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# Microbial co-occurrence network and its key microorganisms in soil with permanent application of composted tannery sludge

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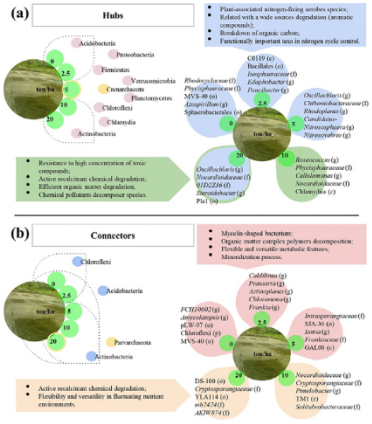
## HIGHLIGHTS

- Permanent application of CTS impacted microbial network structure and modularity.
- Actinobacteria and Acidobacteria are keystones microbial phyla in CTS-treated soil.
- Different CTS levels generate a distinct microbial community in soil.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Soil microbial communities act on important environmental processes, being sensitive to the application of wastes, mainly those potential contaminants, such as tannery sludge. Due to the microbiome complexity, graph-theoretical approaches have been applied to represent model microbial communities interactions and identify important taxa, mainly in contaminated soils. Herein, we performed network and statistical analyses into microbial 16S rRNA gene sequencing data from soil samples with the application of different levels of composted tannery sludge (CTS) to assess the most connected nodes and the nodes that act as bridges to identify key microbes within each community. The network analysis revealed hubs belonging to Proteobacteria in soil with lower CTS rates, while active degraders of recalcitrant and pollutant chemical hubs belonging to Proteobacteria and Actinobacteria were found in soils under the highest CTS rates. The majority of classified connectors belonged to Actinobacteria, but similarly to hubs taxa, they shifted from metabolic functional profile to taxa with abilities to degrade toxic compounds, revealing a soil perturbation with the CTS application on community organization, which also impacted the community modularity. Members of Actinobacteria and Acidobacteria

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Next-generation sequencing
Key microbes

were identified as both hub and connector suggesting their role as keystone groups. Thus, these results offered us interesting insights about crucial taxa, their response to environmental alterations, and possible implications for the ecosystem.



## 1. Introduction

Soil microbial communities drive biological processes, such as nutrient recycling, water supply, and the cycling of carbon and nitrogen, being considered key components of the soil ecosystems (Fierer, 2017; Banerjee et al., 2018). On the other hand, the growing anthropogenic activities, such as industrial wastes disposal, have brought issues on their effect on soil microbial communities (Miranda et al., 2018a). Particularly, some studies have assessed the potential of industrial wastes, such as textile and tannery wastes, on soil microbial properties (Araujo et al., 2016; Silva et al., 2010) and showed that permanent application of these wastes could promote soil pollution and affect the soil microbiome, including those metal-contaminated wastes (e.g., chromium) that can remain in the soil for years (Miranda et al., 2019).

Particularly, tannery sludge (TS) presents a high concentration of chromium and its disposal represents one of the major issues in the management of the tannery industry (Silva et al., 2010). However, alternative treatments to improve TS properties have been proposed, such as composting (Miranda et al., 2018a). Composting has been used as a potential method to treat tannery wastes before their application in the soil as organic fertilizer (Sousa et al., 2017; Ravindran et al., 2019). These studies have reported improvement in soil organic matter and nutrients and, consequently, promoting higher plant growth and changes in the soil microbial properties (Sousa et al., 2017). Specifically, the use of composted tannery sludge (CTS) in agricultural soils raises concerns about the presence of chromium (Cr) and its potential accumulation in soils (Miranda et al., 2018a). Indeed, Miranda et al. (2018a) reported an accumulation of Cr in soils ranged from 29 to 150 mg/kg with the application of CTS for 7 years. Previous studies have shown the effects of CTS application on soil chemical properties (Sousa et al., 2017), microbial biomass and enzymatic activity (Araujo et al., 2020), microbial diversity, and community composition (Miranda et al., 2018a,b). Although these studies have reported changes in the soil microbial properties induced by CTS, there is still an essential question in microbial ecology about how these microbiomes respond to the permanent application of CTS.

