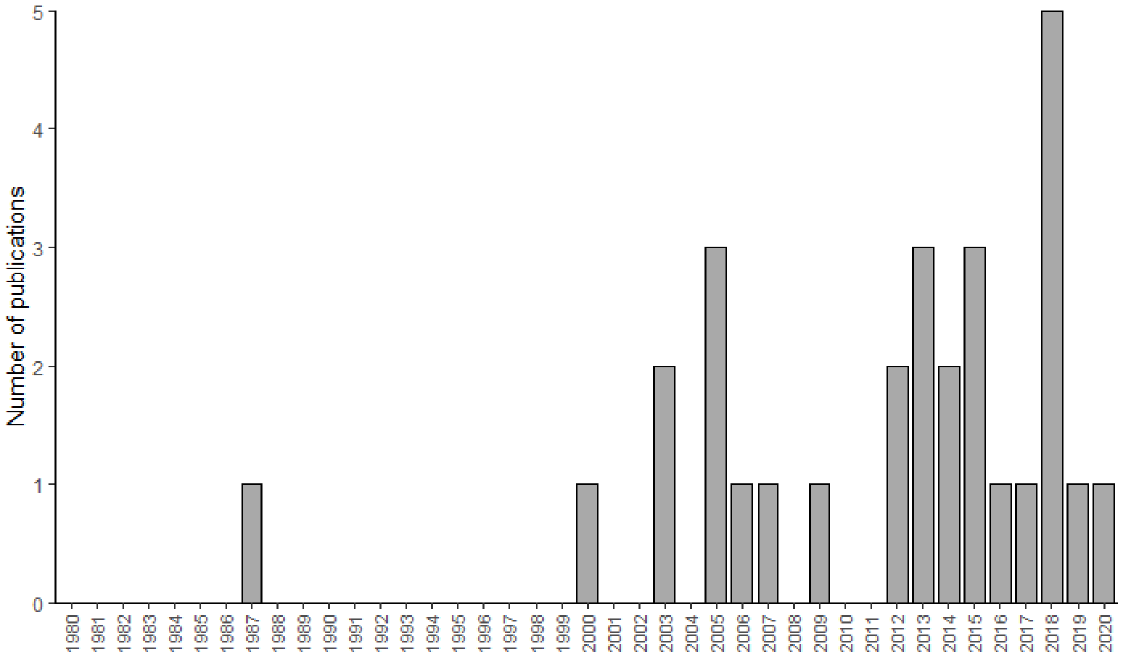

Figure 2. Number of publications focusing on GHG emissions from ant nests by year, compiled through a systematic literature review from 1980 to May 2020 period (n = 29).

The journal Soil Biology and Biochemistry was the one with the most publications and presented the second higher impact factor among the journals included in this review (Figure 3). According to the Journal Citations Reports (JCR), the analyzed journals' impact factors range from 0.5 to 6.5. According to the journals' scope, they are included in the following knowledge areas: soil science, environmental sciences, geosciences, biology, chemistry, biochemistry, and molecular biology, agronomy, meteorology and atmospheric sciences, ecology, zoology, entomology, animal science, evolutionary biology, and microbiology.

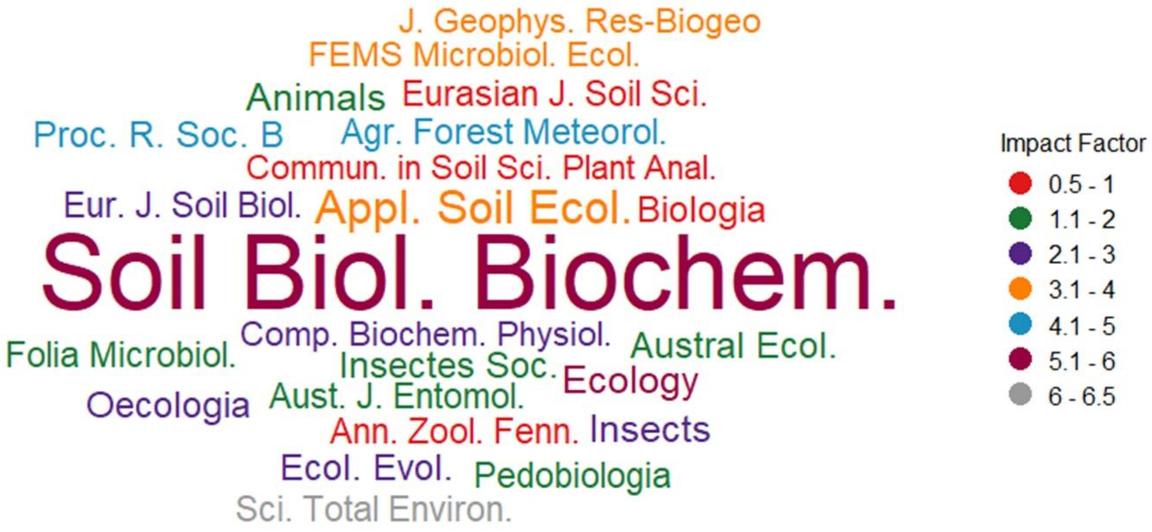

Figure 3. Word cloud representing the number of publications by scientific journals included in the systematic review. Font size represents the frequency of registers. Colors indicate the impact factor in which each journal is fitted.

The 29 selected studies were conducted in 14 countries, listed in descending order: Finland (17.2%), Czech Republic (13.8%), China, Costa Rica (10.3% each), Argentina, Germany, Switzerland, United States (7% each), and Australia, Brazil, Malaysia, Mexico, Russia, Thailand (3.4% each) (Figure 4). In summary, data indicated that Europe presents most of the research, and there is a lack of data for African countries.

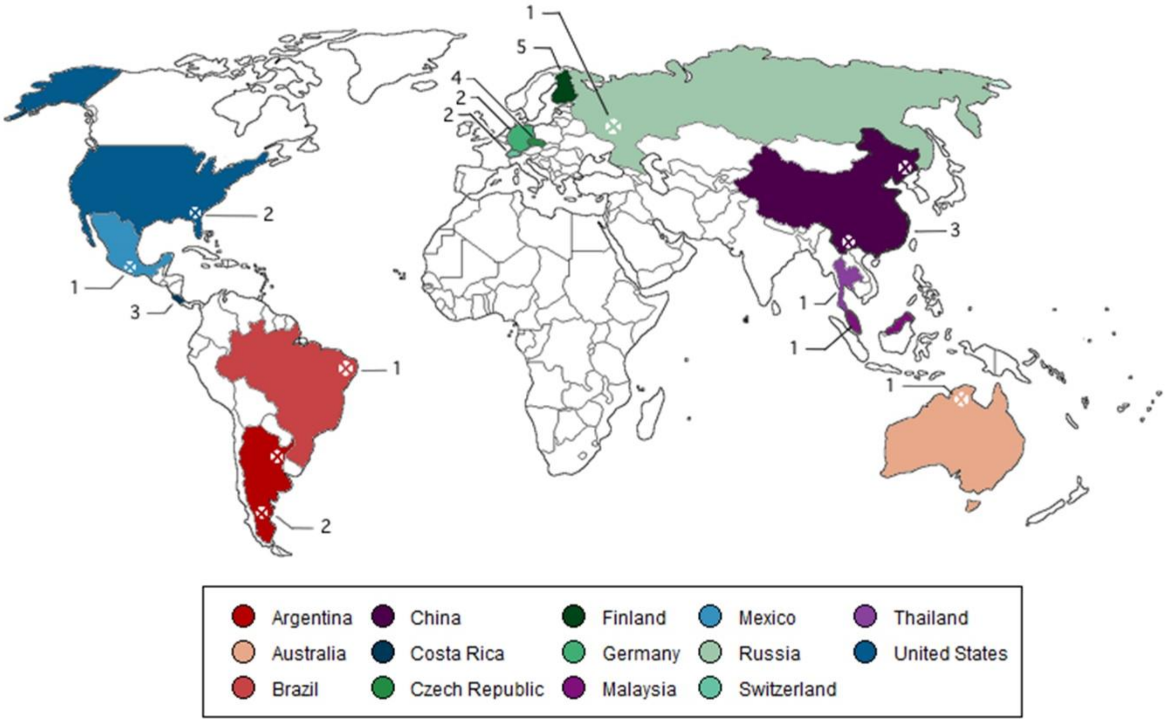

Figure 4. World Map containing geographical distribution and number of publications included in the systematic review. In larger countries, we marked the exact point of the study area of the publications.

Senior author Mizue Ohashi conducted most of the publications (24.1%). The authors' collaboration network revealed that his team's dark green cluster presented a strong interaction to the dark blue cluster, composed by the team led by Timo Domisch (Figure 5). These clusters were also connected to the yellow cluster led by Veronika Jílková and Jan Frouz. We highlight that the author Mizue Ohashi collaborated with Sasitorn Hasin's team, contributing to knowledge advancement on the theme in the Asian continent (dark green cluster). The research teams led by the authors Mizue Ohashi, Timo Domisch, Veronika Jílková, and Anita Risch were responsible for the scientific production in the European continent. All the other clusters were isolated, representing co-authorship in the same publication (Figure 5).

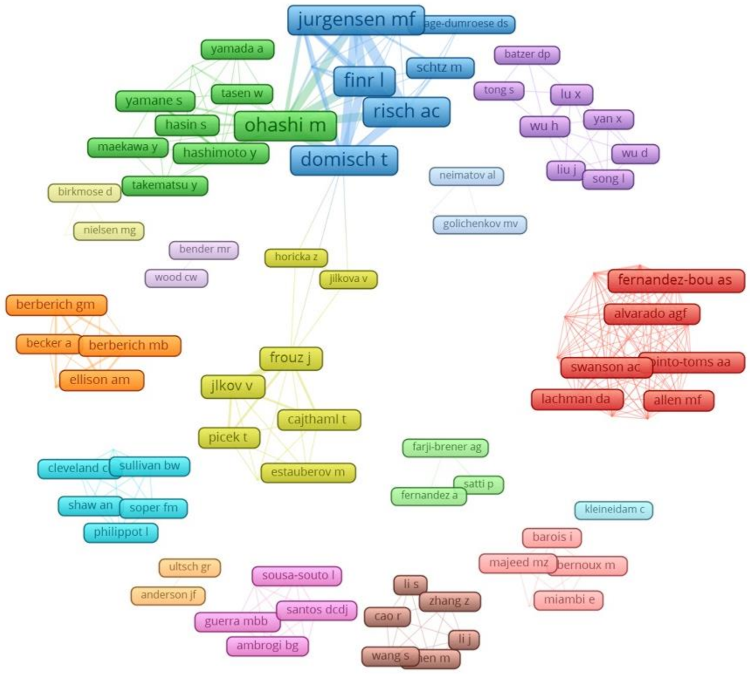

Figure 5. Authors' collaboration network of the publications included in the systematic review. Distinct colors represent the clusters, and the line width is relative to the interaction strength. Some co-authors' names are missing.

3.2 Studied ant species and áreas

In total, 64 ant species had GHG emission examined in their nests. Species from the *Formica rufa* (*F. rufa*) group were the most studied, appearing in 13 publications (44.8%). Some publications considered this ant species under the taxonomic level of species, and others, inside the *F. rufa* groupment. The species *Formica aquilonia* and *Formica polyctena* are part of the *F. rufa* group (Figure 6). The factors used as selection criteria for the ant species were mainly related to the nest's characteristics. Indeed, some ant species share characteristics such as vast and epigeic nests with complex architecture. One publication stood out, as it examined 56.3% of the 64 studied ant species, and the authors randomly chose these. The studied ant species belonged to nine different functional groups, highlighting the cold climate specialists, including 24% of the data (Figure 7, Supplementary Table 1, 2).

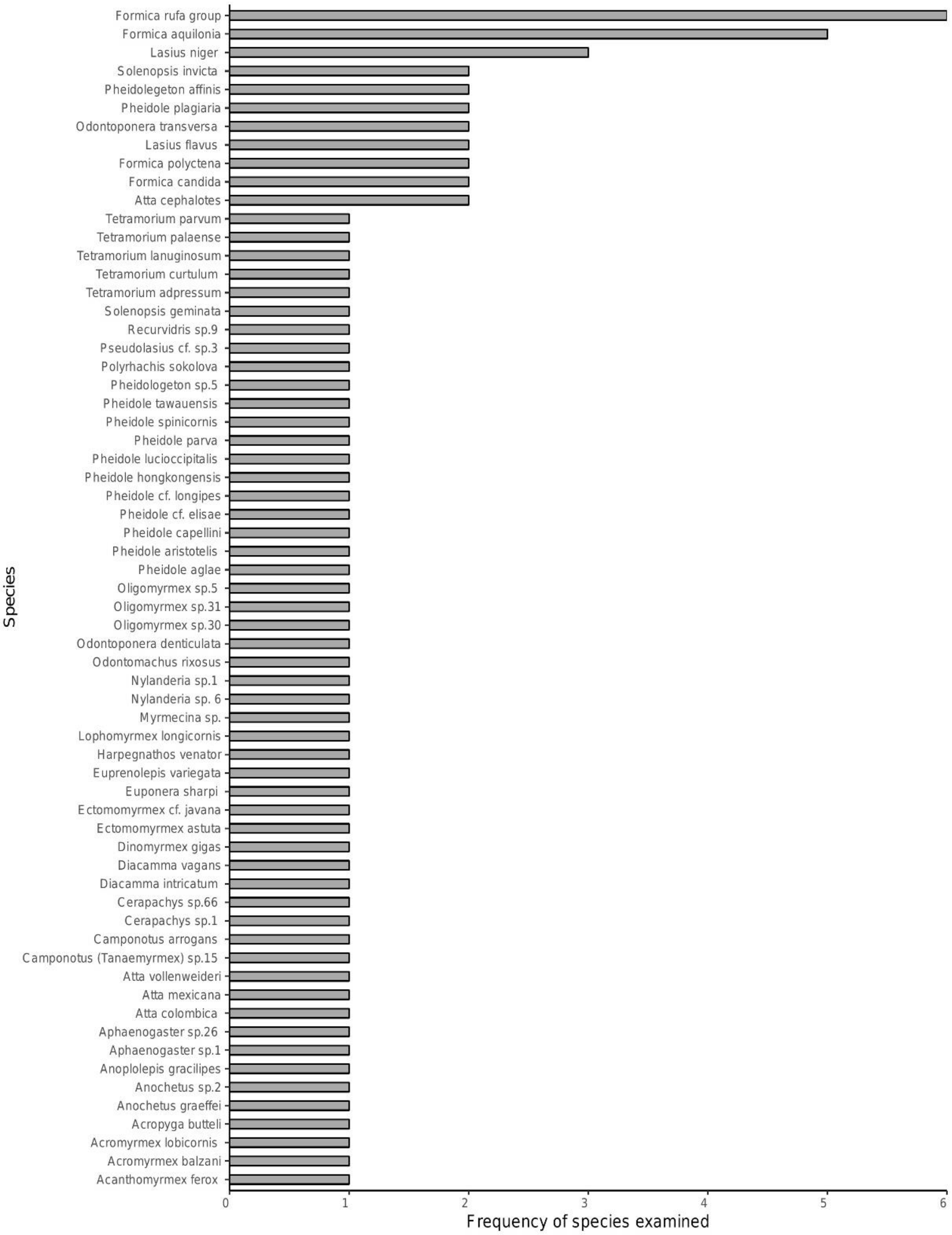

Figure 6. Frequency of ant species investigated about their nest's GHG emission in the analyzed publications.