The knowledge about co-occurrence and co-abundance patterns is a central topic in microbial ecology for understanding community dynamics (Layeghifard et al., 2017; Ghosh et al., 2018). However, the direct evidence on these interactions for ecological interpretations is not possible from sequencing data per se (e.g. 16S rRNA gene and shotgun metagenomics), but they can be well represented and modeled through the utilization of associated metrics for co-occurrence network construction, and it allows making predictions and ecological assumptions (Berry and Widder, 2014). In recent years, the microbial network has been used to analyze co-occurrences and interactions between members of microbial communities, resulting in a large number of microbial co-occurrence patterns in soils (Sun et al., 2017; Ma et al., 2020), plants (Agler et al., 2016; Marasco et al., 2018; Toju et al., 2018), as well as in human microbiome (Fisher and Mehta, 2014). Moreover, the analysis of the architecture of microbial networks made from topological indices allows the identification of connectivity between taxa, community resilience, habitat heterogeneity, and clusters of phylogenetically close species (Mondav et al., 2017). Besides, it allows recognizing potentially relevant microorganisms including hubs, connectors, and keystones that, regardless of their abundance, are considered "central" microbial species and exert a considerable influence on the structure and functioning of the community (Agler et al., 2016).

Currently, there are no other treatable means of identifying important microorganisms in the diverse and largely uncultivated soil microbial communities. Thus, network analysis fulfills a crucial need in the ecology of microbial communities (Berry and Widder, 2014). The interpretation of these networks allows the illustration of how these observed structures can be used to generate testable hypotheses about candidate microbes that affect the health of the community, aiming at the functional optimization of the microbiome in natural and agricultural ecosystems for the increase of soil fertility (Hartman et al., 2018), plant growth (Toju et al., 2018), pathogen and disease control (Schlatter et al., 2017), and even natural ecosystem preservation (Ma et al., 2016).

Taking the considerations presented, this study used statistical and computational tools and techniques to find valuable insights into the impacts caused by CTS application on the organization and maintenance of the soil microbiome and the implications of it for ecosystem stability. Network theory approaches were implemented to access the most connected taxa and identify important microbes of the community in 16S rRNA amplicon data from soil samples after 7 years of application of different rates of CTS.

## 2. Material and methods

### 2.1. 16S rRNA sequencing data from soil treated with CTS samples

This study used a microbial dataset from a study with permanent application of CTS (Miranda et al., 2018a, 2018b). In each CTS rate applied to the soil, the microbial data were used to construct the network and identify key bacterial taxa as well as their responses to the soil physicochemical properties. The general workflow of this study is summarized in Fig. 1.

The relative abundance of operational taxonomic units (OTUs) was obtained from a study that evaluated the dynamics of the soil bacterial community after permanent application of CTS for 7 years (Miranda et al., 2018a, 2018b). The treatments consisted of CTS applied annually for 7 years and incorporated into the soil in five rates: 0 (control), 2.5, 5, 10, and 20 ton/ha. Detailed information about the soil properties, strategies of soil sampling, DNA extraction, PCR amplification, 16S rRNA sequencing, and phylogenetic classification can be found in Miranda et al. (2018a). The data are available at the NCBI Sequence Read Archive under the identification PRJNA646266.

### 2.2. Co-occurrence network construction and analysis

Microbial co-occurrence networks were constructed using the graphical user interface Cytoscape plugin CoNet (http://apps.cytoscape.org/apps/conet), an available network construction method (Faust and Raes, 2012) by which non-random patterns of microbial co-occurrence are detected by combining multiple association methodologies simultaneously and resulting in a consensus network. For each CTS rate, a co-occurrence network was obtained using the respective OTU table: 0 (control), 2.5, 5, 10,

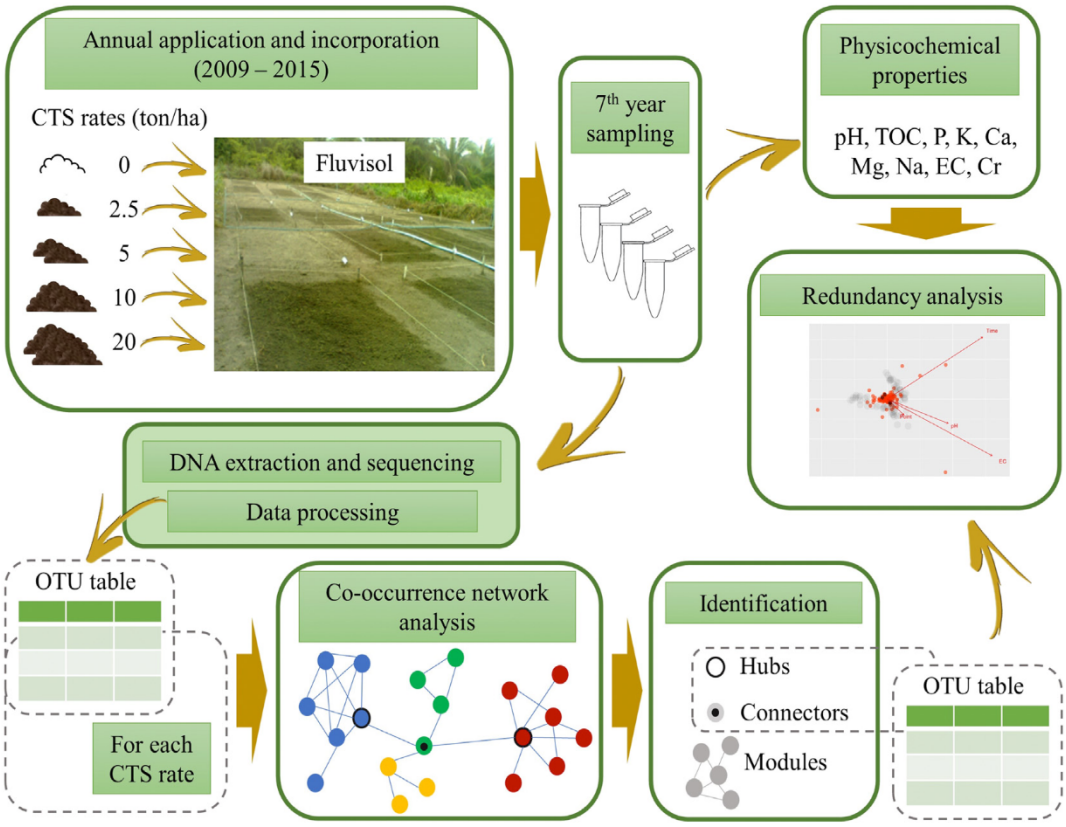


**Fig. 1.** Summary of the general workflow of this study for microbial network construction, analysis, identification of key bacterial taxa and their responses to the soil physicochemical properties. TOC: total organic carbon; EC: electric conductivity.

and 20 ton/ha, totalizing 5 co-occurrence networks. In this process, a combination of four different measures was used: two correlation measures (Pearson and Spearman) and two dissimilarity measures (Bray-Curtis and Kullback-Leibler) (Marasco et al., 2018). Only significant correlations were used to construct the co-occurrence network (*P*-value <0.05) (Faust et al., 2015). Wherein, nodes represent the OTUs (taxa) and the edges represent the most significant correlations between the nodes, in other words, the strongest connections between OTUs (Hartman et al., 2018). Network visualization and computation of centrality indices of each node were performed using Python v3.6. The analysis of network indices was performed using the statistical software R 3.5.1.