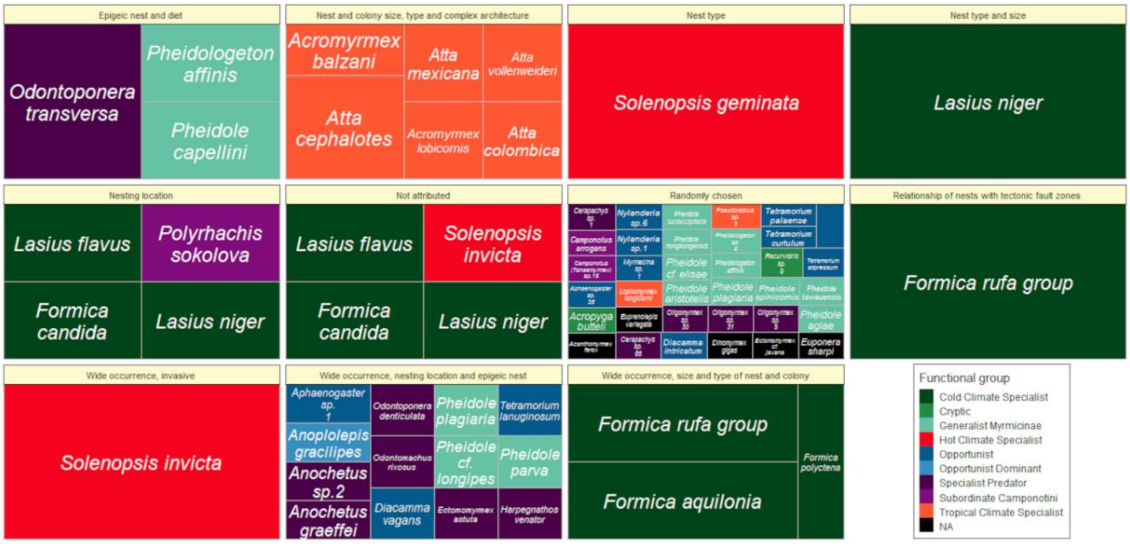

Figure 7. Treemap representing the authors' justificative to choose the ant species for studying GHG emissions of their nest grouped in categories (label of the panels) and the classification of these species according to functional groups (colors). The size of the tiles represents the frequency of each ant species within the authors' justificative category.

The main reported CO_2 drivers (Table 1) were: respiration and metabolic rate of the ants (mentioned in 63% of the publications that studied CO_2 flux); soil properties, which include temperature, moisture, organic matter, nutrient content, dissolved Carbon and Nitrogen content, C:N ratio, and pH (55.5%); the soil biota activity, which include decomposition, and respiration of roots, non-ant invertebrates and microorganisms (51.8%). Regarding CH_4 drivers, microbial activity was the main factor (44.4%), as for N_2O drivers, microbial activity also stood out (42.8%).

Table 1. Drivers of GHG flux from ant nests cited in each publication.

#	Reference	Drivers of GHG flux from ant nests		
		CO_2	CH_4	N_2O
(1)	Anderson and Ultsch, 1987	Moisture content and metabolic rate	NA	NA
(2)	Kleineidam and Roces, 2000	Respiratory exchange ratio of the colony and soil properties	NA	NA
(3)	Bender and Wood, 2003	Respiration by ants and microbial activity within the colonies	NH_4-N concentrations	Temperature, soil water content, nitrifiable N content, organic materials,

				concentrations of soil NO_3-N and NH_4-N
(4)	Nielsen et al., 2003	Soil microbial respiration and density of ants	NA	NA
(5)	Ohashi et al., 2005	Organic matter and nutrient concentration, temperature, respiration of ants, microbes, and plant roots	NA	NA
(6)	Risch et al., 2005a	Respiration of ants and other invertebrates living in the mounds	NA	NA
(7)	Risch et al., 2005b	Changes in air temperature, metabolic rates of ants	NA	NA
(8)	Domisch et al., 2006	Ant respiration and mound temperature	NA	NA
(9)	Ohashi et al., 2007	Ant respiration and temperature and moisture within the mound		
(10)	Golichenkov, Neimatov, and Kiryushin, 2009	Ant respiration	Effect of ants on soil microbiological activity	Effect of ants on soil microbiological activity
(11)	Ohashi et al., 2012	Ant respiration, population size, and mound temperature	NA	NA
(12)	Sousa-Souto et al., 2012	Soil microorganism's activity, water and soil organic matter content	NA	NA
(13)	Jílková et al., 2013	Moisture, carbon content and availability, C:N ratio, and pH	NA	NA
(14)	Wu et al., 2013	Temperature, soil microorganism's activity, soil nutrient concentration, ant respiration, soil organic carbon and dissolved carbon concentrations	Water table height, soil water content, temperature, plant community, soil redox potential, and nutrient content	Higher nitrogen concentration, soil moisture
(15)	Fernandez, Farji-Brener, and Satti, 2013	Respiration rate of soil microorganisms and soil moisture	NA	NA
(16)	Hasin et al., 2014	Species-specific activity of ants, the nest soil environment, and nest structure	NA	NA
(17)	Jílková and Frouz, 2014	Ant and nest material respiration, water content, and ant foraging activities	NA	NA
(18)	Jílková, Cajthaml, and Frouz, 2015	Response of ant and microbial respiration to temperature in nests	NA	NA
(19)	Jílková, Picek, and Frouz, 2015	Ants and microorganisms respiration	Nest material characteristics	NA
(20)	Wu et al., 2015	Species-specific (different foraging	NA	Species-specific (different foraging

		habits) and ant mound soil		habits) and ant mound soil
(21)	Jílková et al., 2016	Substrate properties, differences in available nitrogen and carbon contents and pH	Substrate properties, differences in available nitrogen and carbon contents and pH	NA
(22)	Ohashi et al., 2017	Ant respiration, the soil mixing and soil moisture content	NA	NA
(23)	Berberich et al., 2018a	NA	Microbial activity and tectonic fault-related emissions	NA
(24)	Berberich et al., 2018b	Respiration of ants and other invertebrates, root respiration, and microbial decomposition of nest material	NA	NA
(25)	Majeed et al., 2018	NA	NA	Nitrification and denitrification by microbial processes in ant mounds
(26)	Wang et al., 2018	Ant species abundance and body size, inputs of nest materials, soil micro-environment, microbial and physicochemical properties	NA	NA
(27)	Fernandez-Bou et al., 2018	Nest activities and microbial and root respiration in the soil	NA	NA
(28)	Soper et al., 2019	Substrate availability, abiotic conditions and microbial biomass and activity	Substrate availability, abiotic conditions and microbial biomass and activity	Substrate availability, abiotic conditions and microbial biomass and activity
(29)	Fernandez-Bou, Dierick, and Harmon, 2020	Fungus and ant respiration, refuse decomposition, roots and soil microbes surrounding the nest	Fungus and ant respiration, refuse decomposition, roots and soil microbes surrounding the nest	Fungus and ant respiration, refuse decomposition, roots and soil microbes surrounding the nest

Most studies occurred on natural environments without anthropogenic influence, mainly in forests (59%), specifically the Temperate, Subalpine, Boreal coniferous, Boreal, Wet tropical rainforest, Tropical, Seasonal tropical, Tropical rainforest, and Humid tropical rainforest. Only one study was conducted in an abandoned cropland area (Golichenkov et al., 2009), and another in managed boreal forests (Ohashi et al., 2012) (Supplementary Table 3).

3.3 Sampling methods and equipment

Sampling methods to measure the GHG flux from ant nests presented variations according to research purpose and equipment availability. We grouped them into four types of sampling methods: (i) ant nests samples; (ii) alkali absorption; (iii) air probes; and (iv) closed chamber (Figure 8, Supplementary Table 3).

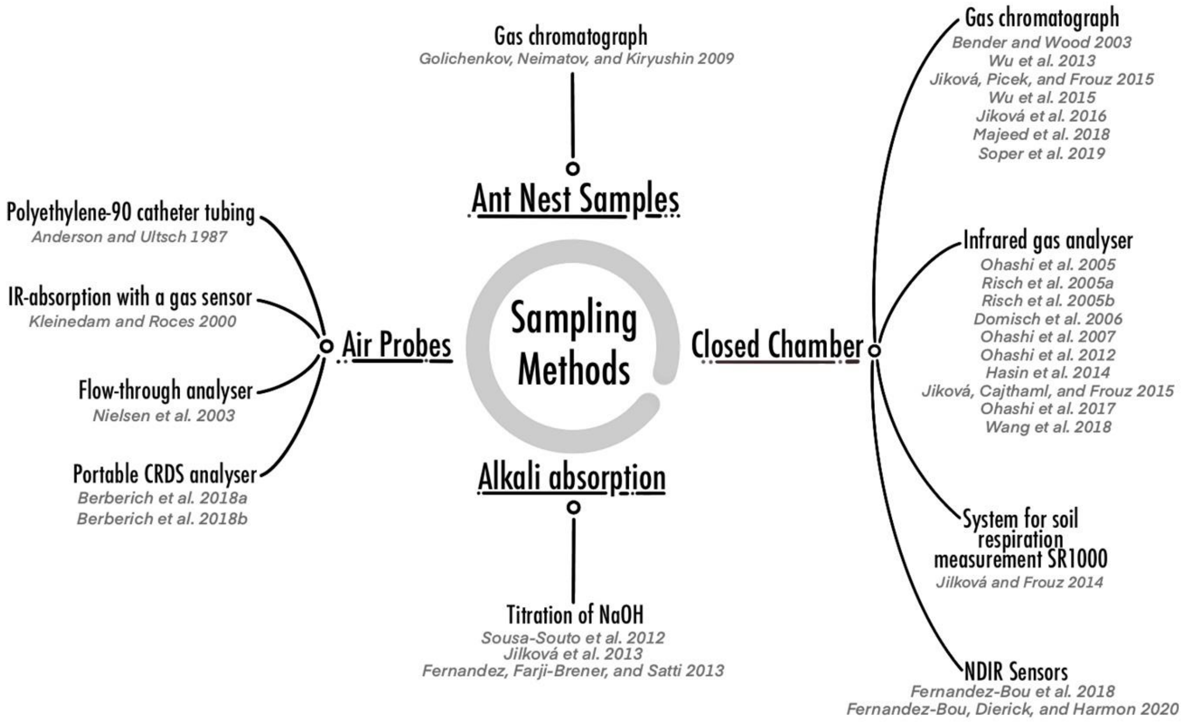

Figure 8. Mind map visualization of the different types of GHG emission sampling methods.

Only one publication relied on taking physical samples from ant nests to be later analyzed in a laboratory (Golichenkov et al., 2009). Three other studies used alkali absorption followed by sodium hydroxide (NaOH) titration as a sampling method (Sousa-Souto et al., 2012; Jílková et al., 2013; Fernandez et al., 2013). Another five studies collected air samples inside the nest using air probes and conducted the analyses either in a laboratory (Kleineidam and Roces, 2000; Nielsen et al., 2003) or immediately in the field, as the air probes were associated with a gas sensor equipment (Anderson and Ultsch, 1987; Berberich et al., 2018a, b), allowing the authors to make continuous gas measurements.

The most used measurement method for GHG emission was the closed chamber system (69%), defined as a GHG analyzer connected to a chamber through a closed system in which the air circulates (Figure 8, Supplementary Table 3). This method presented variations related to dimension, building, sealing material of the respirometric chambers, and the chamber's placement relative to the ant nests (Supplementary Table 3). The closed chamber was often associated with equipment that used Infrared Gas Analyzer (IRGA) technology (35% of the studies) or gas chromatography (24%) (Figure 8, Supplementary Table 3).

4. Discussion

4.1 Systematic review and scientific production

Our analysis provides an overview of what has been globally published about GHG emissions from ant nests, a topic that has not been covered in previous reviews. Summarizing our results, we verified that CO_2 is still the most investigated GHG in ant nests, and the main measurement method was the closed chamber system. Also, our analysis revealed that the studied ant species belong to the cold climate specialists functional group, a reflection of the research teams' affiliation, and these species share some nest characteristics, often being large and populous. These nest traits were the main reported justification for the authors' choice.

Although there was no linear trend in the number of publications over the years, both the technological advances and the researchers' effort to ascertain the importance of different GHG emission sources contributed to increasing the number of publications over the last 20 years. Furthermore, although ant nests are natural sources of GHG emission, the density of some species is higher in anthropogenic areas than in natural ones (Santos et al., 2016; Michlewicz and Tryjanowski, 2017), allowing us to make predictions about how the deforestation process could impact GHG emission.

The 29 studies were published in 23 different journals, which presented a wide range of impact factor values. The two journals with the most publications cover the description and explanation of fundamental biological and biochemical features and processes occurring in soil systems. In comparison, the other 21 journals have distinct scopes, indicating that GHG emission from ant nests is an interdisciplinary topic.

Most of the publications regarding GHG emissions from ant nests were conducted in countries (Finland, Czech Republic, and Germany) where the authors had their professional affiliation. These countries are in the list of countries where CO_2 emission per capita varies between 7.5 to 10 tonnes, which are the highest emission values in Europe (Ritchie and Roser, 2017). The relationship between the number of publications and the higher emission of GHG per capita revealed their commitment to the Paris

Climate Agreement, which identifies the need to enhance and strengthen systematic observation, climate services, and knowledge sharing (UNFCCC, 2015). Some authors of these publications established worldwide collaboration networks, generating a comprehensive investigation of the role of red wood ants (RWAs) in the context of GHG emission. On the other hand, other countries that also presented higher GHG emission per capita (Mongolia, USA, Canada, Australia, Kazakhstan, and Saudi Arabia, according to Ritchie and Roser, 2017) had few or no publications. Thus, there is the need to encourage collaborative research between the authors to advance continental or global scale projects that cover other ant species.

4.2 Studied ant species and areas

As mentioned previously, the most studied ant species are the RWAs, which include six morphologically similar *Formica* species (*F. rufa*, *F. aquilonia*, *F. lugubris*, *F. polyctena*, *F. paralugubris*, and *F. pratensis*) (Goropashnaya et al., 2004; Seifert, 2007; Punttila and Kilpeläinen, 2009; Stockan and Robinson, 2016). This group is characterized by populous colonies, as these can reach millions of workers in a mature stage (Collingwood, 1979; Rosengren and Pamilo, 1983; Seifert, 1991). These ants generally establish epigeic nest building mounds aboveground (Jurgensen et al., 2008; Domisch et al., 2008) and are found in high densities in forests of Eurasia (Risch et al., 2005a, b; Domisch et al., 2006; Jílková and Frouz, 2014). These nest mounds have a heterogeneous composition that promotes temperature and moisture variation (Coenen-Stass et al., 1980; Rosengren et al., 1987). These different conditions provide various microhabitats with high food supply, supporting a high diversity of myrmecophile species (Skinner, 1980; Rosengren et al., 1987; Parmentier et al., 2014), contributing to a higher GHG emission.

Altogether, the high density of ant nests, myrmecophile species, and organic material, along with a high food concentration, results in alterations of the soil properties of both ant nests and the surrounding area (Frouz et al., 2005; Risch et al., 2005a, b; Domisch et al., 2008; Ohashi et al., 2012; Finér et al., 2012), which are the main drivers of the GHG flux from ant nests (Table 1). The alteration of soil properties results in a considerable flux of CO_2 and nutrients from ant nests, especially in colder environments, where CO_2 emission is usually higher than in warmer ones (Begon et al., 2005), as ants must alter their respiration rate to maintain adequate nest temperatures (Jílková et al., 2015a).

Indeed, the cold climate specialists, which includes the RWAs, *Lasius flavus* and *L. niger*, was the most investigated functional group. The cold climate specialists group has in common a high tolerance to low temperatures, and therefore can be dominant in temperate forests (Cuautle et al., 2016). In contrast, tropical climate specialists was the second functional group that stood out in the number of publications. In this functional group are the leaf-cutting ants (LCAs), which include the genera *Atta*, *Acromyrmex*, and the recently described *Amoimyrmex* (Cristiano et al., 2020) (Myrmicinae, Formicidae). The LCAs are of great economic importance due to their strong herbivory impact on cultivated plants (Costa et al., 2008; Leal et al., 2014; Fleury et al., 2015; Knoechelmann et al., 2020). Their nests are large in size, depth and population, and also present dominance in disturbed areas (Costa et al., 2008; Leal et al., 2014), factors that posit LCAs as a significant source of GHG (Kleineidam and Roces, 2000; Sousa-Souto et al., 2012; Fernandez-Bou et al., 2018, 2020; Soper et al., 2019).

Ant nests in natural environments received significantly more attention in the publications, only one study was carried out in abandoned cropland areas (Golichenkov et al., 2009) and another one in managed boreal forests (Ohashi et al., 2012). Although part of the GHG emissions would occur under natural conditions, ant nests are also present in urban areas and should be considered anthropogenic GHG sources. Some ant species, like LCAs, prefer anthropogenic areas as their nest founding sites (Santos et al., 2016; Michlewicz and Tryjanowski, 2017), where their nests and population are more prone to growing (Wirth et al., 2007; Leal et al., 2014). Thus, as nest size and population were an essential justification for the authors' choice of studied ant species, we suggest that future studies investigate the GHG emission from ant nests in disturbed anthropogenic areas.

4.3 Sampling methods and equipment

Regarding the measurement methods for GHG flux from ant nests, the least used was the manual collection of soil samples from ant nests (Golichenkov et al., 2009). In this method, the soil samples must first be sieved to remove objects that are not of interest, such as roots, and then gas samples are collected, usually with syringes, and transported to the laboratory, where they are injected into a gas chromatograph for analysis (Rolston, 1986). Gas chromatography is available in laboratories of chemical analysis, but although it has been considered a precise, robust, and relatively easy method for GHG quantification, the costs of the equipment restrict the analysis to few laboratories.

Alkali absorption (Witkamp, 1966) is a versatile method to measure CO_2 emissions from soil either in the laboratory (Anderson, 1982; Fernandez et al., 2013; Jíková et al., 2013) or in the field (Kirita, 1971; Sousa-Souto et al., 2012). It consists of the incubation of a direct source of CO_2 or a sample of the soil, with a caustic solution, generally sodium hydroxide (NaOH) or potassium hydroxide (KOH), that reacts with the CO_2 (Witkamp, 1966; Kirita, 1971). After the incubation period, the remaining solution is titrated with an acidic substance to determine the concentration of absorbed CO_2. It is a cheap, simple, and frequently adopted method (Freijer and Bouten, 1991). However, some authors report a potential bias in this method which could under or overestimate the real gas emission by suppressing the gas diffusion (Kucera and Kirkham, 1971; Freijer and Bouten, 1991) or accelerating the emission in response to a minor CO_2 concentration inside the chamber (Koizumi et al., 1991; Nakadai et al., 1993).

The air probe method is adopted to collect gas samples internally from the ant nests, with the great advantage of taking air samples at different depths (Kleineidam and Roces, 2000; Nielsen et al., 2003). Besides, it is possible to read the gas concentration immediately in the field if the probe is associated with some gas sensor equipment (Anderson and Ultsch, 1987). The air probe can be used momentarily, quickly analyzing samples, or continuously, monitoring data over a time period and measuring changes in the gas concentrations (Berberich et al., 2018a, b). However, the presence of air passages around the probe hole may cause a potential underestimation of gas concentration, demanding the sealing of the soil surface to prevent an air leakage (Nielsen et al., 2003).

The most used method to measure the GHG flux from ant nests was the closed chamber system (Hutchinson and Mosier, 1981; Rolston, 1986). Generally, air is circulated in a loop between the gas chamber and the external gas analyzer, enabling an observation of the variation of gas concentration over time. The Infrared Gas Analyzer (IRGA) equipment was the most frequently used. This method can be used for measurements in the field and is relatively low in cost, simple to operate, and allows the obtention of many measurements in a short time (Bekku et al., 1995; Rochette et al., 1997; Davidson et al., 2002; Pavelka et al., 2018). However, this method could present biases, as mechanical disturbances derived from the insertion of the chamber can affect the soil diffusion coefficient (Freijer et al., 1991). Furthermore, the measurement time should be short, as gas emissions rates can be affected by changes in the gas concentration gradient (Bekku et al., 1995; Davidson et al., 2002).

Our concern about the use of the closed chamber is that we verified that there are many systematic differences, such as dimension, raw material, and sealing material of the respirometric chambers. These differences point out an urgent need for standardization and validation of the closed chamber method, as also recommended by Janssens et al. (2000).

4.4 Summary and future perspectives

Our systematic review of GHG emissions from ant nests provides a more comprehensive current outlook of this theme to the scientific community. It signalizes the need to investigate GHG flux from ant nests in areas under anthropogenic intervention and countries with higher GHG emissions, thus contributing towards the global effort to inventory GHG sources. Moreover, our data highlight the need to standardize the GHG measurement method, allowing reliable comparisons among ant species and habitats. These imperative advances will only be possible through collaborative research, increasing interaction among researchers through projects on a continental or global scale.

Acknowledgments

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3 | Chapter II

Chamber's size does matter in measurements of CO_2 emission from leaf-cutting ant nests

Luana Caiafa[1,3], Nathan Oliveira Barros[1,2], Juliane Floriano Santos Lopes[1,3]

[1] MirmecoLab, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora 36036–900, Juiz de Fora, Minas Gerais, Brazil

[2] Laboratório de Ecologia Aquática, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora 36036–900, Juiz de Fora, Minas Gerais, Brazil

[3] Programa de Pós-Graduação em Biodiversidade e Conservação da Natureza, Universidade Federal de Juiz de Fora, 36036–900, Brazil

Corresponding author: caiafaluana96@gmail.com

Highlights

- Do different sampling methods interfere in the accuracy of CO_2 flux measurements from ant nests?
- Can the chamber's diameter and depth interfere on the CO_2 flux from the ant nests?
- Chambers much larger than the ant hole could underestimate the real value of CO_2 flux by covering a greater area of soil surrounding the ant hole, promoting a dilution effect.
- The depth of soil insertion had no significant effect on the diffusive CO_2 flux variation.

Abstract: In the scenario of global climate change, accurate measurements of greenhouse gas emissions to the atmosphere are a current and crucial concern. Many authors consider ant nests as hotspots of carbon dioxide (CO_2) emission, with leaf-cutting ants (LCAs) standing out due to their abundance and huge subterraneous nests, which produce more CO_2 than surrounding soil. The most used method to measure CO_2 flux from ant nests is an infrared CO_2 analyzer coupled to a closed chamber. However, there is still no standard methodology regarding the chamber's dimensions and depth of insertion in the soil. Therefore, in order to check if there can be any bias caused by different chamber diameters or depths, we tested three chambers with different diameters and a 10 cm-closed chamber inserted at different depths in the soil. Our data revealed that the diffusive CO_2 flux from ant nest holes reduced as chamber diameter increased, when compared to the 5 cm-diameter chamber, the 10- and 15 cm chamber had, respectively, a reduction of 34.6 and 52.3% in the CO_2 emission. Chamber diameters had no significant effect when tested over soil surface without ant nests. As CO_2 flux represents the release of CO_2 from the total area inside the chamber (soil and ant nest), we suggest that CO_2 emission from soil covered by larger chambers could promote a dilution effect on the total emission. On the other hand, the depth of the chamber's insertion in the soil had no significant effect on CO_2 flux. Our study is the first that proposes testing the potential of two-parameter biases in closed chamber methodology to measure CO_2 flux from ant nests. Since there are significant effects of chamber diameter in the diffusive CO_2 flux measurements, we strongly recommend that variations in the chamber diameter should be carefully chosen. The chambers must have a diameter as close as possible to the holes, and the depth of insertion must consider the characteristics of the soil. Further investigations regarding CO_2 flux measurement are necessary to evaluate other potential sources of bias related to chamber parameters and characteristics, such as distinct raw materials and the use of chamber sealing materials, not covered by our study.

Keywords: CO_2 emission methods, soil biogeochemistry, ant nests emission, soil respiration.

1. Introduction

Current concerns over greenhouse gas emissions have increased the interest in obtaining reliable and accurate measurements of carbon dioxide (CO_2) flux from the soil surface, one of the most significant pathways of CO_2 emission from terrestrial ecosystems. The main drivers of CO_2 emission from the soil include edaphic fauna activities, especially by ants, which represent localized CO_2 sources that increase spatial variation and heterogeneity of CO_2 emission from soil (Davidson et al., 2002, 2006; Risch et al., 2005a, b; Ohashi et al., 2007).

Ant nests induce spatial heterogeneity in the soil's CO_2 emissions by producing more CO_2 than the surrounding soil (Ohashi et al., 2005; Wu et al., 2013; Wu et al., 2015). The CO_2 flux from ant nests originates directly from the ants' respiration (Risch et al., 2005a, b, Jilková and Frouz, 2014) and indirectly from soil disturbances caused by the nests, which cause alterations in microorganisms' activity and, consequently, in soil respiration (Kilpeläinen et al., 2007; Jiménez et al., 2008; Cerdá and Doerr, 2010; Wu et al., 2010; Sousa-Souto et al., 2012).

The contribution to CO_2 flux from ant nests is small compared to the total soil C budget (< 3%) (Ohashi et al., 2012). However, many authors consider ant nests as hotspots of CO_2 emission throughout the world, such as in coastal environments (Sousa-Souto et al., 2012), wetlands (Wu et al., 2013), and pastures (Bender and Wood, 2003). Most studies refer to forests (Jílková and Frouz, 2014; Jílková et al., 2016) and their variations, such as subalpine (Risch et al., 2005a, b), boreal (Ohashi et al., 2005, 2007, 2012; Domisch et al., 2006), tropical (Hasin et al., 2014; Wang et al., 2018; Soper et al., 2019), temperate (Jílková et al., 2015a, 2015b), tropical rainforest (Ohashi et al., 2017), and wet tropical rainforest (Fernandez-Bou et al., 2018, 2020).

The variation of CO_2 flux within ant nests is related to a few factors, such as species-specific activities (Hasin et al., 2014; Wu et al., 2015), feeding habits (Jílková and Frouz, 2014; Wu et al., 2015; Wang et al., 2018), and both individual and population size (Wang et al., 2018; Ohashi et al., 2012). Nevertheless, the ant nests' characteristics stand out as the main driver of CO_2 flux, especially those related to soil environment, structure, and size (Hasin et al., 2014). The leaf-cutting ants (LCAs) present large nests and include the genera *Atta*, *Acromyrmex*, and the recently described genus *Amoimyrmex* (Cristiano et al., 2020) (Myrmicinae, Formicidae). These ants build large underground nests with millions of individuals, presenting the potential to emit a large amount of CO_2.

A structurally complex nest is built to lodge this considerable population, with connected tunnels and internal chambers to keep their brood and symbiotic fungus (Camargo et al., 2004; Moreira et al., 2004a, b; Verza et al., 2007; Forti et al., 2018). The construction of the nest results in sizable external soil mounds resulting from the soil pellets transported from as low as 12 m deep (Moreira et al., 2004a, b). Due to their habit of cutting and transporting fresh leaves, a lot of organic matter is transferred to the nests. Indeed, LCA nests contribute to CO_2 emission variability, producing more CO_2 than the surrounding soil (Kleineidam and Roces, 2000; Sousa-Souto et al., 2012; Fernandez-Bou et al., 2018; Soper et al., 2019; Fernandez-Bou et al., 2020). However, little is known about the CO_2 emission from LCA nests, especially in *Acromyrmex* species, of which there are only two studies (Sousa-Souto et al., 2012; Fernandez et al., 2013).

The main method used for measuring CO_2 emission from ant nests is the insertion of a closed chamber into the ground, coupled with an infrared CO_2 analyzer, one of the most common CO_2 sensors (Yunusa et al., 2014). To date, there is still no standard methodology to measure CO_2 emission from LCA nests, each study presents its own personalized methods, which can differ in raw chamber material, dimensions, and sealing material (Fernandez-Bou et al., 2018, 2020; Caiafa et al., submitted). Thus, it is necessary to verify if these variations cause bias in the obtained measurements, giving a more accurate view of how essential ant nests are in the context of GHG emissions.

We built three low-cost, non-transparent circular Polyvinyl Chloride (PVC) closed chambers to make CO_2 emission measurements *Acromyrmex subterraneus* (Forel, 1893) nests. Each chamber with a different diameter and the chamber with the medium diameter was inserted at different depths. Our study aimed to check if there can be any bias in CO_2 emission measurements caused by different chamber diameters or depth of insertion in the soil. We also provide considerations to the closed chamber methodology for CO_2 flux measurements in LCA nests, describe and discuss a closed chamber design.

2. Material and Methods

We conducted tests with manual chamber measurements to investigate if the pattern of CO_2 flux varies according to different methodological approaches, using two *Acromyrmex subterraneus* (Forel, 1893) nests, each containing three holes (Nests' location: 21°46'29''S, 43°22'06''W). For this, we build three chambers made of Polyvinyl chloride (PVC) opaque cylindrical with three diameters (5, 10, and 15 cm). The chambers had 15 cm of height (resulting volumes of approximately 0.29, 1.17, and 2.65 L, respectively) (Figure 1). Using the 10 cm chamber, we tested if the depth of insertion in the soil had an effect over CO_2 flux. We established three markings, at 1, 3, and 5 cm, with tape on the external surface of the chamber, which allowed us to insert the chamber into the ground at the desired depths (Figure 1) over each tested hole of the ant nests.

Figure 1. Polyvinyl chloride (PVC) opaque cylindrical chambers at sequence, from left to right: (A) Chambers with 5 cm, 10 cm, and 15 cm diameter. (B) PVC chambers depicting the three markings to assist insertion of the 10 cm chamber at depths of 1 cm, 3 cm, and 5 cm.

We connected the chamber system to an Ultraportable Greenhouse Gas Analyzer (UGGA), which uses Integrated Cavity Output Spectroscopy (ICOS) technology (M-GGA-918, Los Gatos Research). We performed a test assay in the laboratory to check if there were gas leakages from the chambers or tubing (Figure 2).