### 2.3. Identification of putative important nodes

Centrality-based approaches were used to access nodes with the highest connectivity for putative hub identification. The centralities of degree, closeness, and betweenness (Agler et al., 2016) were chosen due to their wide use in the literature, not only to identify hub and connector nodes but also keystone species (Lupatini et al., 2014; Hartman et al., 2018; Bonito et al., 2019). Additionally, harmonic centrality, an improved variant of closeness centrality, was also chosen (Boldi and Vigna, 2014). Centrality indices of each node were computed using the networkx v.2.1, and the nodes with the highest centrality values were accessed for each different CTS application network level. Five nodes with the highest values of degree centrality were considered hubs. Nodes with higher betweenness, closeness, and harmonic centralities, obtained through Principal Component Analysis (PCA) (R 3.5.1 Stats Package) were classified as connectors, while identification of the same taxa in both classifications was considered as a potential keystone.

### 2.4. Community identifications

Based on the co-occurrence networks constructed from the measures ensemble approach, communities, clusters, or modules were obtained using the Louvain method of maximizing modularity (Layeghifard et al., 2017). The community detection algorithm was employed using the python-louvain v0.13 package of the PyPI repository (*Python Package Index*) (https://pypi.org/project/python-louvain/), to observe interaction patterns and identification of important groups of nodes in bacterial community networks.

### 2.5. Statistical analysis

All data were standardized before statistical analyses and all statistical tests were performed considering significant values with *p*-value <0.05. Variables exploratory descriptive analysis was carried out with a quantiles approach, which allowed the knowledge of measurement distribution, relative variation, and identification of outliers (Faust et al., 2015). Principal Component Analysis (PCA) was performed using software R to determine the higher variance components, allowing inferences about the compatibility and complementarity of the data obtained in network analysis (Hartmann et al., 2015).

The redundancy analysis (RDA) was performed to show the correlation among treatments, time, and physical-chemical parameters with the OTUs. It was used the OTU table at genus level with relative abundances as response variables, physicochemical parameters as explanatory variables, and time and treatment level as additional environmental variables. Monte Carlo permutation tests (999 permutations) were performed, including the single physicochemical parameters as an explanatory variable, the sampling date as the covariate, with permutation only within the

covariables, to select only the physicochemical variables that are significant for the RDA (Edgar, 2013).

## 3. Results

### 3.1. Microbial network and centrality indexes

The co-occurrence networks presented an average number of 626 nodes (OTUs) and 190,908 edges (co-occurrence relation) (Table 1). The centrality indices approach was performed to determine the highest connected nodes within each constructed network (Fig. 2a). The correlation analysis between the selected centrality indices has shown that the degree centrality had a negative correlation as compared to the other three centralities: closeness, betweenness, and harmonic (Fig. 2b). The quantile box plots, performed in closeness, betweenness, and harmonic centralities, showed non-occurrence of disproportionately centrality values (Fig. 2a), indicating a requirement of variance maximization technique approach (Fig. 2c).

### 3.2. Hub identification

Within identified microbial taxa in each network, the most connected genera belonged to Proteobacteria, Planctomycetes, Acidobacteria, Chloroflexi, Firmicutes, Verrucomicrobia, Actinobacteria, Chlamydia, and Crenarchaeota (Fig. 3a). Regarding bacterial groups, Chloroflexi and Planctomycetes appeared as hubs in all microbial networks, except to soil with CTS at 10 and 5 ton/ha, respectively. Members of Planctomycetes also appeared in all microbial networks, except in the CTS rate of 5 ton/ha. Acidobacteria were classified in control and lower CTS rates, whereas members of Actinobacteria were classified in the two highest CTS rates. Genera belonging to Firmicutes and Chlamydia were classified only once each, in the CTS rate of 2.5 ton/ha and 10 ton/ha microbial network, respectively. Regarding the archaeal community, Crenarchaeota was classified only in one microbial network (5 ton/ha) as a highly connected taxon. The unidentified genus belonging to the Nocardioidaceae family (Actinobacteria) was classified as a hub in both 10 and 20 ton/ha CTS rates microbial networks, and the genus *Oscillochloris* (Chloroflexi) was also identified as a hub in two levels of CTS rates microbial networks (soil 5 and 20 ton/ha), being all others classified microbial genera different in all CTS rates networks, suggesting that hubs were intensively dynamic between different networks (Fig. 3a, Supplementary Table S1).