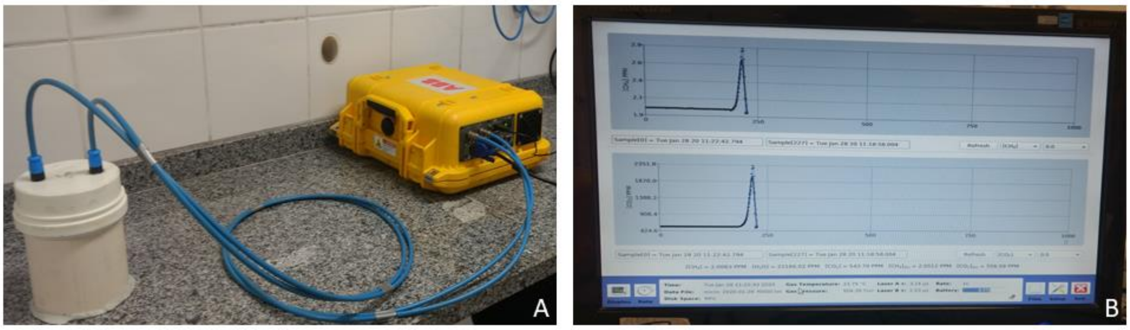

Figure 2. Test assay to check the presence of gas leakages from the chambers and tubing. (A) The UGGA connected in the sealed chamber; (B) UGGA system output without gas lecture alteration. This procedure was done for all three diameter chambers.

After verifying that there was no gas leakage, we carried out the field experiments, inserting the chambers with different diameters directly over an ant nest hole, creating a closed gas circuit. Measurements of CO_2 concentration were done in triplicates for 3 min in each of the three holes from the same nest. We performed the same procedure in three holes from another nest for the insertion depth tests. As the closure period was shorter than 5 min, the air temperature inside the chamber would not increase much, minimizing the soil components' warming effects (Pavelka et al., 2018). We performed all measurements between 9:00 am to 12:00 pm and did not use any sealing method. We also measured soil surface temperature (Minipa MT-350).

We calculated the diffusive CO_2 fluxes (mg CO_2 m^{-2} d^{-1}) using the linear regression slopes of the variation of CO_2 concentration in the PVC chamber at each second:

$$F = (V/A) \times S \times [(P \times F1 \times F2)/(R \times T)] \tag{1}$$

where F = gas flux (mg m^{-2} d^{-1}), V = chamber volume (L), A = chamber surface area (m^2), S is the slope of the linear relationship between CO_2 concentration and time (ppm s^{-1}), P = atmospheric pressure (hPa), $F1$ = molar mass of CO_2 (44 g mol^{-1}), $F2$ = conversion factor of seconds to day, R = gas constant 0,082 (L atm K^{-1} mol^{-1}), and T = temperature (K). We made corrections for temperature and atmospheric pressure. We corrected the chamber volume for the data related to the depth effect, considering the headspace available inside the chamber. We only considered data that presented linearity (R2 > 0.80).

To evaluate the effects of different diameters and depths on CO_2 flux, we log-transformed the CO_2 emission to verify the normality, homoscedasticity, and sphericity assumptions. Then, we applied the repeated measures ANOVA test, considering the nest holes as repeated measures. We used the R Statistical Software version 3.6.0 (R Core Team, 2019) and the "emmeans" (Lenth, 2021) package, assuming $p < 0.05$ as the threshold level of acceptance for all statistical tests. For the graphs, we used the package "ggplot2" (Wickham, 2016).

3. Results and Discussion

We verified that the diffusive CO_2 flux decreased as chamber diameter increased (ANOVA, F = 28.08, $p < 0.001$) (Tukey post hoc test, 5 cm - 10 cm: p = 0.0002; 5 cm - 15 cm: $p < 0.0001$; 10 cm - 15 cm: p = 0.003), when measured over ant holes. On average, when compared to the 5 cm-chamber, there was a reduction of 34.6% in the CO_2 flux using the 10 cm-chamber and of 52.3% for the 15 cm-chamber. On the other hand, when the measurements were made over soil without ant nests, there was no significant effect of chamber diameter (ANOVA, F = 0.29, p = 0.74) (Figure 3).

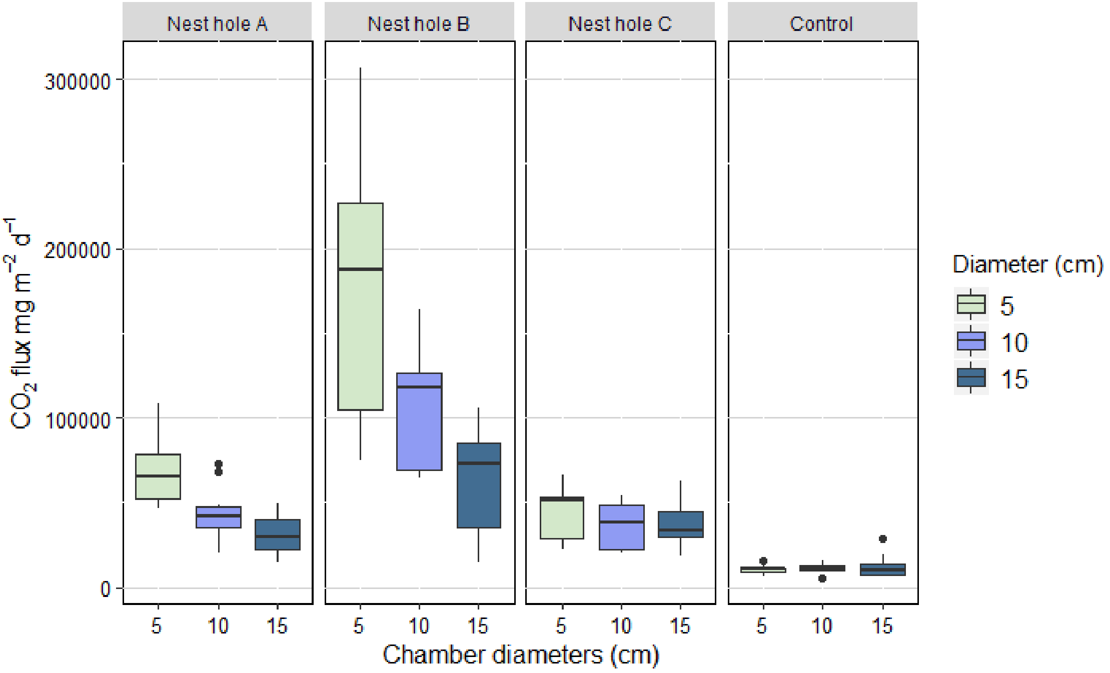

Figure 3. CO_2 diffusive flux in chambers with different diameters. Boxplots presents the CO_2 flux (mg m^{-2} d^{-1}) in chambers with 5 cm, 10 cm, and 15 cm of diameter over each ant nest hole (A, B, and C) and soil surface (Control). Box-and-whisker plots show median (horizontal line), interquartile range (box), distance from upper and lower quartiles (whiskers), and outliers (full black circles).

Our results indicated that chamber diameter should be carefully considered, since its variation provided different values of diffusive CO_2 flux for the same ant nest hole, with the larger diameter providing a lower emission. We suggest that different chamber diameters could promote biases in the measurements of CO_2 flux, which could underestimate the real value. The lower CO_2 flux verified for the 15 cm-chamber, followed by the 10 cm-chamber, was probably due to the larger soil surface area covered by the chamber compared to the 5 cm-chamber, and this surface area has a lower CO_2 flux than the nest hole. As CO_2 flux represents the variation of CO_2 emission inside the chamber, the emission from a larger surface area causes a dilution effect, resulting in a lower total flux (Figure 4A). The total flux was higher in the 5 cm-chamber since it covered a smaller soil area, generating a more concentrated total flux inside the chamber (Figure 4C). On the other hand, our control data revealed no effect of chamber diameter since the chamber only covered soil, and thus CO_2 emissions come from similar sources, reflecting in a homogeneous CO_2 flux inside the chamber.

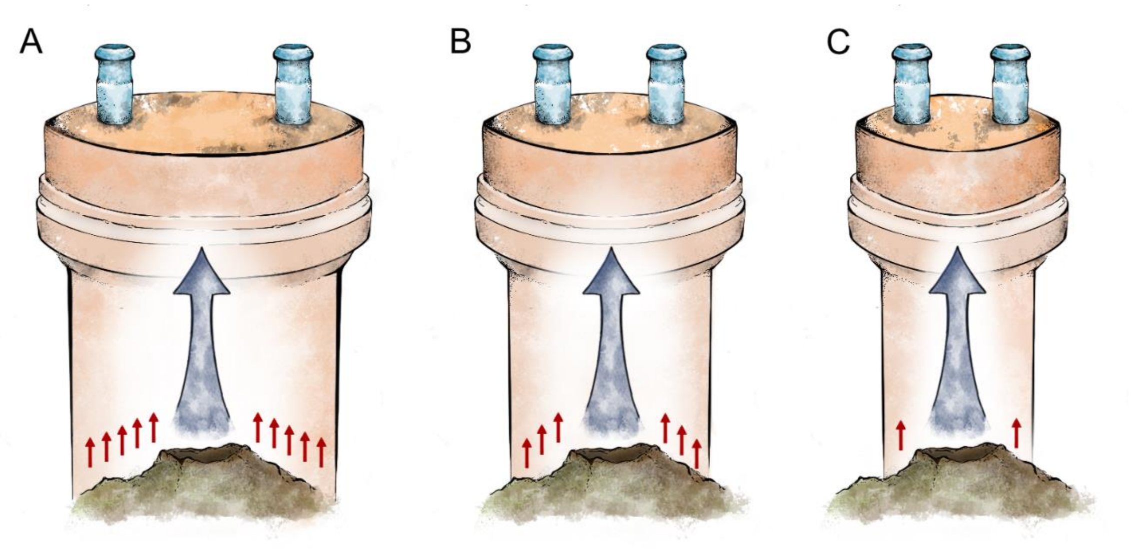

Figure 4. Illustration showing how soil surface area covered by chambers with different diameters (A: 15 cm-chamber; B: 10 cm-chamber; C: 5 cm-chamber) could cause the dilution effect on the total CO_2 flux measurement inside the closed chamber.

Therefore, chamber diameter should be carefully chosen to avoid covering large areas of soil, which interfere with the total flux. Analyzing the description of chamber design for measuring CO_2 emission from ant nests, we observed no pattern (Fernandez-Bou et al., 2020, Caiafa et al., submitted), with the use of chambers whose diameter varied from 10 cm (Risch et al., 2005 a, b; Hasin et al., 2014; Ohashi et al., 2017; Wang et al., 2018, Fernandez-Bou et al., 2018), 12.5 cm (Jílková et al., 2015a), 20 cm (Bender and Wood, 2003; Jílková et al., 2015b), 22 cm (Soper et al., 2019), and up to 169 cm (Ohashi et al., 2005). This problem seems to be avoided, as the different chamber diameters used in other studies considered the area of the target CO_2 source. Indeed, some studies with ant nests use chambers with a large enough diameter to cover the whole nest (Ohashi et al., 2005; 2007; Domisch et al., 2006; Wu et al., 2013), while others use small chambers directly over each ant nest hole (Hasin et al., 2014; Fernandez-Bou et al., 2020).

In contrast, the chamber's depth of insertion in the soil had no significant effect over the diffusive CO_2 flux variation (ANOVA, $F = 2.26$, $p = 0.11$) (Figure 5), thus not representing a source of bias for CO_2 flux measurements. This procedure also varies in studies with ant nests, with some authors not describing how they insert the chamber (Risch et al., 2005a, b). Instead, when a detailed report is found in the papers, they explain that the chamber was directly inserted on the surface of the nest (Bender and Wood, 2003), or that it was placed in a collar previously inserted into the soil (Ohashi et al., 2012; Hasin et al., 2014; Wang et al., 2018). However, chamber insertion may be a potential source of bias depending on soil texture. For example, studies evaluating the closed chamber methodology for soil respiration measurements report that inserting the chamber at a depth of a few centimeters can disturb the diffusion of the gas on the soil surface in fine-textured soil, such as sandy soils (Matthias et al., 1980; Rochette et al., 1997; Janssens et al., 2000). In contrast, chamber insertion in organic or clay soils generally does not significantly bias the gas flux measures (Rochette et al., 1997). Besides, there are studies in which there is no chamber sealing, and others that use some kind of sealing material such as clay or nylon to prevent gas leakage (Ohashi et al., 2005, 2017).

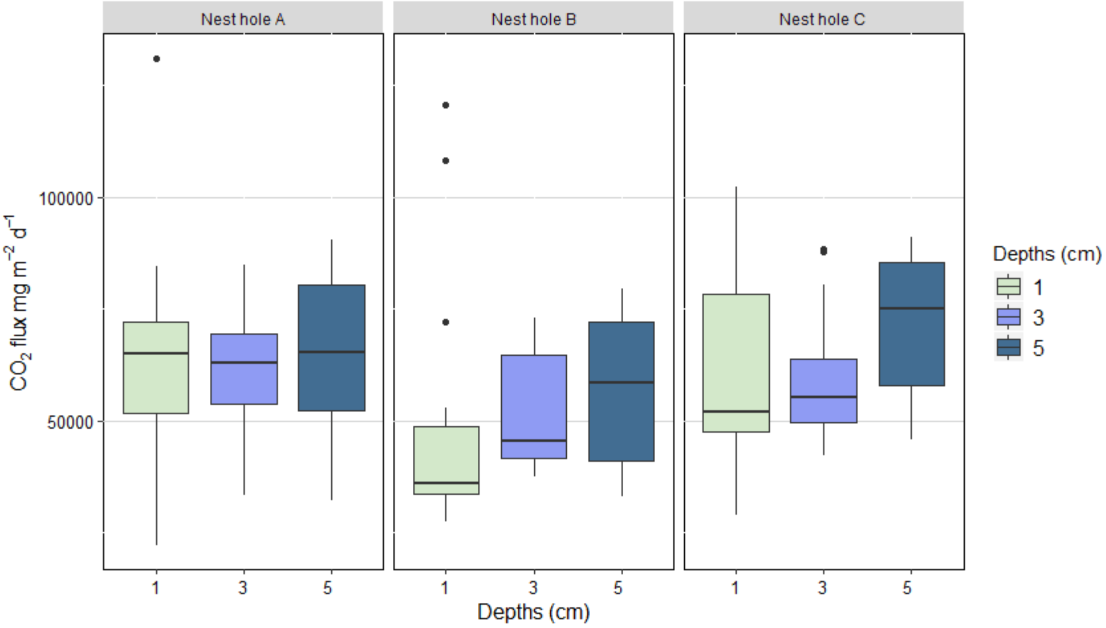

Figure 5. CO_2 diffusive flux in different depths in the soil. Boxplots present the CO_2 flux (mg m^{-2} d^{-1}) measured in chambers inserted in the ground at 1 cm, 3 cm, and 5 cm depths in each ant nest hole (A, B, and C). Box-and-whisker plots show median (horizontal line), interquartile range (box), distance from upper and lower quartiles (whiskers), and outliers (full black circles).