### 3.3. Connectors identification

Within identified bacterial taxa present in each CTS rate network, it was found connector genera belonging to Chloroflexi, Actinobacteria, and Acidobacteria from Bacteria, and Parvarchaeota from Archaea (Fig. 3b). As noticed, different genera belonging to the same phylum were classified as connectors microorganisms considering together all different microbial networks (control, 2.5, 5, 10, and 20 ton/ha), with Actinobacteria being the phylum with the higher number of classified members genera (11 in total). Chloroflexi and Acidobacteria had five and four classified genera, respectively. Although only three phyla were found as connector taxa in all networks, all taxa were classified in different genera, suggesting that these connector taxa were also intensively dynamic between different networks (Fig. 3b, Supplementary Table S2).

Genera belonging to Chloroflexi appeared among the connectors taxa only in control and in the lowest CTS rate, whereas Actinobacteria members dominated most of the classified taxa in the highest CTS rates (5, 10, and 20 ton/ha). Members of Acidobacteria appeared in all microbial networks, except in the CTS rate of 2.5 ton/ha network, and Archaea members appeared as connector taxon only in the highest CTS rate microbial network. Between the two types of classification performed (hub and connector), an unidentified genus belonging to uncultured bacteria of MSV-40 order (Acidobacteria) was found in the control microbial network, and the unidentified member of the Nocardioidaceae family (Actinobacteria) also was found as hub and connector in the 10 ton/ha CST rate network (Fig. 3, Supplementary Table S1, S2). It is noteworthy that many different members belonging to the Actinomycetales order (Actinobacteria) were identified as both hub and connector, suggesting the significant importance of this group to community control, organization, and functioning (Supplementary Table S1, S2).

**Table 1**
Number of nodes and edges found in constructed soil microbial network in each CTS rate.

| CTS rate (ton/ha) | Nodes (n°) | Edges (n°) |
|---|---|---|
| 0 | 595 | 172,489 |
| 2.5 | 632 | 194,293 |
| 5 | 624 | 189,155 |
| 10 | 648 | 203,830 |
| 20 | 633 | 194,775 |

### 3.4. Network community identification and organization of key microbial members

The organization of communities within the network (modules) was observed in each CTS rate microbial network, and the number of modules formed varied through each CTS rate, from three to six modules (Fig. 4a, b). In the control network, the genera were divided into 4 groups, where the number of grouped elements ranged from 80 to 203. In the 2.5 ton/ha CTS rate microbial network, the genera were divided into six groups where the number of grouped elements ranged from 44 to 155. The number of modules decreased for higher CTS rate microbial networks. In 5 ton/ha CTS rate microbial network, the genera were divided into four groups where the number of elements ranged from 112 to 243, while 10 and 20 ton/ha CTS rates microbial networks genera were divided into three groups where the number of elements ranged from 112 to 243 and 89 to 340, respectively (Fig. 4a, b, Supplementary Table S3).

The distribution of identified hubs between the modules in the microbial network was not homogeneous, being almost the majority of identified hubs distributed within one module (Fig. 4a). This pattern was observed in the control microbial network and the two highest CTS rates networks, indicating that these hubs are highly connected between them. Highlighted interactions originating at the hubs showed that they interact with all other modules, varying in intensity (Fig. 4a). Similar heterogeneous distribution of genera classified as connectors were observed (Fig. 4a).

### 3.5. Effects of physicochemical properties on identified key microbial members

The RDA analysis was performed using the OTUs as response variables and physicochemical parameters as explanatory variables. Only significant physicochemical parameters that explained the variation of OTUs between samples were used. RDA was done to highlight each set of identified hubs and connectors (Fig. 4c). The RDA showed almost no relationship between the significant physicochemical properties and most of the identified genera as hubs, due to their central diagram clustering (Fig. 4ci–v). Unidentified genera hubs belonging to Chloroflexi and Planctomycetes had a negative relationship with soil pH and electric conductivity, and an unidentified genus hub belonging to Firmicutes appeared to have a positive relationship with localization of soil sampling sites in soil with the lowest CTS level (2.5 ton/ha) (Fig. 4cii). Moreover, the *Rhodoplanes* genus (Proteobacteria) hub had a negative relationship with physicochemical variables in soil with 5 ton/ha of CTS (Fig. 4ciii).