Our study is the first that proposes to test the potential for biases of two parameters (diameter and depth) in chamber methodology while measuring CO_2 emission from ant nests and adds some warnings that must be considered when designing the closed chamber system. We custom-manufactured portable chambers with PVC, which is easy to manipulate and cheaper than commercial chambers. Furthermore, it allows the manufacturing of chambers with dimensions as close as possible to the hole or ant nest, thus avoiding the dilution effect of the soil surface over the measurements. The lack of standardization of the closed chamber methodology and our results alert caution when comparing data between studies. Regarding the chamber's depth of insertion in soil, we advise researchers to consider the properties of the local soil. Also, we recommend additional investigations to test if other parameters that not covered by our study, such as chamber material and sealing, could also be a source of bias for the measurement of CO_2 flux.

Acknowledgments

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4 | Chapter III

Ant nests as CO_2 sources in urban areas: How are the physical features of *Acromyrmex subterraneus* nests related to CO_2 emission?

Luana Caiafa[1,3], Nathan Oliveira Barros[1,2], Juliane Floriano Santos Lopes[1,3]

[1] MirmecoLab, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora 36036–900, Juiz de Fora, Minas Gerais, Brazil

[2] Laboratório de Ecologia Aquática, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora 36036–900, Juiz de Fora, Minas Gerais, Brazil

[3] Programa de Pós-Graduação em Biodiversidade e Conservação da Natureza, Universidade Federal de Juiz de Fora, 36036–900, Brazil

Corresponding author: caiafaluana96@gmail.com

Highlights

- Different functions of nest holes were related to variations of the CO_2 flux from *Acromyrmex* nests.
- The external nest area and the number of holes positively affected the CO_2 emissions.
- The ant nest hole diameter had no significant effect on the CO_2 emissions.
- The magnitude of CO_2 emission from nests of some ant species was on a par with important anthropogenic CO_2 sources.

Abstract: Underground ant nests are commonly considered hotspots for CO_2 emission, promoting spatial variability of the total CO_2 flux from the soil due to their respiration and alterations of soil properties. Although the CO_2 emissions from ant nests are part of the natural carbon cycle, human intervention in natural areas facilitates the installation and development of more nests, increasing the CO_2 emission. The CO_2 flux from ant nests increases with population and nest size; thus, the features of nest architecture could be an indicator to evaluate their importance as sources of CO_2 emission. We suppose that the nest hole function could be a source of variation of CO_2 emission because different activities performed by ant workers reflect different CO_2 emission rates. *Acromyrmex subterraneus* (Forel, 1893) meet some conditions to check for the effect of varying nest characteristics in CO_2 emissions, such as preference for nesting in urban areas and construction of large nests with many holes; thus, we focused on the investigation of how its nest structure influences the CO_2 flux. We also compared the data obtained with CO_2 emissions from nests of other ant species and anthropogenic sources. We created a ranking to provide an overview of the information available worldwide. We selected twenty-eight nests near anthropogenic structures and measured the CO_2 emissions, the external mound area, diameter, and function of nest holes. We verified that the average CO_2 flux from ventilation holes was 48% higher than that from foraging holes and 62% higher than the one from non-identified function holes, but there was no significant effect of nest hole diameter. Regarding the characteristics of the nest, we verified that the larger the nest area and the number of holes per nest, the higher is the CO_2 flux. Also, we found that CO_2 flux from ant nests was higher than from the soil and that both increased in the function of the relative air humidity. The magnitude of CO_2 emission from some ant nests was similar to recognized anthropogenic sources and considering that disturbed areas facilitate the installation of the two ant species with the highest CO_2 emission values, we call attention to this amplified effect of leaf-cutting ants and deforestation, as important agents of climate change. Our investigation of CO_2 emissions from ant nests revealed that the different functions of nest holes contribute to distinct CO_2 emissions, which opens the way to further studies about the influence of the nest structures in CO_2 flux in other ant species.

Keywords: nest structure, leaf-cutting ants', respiration, soil bioturbation, nest hole function, CO_2 flux.

1. Introduction

Ant nests are important carbon dioxide (CO_2) emission sources to the atmosphere. Inside the nests, especially the large ones, there is a huge population of individuals, whose respiration and activity in the soil are the main drivers for the CO_2 emission (Bender and Wood, 2003; Ohashi et al., 2005, 2007, 2017; Fernandez-Bou et al., 2018). Although the CO_2 emissions from ant nests are part of the carbon cycle, human intervention in natural areas facilitates the installation and development of more nests since their survival improves due to the presence of fewer predators and better conditions for food acquisition (Wirth et al., 2008; Leal et al., 2014).

Underground ant nests are commonly considered hotspots for CO_2 emission, promoting spatial variability of the total CO_2 flux from the soil (Ohashi et al., 2017; Fernandez-Bou et al., 2018). The impact of ant nests in the total CO_2 flux from the soil is related to the respiration of the nest population and alterations of soil properties (Ohashi et al., 2007; Sousa-Souto et al., 2012). Therefore, the CO_2 flux from ant nests increases with population and nest size.

In this scenario, the size and number of entrance holes are good predictors of the internal architecture of ant nests (Tschinkel, 2003), with reports that the diameter of nest entrance holes affects nest CO_2 efflux (Hasin et al., 2014; Fernandez-Bou et al., 2018) as well as the nest external area (Sousa-Souto et al., 2012; Fernandez-Bou et al., 2018). To our knowledge, no study has investigated if the function of the nest hole also interferes with CO_2 efflux to the atmosphere, although there are reports related to their role in the internal gas circulation in the ant nests (Kleineidam et al., 2001; Bollazzi et al., 2012; Halboth and Roces, 2017). This is a particular problem of underground ant nests since in this kind of ant nest the air exchange between the nest environment and the atmosphere is compromised (Halboth and Roces, 2017). To avoid hypercapnic (high carbon dioxide) conditions, some nest holes are explicitly constructed to allow gas exchange and maintain microclimatic conditions (Bollazzi et al., 2008).

In this context, leaf-cutting ants (LCAs) - which includes the genera *Atta* Fabricius, 1805, *Acromyrmex* Mayr, 1865, and the recently described genus *Amoimyrmex* Cristiano, Cardoso & Sandoval, gen. nov. (Myrmicinae, Formicidae) (Cristiano et al., 2020) - stood out since they continually adjust the nest environment and architecture according to internal climate conditions (Bollazzi and Roces, 2010; Kelber et al., 2009). For example, workers of *Atta vollenweideri* Forel, 1893 increased the height and number of ventilation turrets in response to high CO_2 levels inside the nests (Halboth and Roces, 2017). This adjustment of the nest architecture allows the LCAs to deal with unfavorable climatic conditions inside the nest, facilitating the ventilation of the nest and, therefore, the exchange of gases with the atmosphere (Bollazzi et al., 2008; Bollazzi et al., 2012; Halboth and Roces, 2017).

These reports are clues to suppose that the function of the nest hole could be a source of variation of CO_2 emissions. Also, we must consider that different activities performed by ant workers in the holes could result in different CO_2 emission rates since these activities imply other energetic costs. For example, during foraging the *Rhytidoponera aurata* (Roger, 1861) the mean cost of load carriage was just 7.26% more than the mean cost of locomotion (Nielsen, 2001). These data contrast with that from nest excavation activity, during which *Atta sexdens* (Linnaeus, 1758) workers produced, on average, 32.42% more CO_2 than workers that are not performing this activity (Camargo et al., 2013a).

The species *Acromyrmex subterraneus* (Forel, 1893) match many specific conditions, which allows us to check the effect of different variables in CO_2 emission. This species constructs underground nests with connected tunnels and internal chambers (Camargo et al., 2004; Verza et al., 2007), presenting soil mounds in areas with human intervention and possessing a vast population. Additionally, their habit of cultivating a symbiotic fungus garden inside the nest adds one more point of interest in the scene since the fungus contributes to CO_2 emissions in different ways, for example, through its own respiration and as it serves as an allocation place for queens and their brood. Also, the fungus digests fresh leaves requiring 72.1% of a colony's energy demands, rising CO_2 production (Shik et al., 2014).

Here, we focus on analyzing the CO_2 emissions from *A. subterraneus* nests in urban areas to verify the influence of the physical features of the ant nests on variations of CO_2 emissions, with a particular focus on the nest hole functions. Furthermore, we performed a parallel between the obtained data with those about CO_2 emission from other ant species nests and anthropogenic sources to provide an overview of the available information worldwide.

2. Material and Methods

2.1 Study species and methodological approaches

We selected twenty-eight nests with different sizes of *Acromyrmex subterraneus* at two locations: (i) Universidade Federal de Juiz de Fora (UFJF) (21°46'29"S, 43°22'06"W) and (ii) Jardim Botânico da UFJF (21°44'07.8"S, 43°22'09.4"W), Minas Gerais, Brazil. We conducted tests to investigate how the CO_2 fluxes vary in these leaf-cutting ant nests, using Polyvinyl Chloride (PVC) closed chambers (5, 10, or 15 cm diameter x 15 cm height). We placed the PVC chamber over each hole from each ant nest to allow comparisons for the measurements. We repeated the measurements on the surrounding soil without any sign of ants' presence as a control measurement.

2.2 Description of the ant nests localization

We selected the ant nests next to anthropogenic structures (e.g., buildings) since human intervention facilitates the installation and development of *A. subterraneus* nests. The nests were in some openings between disruptions close to concrete structures (1C, 1D), concrete channel water drains (1F), paved roads (Figure 1A, 1B, 1E, 1H), and close to a gully (Figure 1G).

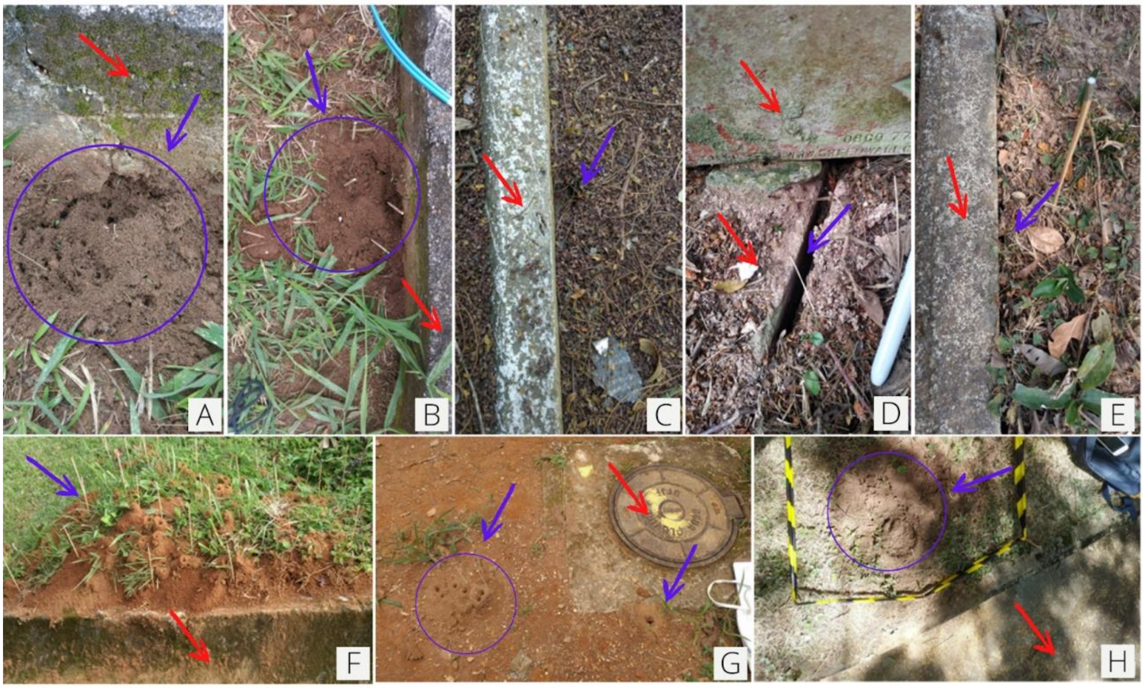

Figure 1. Localization of some ant nests and holes close to anthropogenic structures. Purple arrows indicate the nest and holes, and the red arrows the anthropogenic structures. Note that in panels A, B, G, and H, the presence of many nest holes inside the purple circle. In panel H it is possible to see the tunnels with the entrance holes.

2.3 CO_2 measurements

For the CO_2 measurements, we used a micro portable greenhouse gas analyzer (LGR-ICOS™ M-GGA-918, Los Gatos Research). We carefully centered the chambers over the ant nest holes, minimizing soil disturbances while ensuring continuous contact between the bottom edge and the soil surface to avoid leakage during the measurements. We placed the chambers over all the nest holes in each nest and performed three measurements of CO_2 emissions for three minutes at each entrance hole. We checked the linear increase of the measurements and verified the slope that was used to calculate the CO_2 flux. We did the same at the surrounding control soil, which showed no sign of ants' presence. Also, we measured the soil and air temperature, air relative humidity (THAL-300-INSTRUTHERM) locally over the nest hole and surrounding control soil. All data collections were performed between 9:00 am to 12:00 pm.

2.4 Nest structure characterization

To characterize the nest structure, we evaluated the external mound area (greatest width x length of the nest), the diameter of nest holes, and their respective functions, which was assessed by digging after performing the CO_2 emission measurements. We also registered whether the ant hole was at the soil surface or above the nest mound. The observation of workers' activity classified the function of the hole, providing four categories: (i) foraging when workers transporting leaf fragments were observed; (ii) excavation, when we observed the workers carrying soil pellets; (iii) ventilation, when we observed that the hole was above the fungus garden chamber and (iv) non-identified when there were no workers traffic through the hole, or it was not over the fungus garden chamber.

2.5 Preliminary treatment of data and statistical analysis

We calculated the diffusive CO_2 fluxes (mg CO_2 m^{-2} d^{-1}) using the linear regression slopes of the variation of CO_2 concentration in the PVC chamber at each second:

$$F = (V/A) \times S \times [(P \times F1 \times F2)/(R \times T)] \tag{1}$$

where F = gas flux (mg m^{-2} d^{-1}), V = chamber volume (L), A = chamber surface area (m^2), S is the slope of the linear relationship between CO_2 concentration and time (ppm s^{-1}), P = atmospheric pressure (hPa), $F1$ = molar mass of CO_2 (44 g mol^{-1}), $F2$ = conversion factor of seconds to day, R = gas constant 0,082 (L atm K^{-1} mol^{-1}), and T = temperature (K). We made corrections for air temperature and atmospheric pressure and considered only data that presented linearity (R2 > 0.80), where positive flux values indicate CO_2 emissions to the atmosphere. After that, we log-transformed the CO_2 flux data, our response variable, to meet the assumptions of normality and homogeneity of the variances.

In the first analysis, we used a Generalized Linear Mixed Model (GLMM) to evaluate the effect of the fixed variables: diameter and nest hole's function on CO_2 flux. The nest identification was considered as the random factor. We started with the full model and followed all the steps to simplify the random structure of the model to represent our data. The AIC (Akaike Information Criterion) value was used to choose the best fit model. After this, we validated the model checking the normality and the homogeneity of the residual variances. We applied the Kenward-Roger approximation (Kenward and Roger, 1997), a multivariate comparison method, to quantify the differences among the levels of the significant fixed terms.

In the second analysis, we followed the same procedures to test the effects of the nest area and holes on CO_2 flux. We used a Generalized Linear Model (GLM) with the gaussian family since our response variable presented the normal distribution. After that, we validated the best fit model representing our data according to AIC values.