Regarding the relationship between the identified connectors and the significant physicochemical properties, it was found almost no

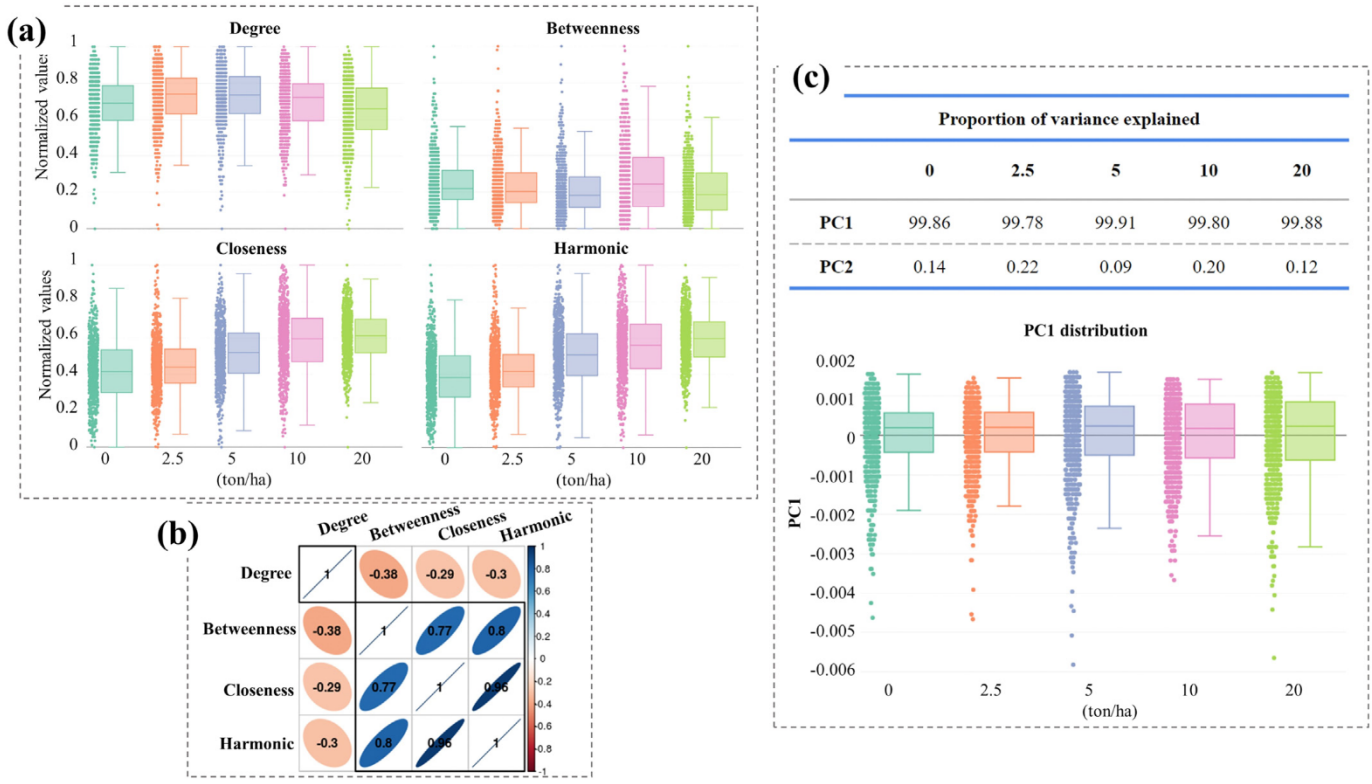


**Fig. 2.** Computed centrality indexes quartile distribution in different CTS rates (0, 2.5, 5, 10, 20 ton/ha) (a). Representation of centrality indexes linear correlations (b). Proportion of variance explained and distribution of first component values obtained for each CTS rate (c).

relationship between them, due to their tight central diagram clustering (Fig. 4cvi–x), as similarly were found in hubs diagrams. However, an unidentified genus belonging to the uncultivated mb2429 family (Acidobacteria) appeared to have a positive relationship mostly with the localization of soil sampling site in the experimental block in soil with 20 ton/ha of CTS (Fig. 4cx).