To estimate the relationship between CO_2 flux and abiotic parameters (air temperature, soil temperature, and relative air humidity) for both nest holes and control soil, we applied a GLM. After considering the mean value of relative humidity, we compared the slopes by using a T-test. The intercepts were compared to verify the overlapping of the confidence intervals. We assumed $p < 0.05$ as the threshold level of acceptance for all statistical tests.

Finally, we selected papers that used the same CO_2 sampling method (closed chamber) and extracted the data relative to the maximum CO_2 flux measured from ant nests of different species and their global localization. Then, we transformed the units of CO_2 flux into a common unit (mg CO_2 m^{-2} d^{-1}), and, using the quartile values, classified the CO_2 flux in Low, Medium, High, and Very High levels of CO_2 emission. Following, we performed the same procedure with available data about CO_2 flux from some anthropogenic sources (Carvalho et al., 2009; Barros et al., 2011; Owen and Silver, 2015) and ranked these data together with that from ant nests.

All statistical analyses, model validation procedures, and graphs were performed in the R Statistical Software version 3.6.0 (R Core Team, 2019) using the packages “afex” (Singmann et al., 2021), “lme4” (Bates et al., 2015), “emmeans” (Lenth, 2021), “multcomp” (Hothorn et al., 2008), “interactions” (Long, 2019), “ggbeeswarm” (Clarke and Sherrill-Mix, 2017), “effects” (Fox and Hong, 2009), “lsmeans” (Lenth, 2016), “ggplot2” (Wickham, 2016), “ggpubr” (Kassambara, 2019), “rworldmap” (South, 2011), and “maps” (Minka and Deckmyn, 2018).

3. Results

We verified a significant variation of CO_2 flux according to the nest hole’s function (GLMM: F = 3.99, p = 0.009), while there was no significant effect of nest hole diameter (F = 2.45; p = 0.120). Through the multivariate comparison, we verified that the average CO_2 flux from ventilation holes was 48% higher than that from foraging holes (t-ratio = -2.98; p = 0.019) and 62% higher than the one from non-identified holes (t-ratio = -2.53; p = 0.062). On the other hand, the average CO_2 flux from excavation holes did not differ of those from holes with any other function (excavation - foraging: t-ratio = 1.28, p = 0.575; excavation - non-identified: t-ratio: 1.15, p = 0.662; excavation - ventilation: t-ratio: -2.04, p = 0.183) (Figure 2).

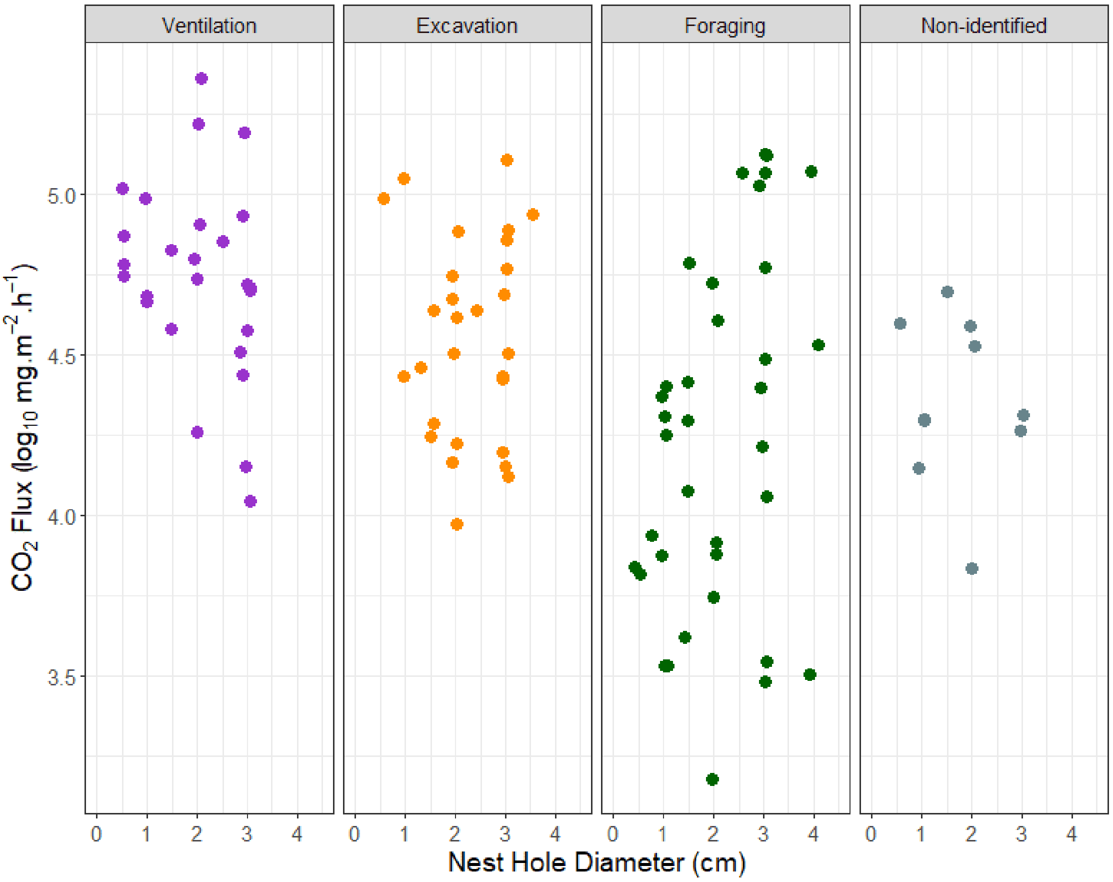

Figure 2. CO_2 flux (mg m^{-2} h^{-1}) from *Acromyrmex subterraneus* nest holes, according to the hole function and diameter.

Regarding the characteristics of the nest, we verified that the larger the nest area (F = 1.75, p = 0.093) and the number of holes per nest (F = 4.39, p = 0.0002), the higher the CO_2 flux (Figure 3).

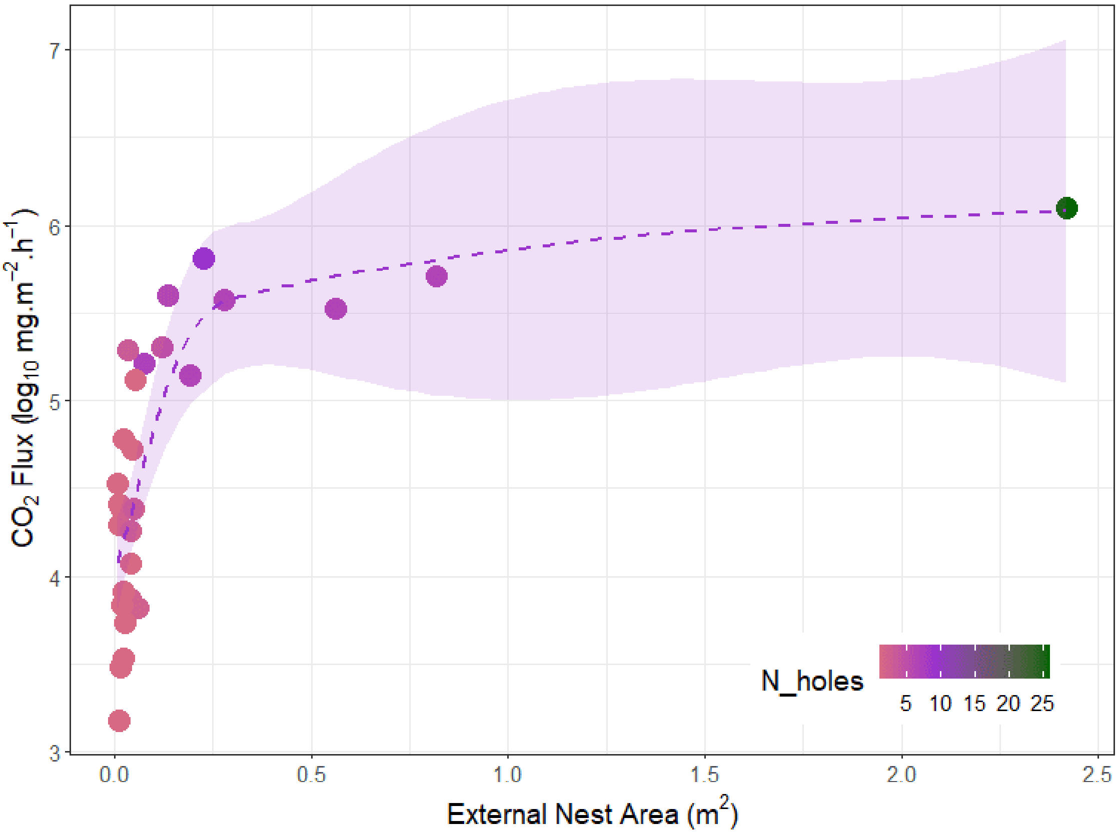

Figure 3. CO_2 flux (mg m^{-2} h^{-1}) from *Acromyrmex subterraneus* nest holes according to the number of holes and external nest area.

Comparing the CO_2 flux measured over the holes with their respective controls (soil without ant presence) and considering the effect of abiotic factors, we found that CO_2 flux from ant nests was higher than from the soil ($F = 20.85$, $p < 0.001$) and that both increased in the function of higher relative air humidity ($F = 23.39$, $p < 0.001$) (Figure 4). Considering the mean value of relative air humidity (mean value = 58.35%), there was no difference between the slopes ($t = -0.75$, $p = 0.456$), indicating no significant interaction. Also, there are no overlapping confidence intervals of the predicted intercepts (Ant nest = 4.41 - 4.85, Control soil = 3.73 - 4.18), indicating that the CO_2 flux was higher from ant nests than from control soil. The model predicted that the CO_2 flux from ant nests is 0.68 higher than that from control soils considering the average value of the relative air humidity.

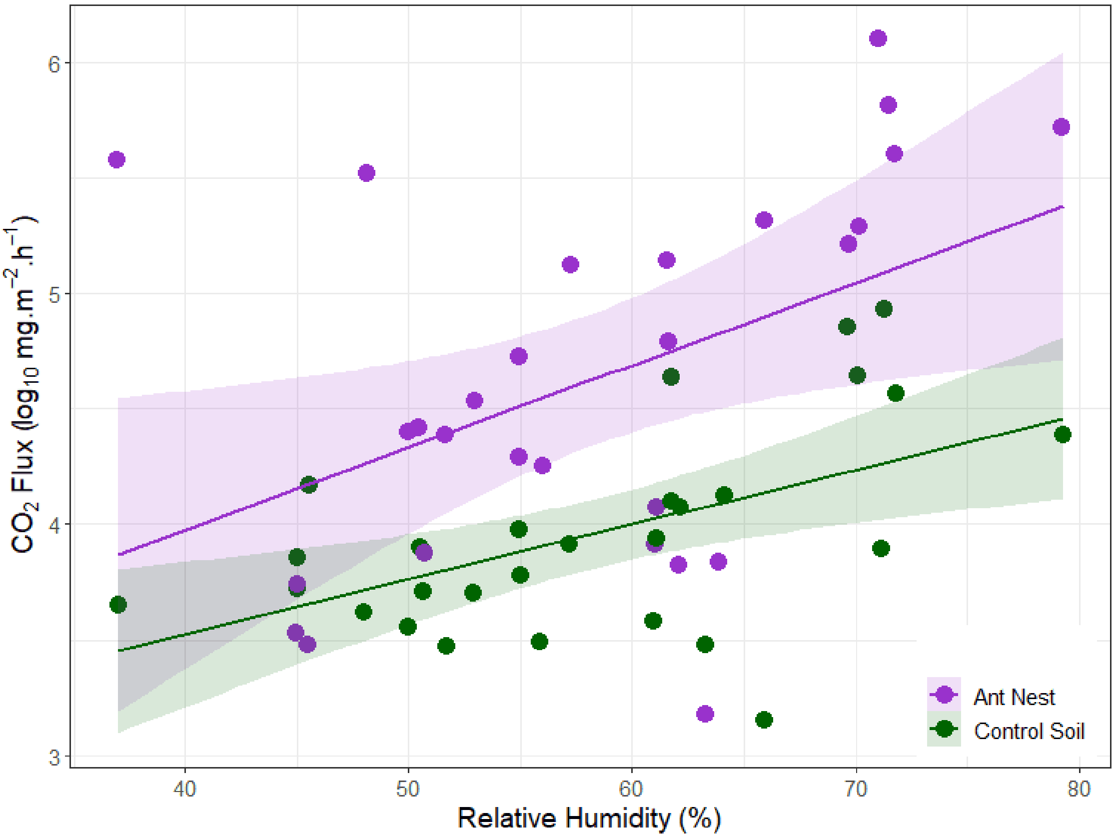

Figure 4. CO_2 flux (mg m^{-2} h^{-1}) from *Acromyrmex subterraneus* nest holes and respective controls in soil without ant presence according to the relative air humidity.

The maximum CO_2 flux determined for nests from 45 ant species showed great variability (6.196 - 126.213.120 mg CO_2 m^{-2} d^{-1}), with the lowest value registered for *Solenopsis invicta* and the highest for *Atta cephalotes* (Figure 5A, Supplementary Table 1). The flux of CO_2 from ant species can be as high as the flux from different reported anthropogenic sources. Most of the registered CO_2 flux from anthropogenic sources were classified in the very high CO_2 emission level, except for water reservoirs in temperate areas, barn floors, and non-Amazonian reservoirs (Figure 5A). Also, the classification of CO_2 emission from ant nests in these four levels of magnitude revealed no pattern related to their distribution, with very high fluxes in both temperate and tropical areas. In Malaysia, for example, there were registers from low to very high CO_2 emissions (Figure 5B).

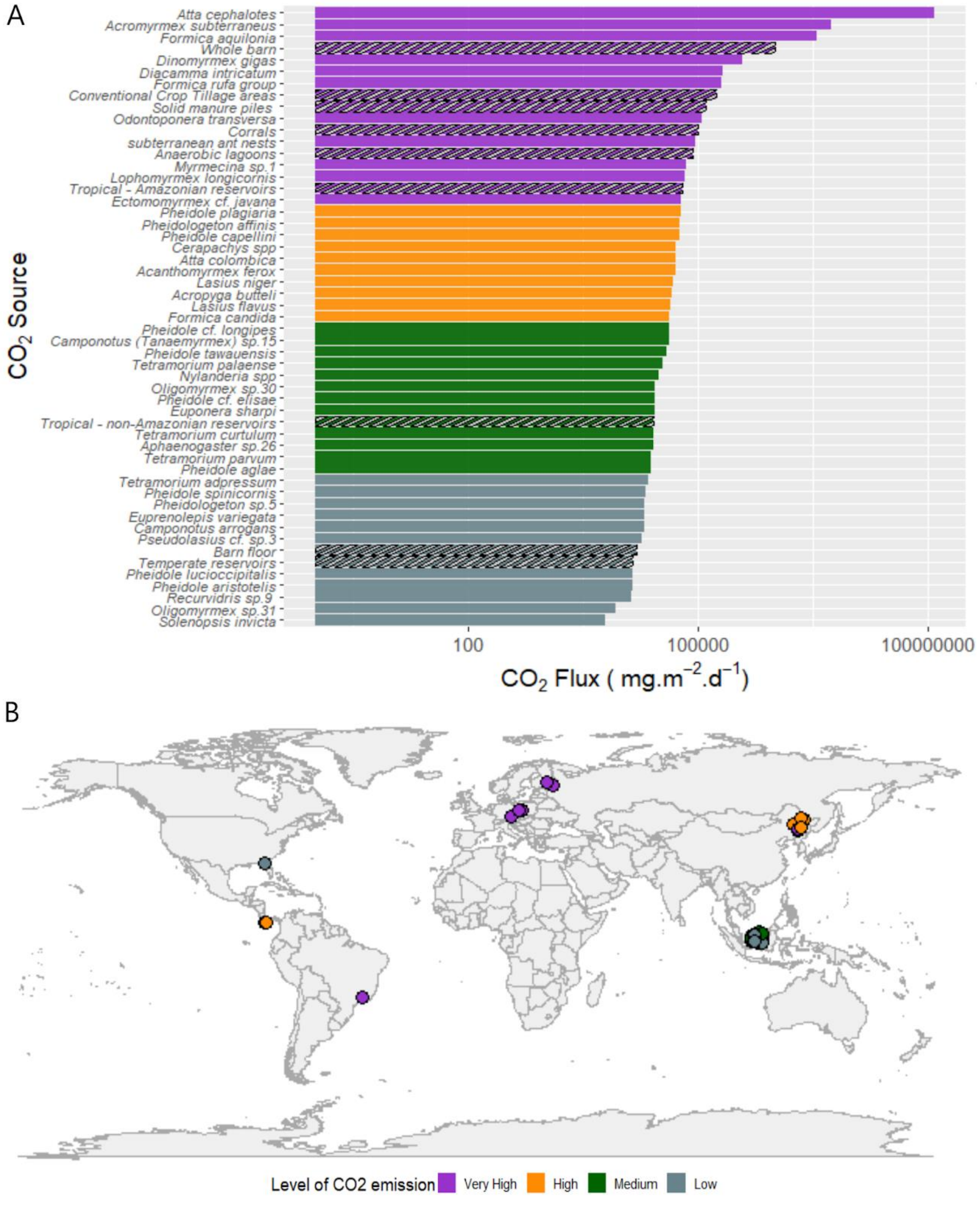

Figure 5. Ranking of CO_2 emission from ant nests and anthropogenic sources based on the quartile values of maximum CO_2 flux from nests of 45 ant species (A) and global localization of the evaluated ant species (B). Hatched bars represent the registered CO_2 flux from anthropogenic sources.

4. Discussion

Our analysis revealed that the CO_2 flux presented a significant variation according to the hole function, with the ventilation holes standing out with higher emission values.

The hole diameter used as an indicator to evaluate their influence on CO_2 flux did not exert any significant effect. On the other hand, considering the physical features of the whole nest, we verified that the greater the external nest area and the number of holes, the higher the CO_2 flux. Evaluating the influence of abiotic factors, we found a positive relationship between relative air humidity and CO_2 emission for both ant nests and control soil. However, the emission from ant nests was higher. Surprisingly, the magnitude of CO_2 emission from some ant nests was on a par with recognized anthropogenic sources but presented no pattern regarding their location in tropical or temperate zones.

The higher CO_2 emission from ventilation holes in comparison to that from the holes with other functions could be related to their localization over the fungus garden. These ventilation holes were directly connecting the fungus chamber to the external environment through a short path, which could facilitate the CO_2 release. Indeed, CO_2 flux from straight and short tunnels is higher than that from long narrow tunnels connecting different chambers (Hasin et al., 2014; Drager et al., 2016). Besides, it is observed that the higher CO_2 concentration inside some leaf-cutting ant nests (Kleineidam and Roces, 2000; Bollazzi et al., 2012) is due to the sheer mass of the cultivated fungus garden rather than the fungal respiration (Shik et al., 2014).

Excavation holes also presented higher values of CO_2 flux, which were not significantly lower than that from the ventilation holes, but also not higher than that from foraging or non-identified ones. The increased CO_2 emission from excavation holes could be related to the excavation activity itself, which requires a lot of energy from the workers (Camargo et al., 2013a, b) and consequently induces an increase in ventilation rates of the workers. Another factor might be the impact of the nest structure on the soil CO_2 concentrations. The ventilation network of ant nests provides an alternative pathway for the entrapped gas in the surrounding soil (Fernandez-Bou et al., 2018). Also, it is verified for some *Formica* species that the nest excavation reduces the soil bulk density, facilitating the soil gas diffusion (Drager et al., 2016).

Despite presenting a higher variability of CO_2 flux, the foraging holes had lower CO_2 emissions than the ventilation holes. The foraging activity has a lower energetic cost and thus does not necessarily imply a high respiration rate of workers (Lighton et al., 1987; Duncan and Lighton, 1994; Nielsen, 2001; Lighton and Duncan, 2002). Comparisons between CO_2 emission of foraging workers with and without load demonstrate an increase of CO_2 emission of about 43% for heavy and 24% for light leaf fragments in *Atta vollenweideri* (Moll et al., 2012). In *Camponotus rufipes*, laden workers present a higher metabolic rate than the unladen, emitting an average of 5 mL more CO_2 (Schilman and Roces, 2006). On the other hand, increases in the CO_2 emission are not verified for loaded workers of *Atta colombica* (Lighton et al., 1987), *Myrmecocystus mendax* (Duncan and Lighton, 1994), and *Rhytidoponera aurata* (Nielsen, 2001), indicating that the cost of carrying a load could be similar to the cost of locomotion.

Also, we must consider that although the leaf-cutting workers transport the fresh leaf material into the nest, their decomposition could not represent a pronounced CO_2 source. According to Soper et al. (2019), the decomposition of leaf fragments results in lower CO_2 emissions than that registered for refuse material. This low value is probably because their degradation is mainly carried out by the fungus garden, which can degrade 74.9% of the cellulose (Nagamoto et al., 2019) used for the hyphae respiration in the garden (Grell et al., 2013). Thus, the produced CO_2 from leaf decomposition would not be released in the foraging holes but would be added to the CO_2 from the metabolism of the fungus garden, which we believed in this study was captured at the ventilation holes.

Regarding the holes with non-identified function, which present the lower CO_2 flux values, we suggest that they could be related to O_2-rich air inflow, as most of them are centrally located in the external nest mound. The ventilation inside leaf-cutting ant nests occurs passively and is wind-induced through the nest holes and facilitated by the internal architecture (Kleineidam et al., 2001; Bollazzi et al., 2012). It is reported that the height of nest holes has an essential role in the internal gas circulation in *Atta laevigata* nests (Bollazzi et al., 2012), as well as their location in the nest mound, with O_2-rich air inflow holes at the outer mound region and CO_2-rich air outflow holes at the central region in *Atta vollenweideri* (Kleineidam et al., 2001).

In contrast to other subterranean ant species where the diameter of the nest hole has a positive relationship with the CO_2 flux (Hasin et al., 2014), we did not find an effect of hole diameter on CO_2 flux. One possible explanation is that the range of hole diameters analyzed here is smaller than the one analyzed by Hasin et al. (2014). Additionally, only one ant species was sampled, while Hasin sampled 13 species. The air outflow of our sampled nests arose from straight and short tunnels, which did not have a connection with different chambers since *Acromyrmex* nests commonly present just one chamber (Gonçalves, 1961; Verza et al., 2007). The nest area and the number of nest holes were a source of CO_2 flux variation since more nest openings represent more points of CO_2

emission from the nest, supporting the hypothesis that ant nest architecture is an important factor that causes CO_2 flux variations (Kleineidam et al., 2001).

Among the abiotic factors, such as soil temperature, conductivity, bulk density, porosity, and amount of water are commonly reported as drivers of CO_2 emissions from ant nests (Hasin et al., 2014; Jílková et al., 2015a, 2015b; Ohashi et al., 2017; Wang et al., 2018). However, to our knowledge, the direct association between air relative humidity and CO_2 emissions has not been reported yet. On the other hand, it is reported that the relative air temperature affects the building nest behavior of workers in many leaf-cutting ants' species. For example, workers of *Acromyrmex heyeri* close some of the entrances on their nest mound when the relative air humidity drops from 98% to 50% to avoid the desiccation inside the nest (Bollazzi and Roces, 2010). The same is also suggested to occur in *Acromyrmex ambiguus* (Bollazzi and Roces, 2007), which could indirectly affect CO_2 emissions. Our results validated that relative air temperature presents a positive effect on CO_2 emissions from ant nests. Another consequence of a higher air relative humidity is that this condition facilitates turret constructions in nests since soil pellets desiccate at a slower rate and stick to each other easily, which was observed in *Atta vollenweideri* (Halboth and Roces, 2017).

It is commonly described in the literature that soil with ant nests have higher CO_2 emissions compared to soil without ant nests (Risch et al. 2005; Ohashi et al., 2005, 2007; Domisch et al., 2006; Wu et al., 2013; Hasin et al., 2014) and our study corroborates this information. The CO_2 flux from ant nests presents a massive variability among ant species, presenting no pattern related to their global localization. It is verified for hydroelectric reservoirs, where the CO_2 emissions are negatively correlated to latitude (Barros et al., 2011). Considering our data and that from other reports, we suppose there is a positive correlation between CO_2 emission and nest size, but unfortunately, there is no available information about nest size for all the studied ant species. We recommend that further research which aims to measure the contribution of ant nests to CO_2 emission also consider the internal or even just the external architecture of the nests to fill this gap.

Comparing the maximum values of CO_2 emission from ant nests and anthropogenic sources revealed that some ant species are at the top of the ranking. The CO_2 emissions from ant nests occur naturally and are part of the carbon cycle. However, the urbanization process and human-induced disturbance, which are recognized drivers of the expansion of CO_2 emissions (McGee and York, 2018), are related to increases in the abundance and herbivory rates of the leaf-cutting ants (Costa et al., 2008; Leal et al., 2014), of which two species showed the highest values of CO_2 emissions in our study. In this context, the increased urbanization and deforestation associated with the increase of leaf-cutting ants' density could intensify the CO_2 emissions and thus must receive special attention within environmental and climate changes.

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5 | Final Considerations

The present study expanded the knowledge regarding the magnitude of GHG emissions, especially the CO_2 flux, from ant nests, presenting unpublished data about the nest hole function as a source of variability and about the risk of bias on CO_2 flux when larger soil surfaces are included inside the respirometric chamber. The broad systematic review improved the understanding of GHG emissions from ant nests, showing the need to sample GHG flux from ant nests in anthropogenic habitats. Furthermore, we highlight the need to standardize the measurement method to allow reliable comparisons among ant species and habitats. The analysis of physical parameters in the closed chamber system methodology added warnings related to the unreported effect, which we named as dilution effect, that occurs when the soil surface below the respirometric chamber is too larger than the nest hole, generating a bias in the real values of the CO_2 flux measurements. Based on this result, we recommend that chamber dimensions must be as similar as possible from the nest hole or from the emission source subject. Finally, the investigation of the magnitude of CO_2 emissions from *A. subterraneus* nests shows the importance of nest architecture, especially of the nest hole function. Also, our study put the ant nests on a par with recognized anthropogenic CO_2 sources, adding particular importance to the ants as CO_2 emission sources when we consider the facility of installation of some ant species in urban and disturbed areas.

6 | Supplementary Material

Chapter I

Supplementary Table 1. Classification of ant species for studying GHG emission according to functional groups.

Functional Group	**Species**
Generalist Myrmicinae (15 occurrences)	*Pheidole aglae* (1), *Pheidole aristotelis* (1), *Pheidole capellini* (1), *Pheidole cf. elisae* (1), *Pheidole cf. longipes* (1), *Pheidole hongkongensis* (1), *Pheidole lucioccipitalis* (1), *Pheidole parva* (1), *Pheidole plagiaria* (2), *Pheidole spinicornis* (1), *Pheidole tawauensis* (1), *Pheidologeton affinis* (2), *Pheidologeton* sp.5 (1)
Specialist predator (13 occurrences)	*Anochetus* sp.2 (1), *Anochetus graeffei* (1), *Cerapachys* sp.1 (1), *Cerapachys* sp.66 (1), *Ectomomyrmex astuta* (1), *Harpegnathos venator* (1), *Odontomachus rixosus* (1), *Odontoponera denticulata* (1), *Odontoponera transversa* (2), *Oligomyrmex* sp.30 (1), *Oligomyrmex* sp.31 (1), *Oligomyrmex* sp.5 (1)
Opportunist (12 occurrences)	*Aphaenogaster* sp.1 (1), *Aphaenogaster* sp.26 (1), *Diacamma intricatum* (1), *Diacamma vagans* (1), *Myrmecina* sp.1 (1), *Nylanderia* sp.1 (1), *Nylanderia* sp.6 (1), *Tetramorium adpressum* (1), *Tetramorium curtulum* (1), *Tetramorium lanuginosum* (1), *Tetramorium palaense* (1), *Tetramorium parvum* (1)
Tropical climate specialist (9 occurrences)	*Acromyrmex balzani* (1), *Acromyrmex lobicornis* (1), *Atta cephalotes* (2), *Atta colombica* (1), *Atta mexicana* (1), *Atta vollenweideri* (1), *Lophomyrmex longicorni* (1), *Pseudolasius* sp. 3 (1)
Cold climate specialist (20 occurrences)	*Formica rufa* group (6), *Formica aquilonia* (5), *Formica candida* (2), *Formica polyctena* (2), *Lasius niger* (3), *Lasius flavus* (2)
Subordinate Camponotini (3 occurrences)	*Camponotus (Tanaemyrmex)* sp.15 (1), *Camponotus arrogans* (1), *Polyrhachis sokolova* (1)
Hot climate specialist (3 occurrences)	*Solenopsis geminata* (1), *Solenopsis invicta* (2)
Cryptic (2 occurrences)	*Acropyga butteli* (1), *Recurvidris* sp.9 (1)
Opportunist dominant (1 occurrence)	*Anoplolepis gracilipes* (1)
NA (7 occurrences)	*Acanthomyrmex ferox* (1), *Dinomyrmex gigas* (1), *Ectomomyrmex cf. javana* (1), *Euponera sharpi* (1), *Euprenolepis variegata* (1)

Supplementary Table 2. Authors' justification for choosing the ant species for studying GHG emission of their nest.