## 4. Discussion

In this study, we targeted the microbial association within different CTS rates to evaluate their effects on the dynamics and structure of the soil microbial community. Using a network thinking in microbiome and 16S rRNA gene sequencing data of soil samples, microbial co-occurrence networks were constructed to infer networks based on abundance profiles.

Previous studies have been used the microbial co-occurrence network analysis for the identification of the most important nodes that, in microbiome analysis studies, can be the most influential and essential member in the soil or rhizosphere microbial community, emerging the identification of putative hubs, connectors, and keystone microbes (Hartman et al., 2018; Qi et al., 2019; Banerjee et al., 2019). For instance, Hartman et al. (2018) observed dissimilarities in soil highly connected bacteria due to tillage practice, with few exceptions belonging to Firmicutes, Chloroflexi, and Actinobacteria, while fungal communities were additionally and more greatly influenced by fertilization type, suggesting that manure additions to soil play a key role introducing taxa with keystone functions.

Our network analysis revealed that the level of CTS application directly affected the dynamics of the bacterial and archaeal communities and its most critical taxa. Interestingly, in lower CTS rates (2.5 and 5 ton/ha) and the control networks, we found hubs with functional groups related to nitrogen metabolism, such as nitrogen-fixing bacteria from the Rhodocyclaceae family and *Azospirillum*, and ammonia-oxidizing genera *Candidatus-Nitrososphaera* and *Nitrosovibrio* (Oren, 2014; Zhalnina et al., 2014). On the other hand, the hubs found in the highest CTS rates (10 and 20 ton/ha) microbial networks were composed of microbial groups with resistance to toxic compounds and active degradation of recalcitrant and pollutant chemicals, such as the genera *Roseococcus*, *Steroidobacter*, and members of the Nocardioidaceae family (Tóth and Borsodi, 2014). For instance, *Roseococcus thiosulfatophilus* was found to be effective in reducing some metals, such as Cr (Maltman and Yurkov, 2019). Recently, Ma et al. (2020) distinguished key microbial populations belonging to Proteobacteria, Acidobacteria, Actinobacteria, and Chloroflexi while analyzing the bacterial network of damaged mining soils. Particularly to metal contaminated soils, Hur and Park (2019) identified Acidobacteria, Actinobacteria, Chloroflexi, Firmicutes, Nitrospirae, Planctomycetes, and Proteobacteria as key microbial groups. In our study, most of the selected genera classified as connectors were branched filamentous mycelia-shaped bacterium, with flexible and versatile metabolic features and secondary metabolites producers, such as *FCH10602*, *Amycolatopsis*, *Prauseria*, and *Actinoplanes*, most belonging to Actinobacteria, which is known due to its phylogenetic and physiologic diversity composition that supports Earth's ecosystems functioning (Stackebrandt et al., 2014).

Moreover, it was observed bacterial connectors that are active in recalcitrant chemicals degradation (Tóth and Borsodi, 2014), such as members of the Nocardioidaceae family, as well flexible and versatile metabolism (Eichorst et al., 2018), i.e. Solirubrobacteraceae and mb2424 family member, in higher CTS rates (10 and 20 ton/ha), which may contribute to their occurrence. Similarly, co-occurrence network analysis of archaeal community found by Miranda et al. (2019) revealed a direct effect of CTS application by identifying as a key taxon the *Nitrososphaera* genus, which is an important player in nitrogen transformation with high metabolic versatility and enzymatic feature that contribute to Cr resistance. Also, our data showed