Author Justificative	**Species**
Random (36 occurrences)	*Acanthomyrmex ferox* (1), *Acropyga butteli* (1), *Aphaenogaster* sp.26 (1), *Camponotus (Tanaemyrmex)* sp.15 (1), *Camponotus arrogans* (1), *Cerapachys* sp.1 (1), *Cerapachys* sp.66 (1), *Diacamma intricatum* (1), *Dinomyrmex gigas* (1), *Ectomomyrmex cf. javana* (1), *Euponera sharpi* (1), *Euprenolepis variegata* (1), *Lophomyrmex longicorni* (1), *Myrmecina* sp.1 (1), *Nylanderia* sp.1 (1), *Nylanderia* sp.6 (1), *Odontoponera transversa* (1), *Oligomyrmex sp.30* (1), *Oligomyrmex* sp.31 (1), *Oligomyrmex* sp.5 (1), *Pheidole aglae* (1), *Pheidole aristotelis* (1), *Pheidole cf. elisae* (1), *Pheidole cf. longipes* (1), *Pheidole lucioccipitalis* (1), *Pheidole plagiaria* (1), *Pheidole spinicornis* (1), *Pheidole tawauensis* (1), *Pheidologeton cf. affinis* (1), *Pheidologeton* sp.5 (1), *Pseudolasius* sp. 3 (1), *Recurvidris* sp.9 (1), *Tetramorium adpressum* (1), *Tetramorium curtulum* (1), *Tetramorium palaense* (1), *Tetramorium parvum* (1)
Wide occurrence, nest location and type of nest (underground) (13 occurrences)	*Anochetus* sp. 2 (1), *Anochetus graeffei* (1), *Anoplolepis gracilipes* (1), *Aphaenogaster* sp.1 (1), *Diacamma vagans* (1), *Ectomomyrmex astuta* (1), *Harpegnathos venator* (1), *Odontomachus rixosus* (1), *Odontoponera denticulata* (1), *Pheidole hongkongensis* (1), *Pheidole parva* (1), *Pheidole plagiaria* (1), *Tetramorium lanuginosum* (1)
Wide occurrence, size and type of nest and colony (12 occurrences)	*Formica rufa* group (5), *Formica aquilonia* (5), *Formica polyctena* (2)
Nest and colony size, type and complex architecture (7 occurrences)	*Acromyrmex balzani* (1), *Acromyrmex lobicornis* (1), *Atta cephalotes* (2), *Atta colombica* (1), *Atta mexicana* (1), *Atta vollenweideri* (1)
Nesting location (4 occurrences)	*Formica candida* (1), *Lasius flavus* (1), *Lasius niger* (1), *Polyrhachis sokolova* (1)
Nest type (underground) and diet (3 occurrences)	*Odontoponera transversa* (1), *Pheidole capellini* (1), *Pheidologeton affinis* (1)
Wide occurrence, invasive (1 occurrence)	*Solenopsis invicta* (1)
Nest type and size (1 occurrence)	*Lasius niger* (1)
Nest type (1 occurrence)	*Solenopsis geminate* (1)
Relationship of nests with tectonic fault zones (1 occurrence)	*Formica rufa group* (1)
Without a reason (4 occurrences)	*Formica candida* (1), *Lasius flavus* (1), *Lasius niger* (1), *Solenopsis invicta* (1)

Supplementary Table 3. Methodology and equipment used to measure GHG emissions from ant nests in the publications analysed in this systematic review.

#	**Reference**	**Sampling methods**	**Type of equipment**	**Habitat**	**GHG evaluated**
(1)	Anderson and Ultsch, 1987	Polyethylene-90 catheter tubing	Microgas analyzer (Scholander 1947)	Not described	CO_2 and O_2
(2)	Kleineidam and Roces, 2000	Iron pipe; air probes	IR-absorption with a gas sensor type GS 20 ED/CO_2 (Sensor Devices, Germany)	Not described	CO_2
(3)	Bender and Wood, 2003	Closed chamber, Polyvinyl Chloride (PVC) pipe (20 cm diameter x 16 cm headspace); Syringe - to collect gas samples from the chamber headspace	Gas chromatograph (Varian, Walnut Creek, CA)	Bermudagrass pasture	CO_2, CH_4, and N_2O
(4)	Nielsen et al., 2003	Air probes (30 cm long plastic pipette -1 mL - with injection needle into the upper end)	Flow-through analyser model LI-6251 (Sable System International, Nevada, USA, using Datcan V software)	Mangroves among tides	CO_2
(5)	Ohashi et al., 2005	Closed-loop flux measuring system; Chamber placed on a circular stainless-steel collar and fastened with a nylon band to make an airtight seal	Infrared gas analyser (IRGA-EGM-3, PP Systems, UK)	Forest and understory	CO_2
(6)	Risch et al., 2005a	Closed system soil respiration chamber (SRC-1, 15 cm high, 10 cm diameter)	PP-Systems EGM-4 infrared gas analyser (IRGA-PP-Systems, Hitchin, Hertfordshire, UK)	Subalpine forests	CO_2
(7)	Risch et al., 2005b	Closed system soil respiration chamber (SRC-1, 15 cm high, 10 cm diameter)	PP-Systems EGM-4 infrared gas analyser (IRGA-PP-Systems, Hitchin, Hertfordshire, UK)	Subalpine forests	CO_2
(8)	Domisch et al., 2006	Closed chamber system; with a metal collar of height 20 cm inserted around the base of each ant nest mound	Infrared gas analyser (IRGA-EGM-3, PP Systems, Hitchin, UK)	Boreal coniferous forests	CO_2
(9)	Ohashi et al., 2007	Dynamic closed-loop flux measuring system; Aluminium frame and UV-proof plastic chambers; Chamber placed on a circular stainless-steel collar	Infrared gas analyser (IRGA-EGM-3, PP Systems, Hitchin, UK)	Boreal coniferous forests	CO_2
(10)	Golichenkov et al., 2009	Samples taken from anthills; soil samples sifted	Gas chromatography (Khromatograf 3700, Moscow and Khrom-41 chromatograph with a flame ionization detector)	Abandoned cropland	CO_2, CH_4, and N_2O

(11)	Ohashi et al., 2012	A special chamber made of aluminum frame draped with an UV-proof plastic; Chamber placed on a circular steel collar inserted at the base of the ant nest mound	Infrared gas analyser (IRGA-EGM-3, PP Systems, Hitchin, UK)	Managed boreal forests	CO_2
(12)	Sousa-Souto et al., 2012	Alkali absorption method (Sundaravalli and Paliwal, 2000); Beakers containing NaOH solution placed in the nest entrances; Closed PVC chambers inverted over the beakers and inserted 10 cm into the soil; Beakers containing the NaOH solutions immediately sealed	Titration of NaOH	Restinga	CO_2
(13)	Jílková et al., 2013	Two-day incubation of 5g of fresh materials at 20°C with NaOH	Titration of NaOH (Page, 1982)	Forest	CO_2
(14)	Wu et al.. 2013	Opaque closed static chamber technique; Stainless steel base (with an open top and bottom) and a removable stainless-steel top chamber, covering the entire ant mound	Gas chromatograph (Agilent 4890) (Wang and Wang, 2003)	Wetlands	CO_2, CH_4, and N_2O
(15)	Fernandez et al., 2013	Alkali absorption method; Samples incubated in sealed flasks at 25°C with vials containing NaOH	Titration of NaOH	Dry environment	CO_2
(16)	Hasin et al., 2014	Commercial respiration chamber (SRC-1, PP-system; Amesbury, MA)	Infrared gas analyzer (EGM-4, PP-Systems, UK)	Seasonal tropical forest	CO_2
(17)	Jílková and Frouz, 2014	Cylindrical plastic containers (volume 4L, 10 cm diameter, 13 cm high); One container had holes (2 cm diameter) in its side and remains uncovered while the second had no holes	System for soil respiration measurement SR1000 (ADC UK)	Forest	CO_2
(18)	Jílková et al., 2015a	Chamber (volume 4 L), placed on a collar (diameter 20 cm) inserted into the soil	Infrared (IR) gas analyzer (Automated Soil CO_2 Flux System, LI-8100A, LI-COR®)	Temperate forest	CO_2
(19)	Jílková et al., 2015b	Static chamber technique; Two PVC pipes (diameter 12.5 cm; height 30 cm) inserted 20 cm deep into the sloping sides of each ant nest mound; Gas samples withdrawn from the chambers using a 20-mL syringe	Gas chromatograph (Agilent HP 7820, USA)	Temperate forest	CO_2 and CH_4
(20)	Wu et al., 2015	Soil samples collection from the ant mounds and used to prepare microcosm; Gas samples extracted from the closed chamber headspace using syringe and needle	Gas chromatograph (Agilent 4890)	Marsh soil	CO_2 and N_2O
(21)	Jílková et al., 2016	Soil samples collected in the profile of ant nests; Gas samples extracted from vessels hermetically sealed using a syringe	Gas chromatograph (Agilent HP 7820, USA)	Forest soil	CO_2 and CH_4
(22)	Ohashi et al., 2017	Commercial respiration chamber (SRC-1, PP-system, Hitchin, UK); PVC collars (height 3–4 cm, diameter 10 cm) inserted at each measurement hole point at least 0.5-cm deep to mount the commercial respiration chamber; Clay soil put between the collars and soil to make them airtight during the measurement	Infrared gas analyser (EGM-4, PP-systems)	Tropical rainforest	CO_2
(23)	Berberich et al., 2018a	A stainless-steel probe (inner diameter 0.6 cm)	Portable CRDS analyser (G2201-i; Picarro, Santa Clara, CA, USA)	Volcanic field	CH_4

(24)	Berberich et al., 2018b	A stainless-steel nest-gas probe (inner diameter 0.6 cm), inserted 1 m into the nest	CO_2: Portable CRDS analyser (G2201-i; Picarro, Santa Clara, CA, USA)	Volcanic field	CO_2
(25)	Majeed et al., 2018	Samples of worker ants and mound soils placed separately in 10 mL sterilized serum vials (BD Vacutainer® Systems, Plymouth, UK) hermetically sealed with butyl-rubber stoppers and incubated; A sample of headspace gas was drawn, with a syringe; headspace gas samples transferred to sterilized pre-vacuumed Exetainer® vials (Labco Ltd., High Wycombe, England), sealed with silicone gel	Vial headspace gas samples were analyzed for N_2O concentration using a gas chromatograph (CP-3800 VARIAN® STAR; Agilent Technologies, Santa Clara, CA, USA)	Humid tropical rainforest	N_2O
(26)	Wang et al., 2018	Li 6400-09 soil respiration chamber (Li Inc Lincoln, NE, USA); CO_2 emission measured in a PVC collar (10 cm in diameter and 5 cm in height) installed about 3 cm into the soil	Portable infrared gas analyzer (Li Inc.)	Tropical forest	CO_2
(27)	Fernandez-Bou et al., 2018	Surface efflux: 10 cm diameter PVC collars on the ground surface one hour prior to sampling for five minutes using low-cost CO_2 flux chambers; CO_2 efflux estimated using the vent cross-sectional area as effective CO_2 emitting area	NDIR CO_2 sensors (model MH-Z16, Winsen Technology Co., Henan, China)	Wet tropical rainforest	CO_2
(28)	Soper et al., 2019	Static chamber technique; Three 22 cm diameter PVC collars per pile were placed approximately 3 cm into the substrate 10 min prior to sampling and topped with chamber lids	Gas chromatograph fitted with a methanizer, flame ionization, and electron capture detectors (ECD; Shimadzu GC-2014, Shimadzu, Kyoto, Japan)	Tropical forest	CO_2, CH_4, and N_2O
(29)	Fernandez-Bou et al., 2020	Flow-through CO_2 efflux detecting chambers (clear acrylic tubing, 50 mm in diameter and 100 mm in height with a 5 mm diameter hole placed 52.5 mm from the bottom); Gas samples collected with a syringe introduced in the chambers	A low-cost CO_2 sensor (non-dispersive infrared, model MH-Z16, Winsen Technology Co., Henan, China) was located inside of each chamber; Gas chromatography (Shimadzu GC-2014, Kyoto, Japan)	Wet tropical rainforest	CO_2, CH_4, and N_2O

Chapter III

Supplementary Table 1. Number of sampled nests and CO_2 flux (minimum and maximum values) from nests of 45 ant species extracted from 17 papers.

Reference	Ant species	Number of sampled nests	CO_2 flux (mg CO_2 m^{-2} d^{-1})	
			Minimum	**Maximum**
Bender and Wood 2003	*Solenopsis invicta*	3	71.37	6,195.64
Ohashi et al., 2005	*Formica rufa* group	1	14,400	32,880
Risch et al., 2005a	*Formica rufa* group	16	19,200	206,400
Risch et al., 2005b	*Formica rufa* group	16	19,200	206,400
Domisch et al., 2006	*Formica rufa* group	3	7,200	150,960
Ohashi et al., 2007	*Formica aquilonia*	4	22,560	105,120
Ohashi et al., 2012	*Formica rufa* group	15	3,984	136,248
Wu et al., 2013	*Lasius flavus*	3	21,840	4,3920
	Lasius niger	3	28,080	46,800
	Formica candida	3	20,160	42,960
Hasin et al., 2014	13 subterranean ant species	61	30,41.28**a**	240,261.12**a**
Jílková, Cajthaml and Frouz 2015	*Formica aquilonia*	10	10,151.04	152,773.20
Jilková, Picek and Frouz 2015	*Formica aquilonia*	6	14,385	3,666,556.80
Ohashi et al., 2017	*Lophomyrmex longicornis*	14	49,42.08	66,908.106
	Odontponera transversa	10	12,925.44	113,287.68
	Diacamma intricatum	8	31,173.12	212,509.44
	Dinomyrmex gigas	7	44,478.72	377,118.72
	Pheidole tawauensis	7	7,983.36	39,156.48
	Acanthomyrmex ferox	5	8,743.68	50,941.44
	Myrmecina sp.1	5	13,305.6	70,709.76
	Nylanderia sp.1	4	6,082.56	30,412.80
	Nylanderia sp.6	4	7,603.2	12,545.28
	Ectomomyrmex cf. javana	4	18,247.68	60,065.28

	Recurvidris sp.9	4	4,561.92	13685.76
	Tetramorium palaense	4	13,685.76	34,974.72
	Tetramorium adpressum	4	8,363.52	22,809.60
	Camponotus (Tanaemyrmex) sp.15	3	12,545.28	42,577.92
	Pheidole plagiaria	3	15,206.4	59,685.12
	Pheidolegeton cf. affinis	3	9,123.84	25,850.88
	Oligomyrmex sp.31	2	4,942.08	8,363.52
	Pheidole aglae	2	23,950.08	24,710.4
	Pheidole cf. longipes	2	18,627.84	42,577.92
	Tetramorium curtulum	2	15,206.4	26,231.04
	Acropyga butteli	1	*	45,999.36
	Aphaenogaster sp.26	1	*	26,231.04
	Camponotus arrogans	1	*	19,768.32
	Cerapachys sp.1	1	*	91,23.84
	Cerapachys sp.66	1	*	52,081.92
	Euprenolepis variegata	1	*	19,768.32
	Oligomyrmex sp.30	1	*	27,371.52
	Oligomyrmex sp.5	1	*	18,247.68
	Euponera sharpi	1	*	26,991.36
	Pheidole aristotelis	1	*	14,065.92
	Pheidole cf. elisae	1	*	26,991.36
	Pheidole lucioccipitalis	1	*	14,065.92
	Pheidole spinicornis	1	*	20,908.80
	Pheidologeton sp.5	1	*	19,768.32
	Pseudolasius cf. sp.3	1	*	18,627.84
	Tetramorium parvum	1	*	24,710.40
Wang et al., 2018	*Pheidole capellini*	5	16,815.60	57,078.72
	Odontoponera transversa	5	13,263.12	51,631.44
	Pheidologeton affinis	5	43,815.60	57,078.72

Fernandez-Bou et al., 2018	*Atta cephalotes*	3	1,862.78	38491.20
Soper et al., 2019	*Atta colombica*	22	51,118.64	90595.88
Fernandez-Bou, Dierick and Harmon, 2020	*Atta cephalotes*	3	5,702.40	126,213,120
This paper	*Acromyrmex subterraneus*	28	36,216	5,548,080

a Range from all ant nests. There is no available data about separated ant species.

* For some species, there is just available data about the maximum CO_2 flux.