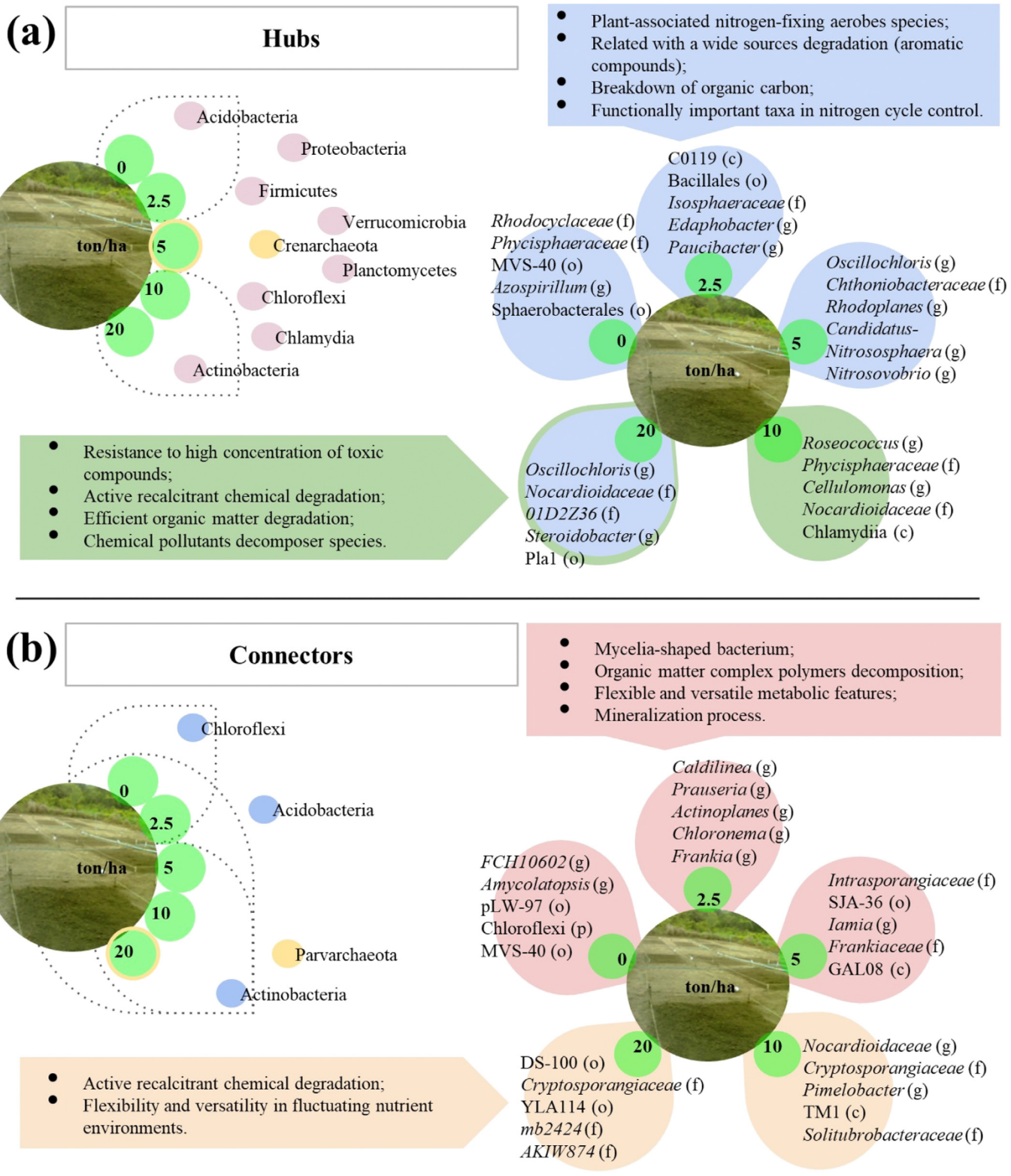


**Fig. 3.** Classified hubs phyla, all hubs lowest taxonomic classification in each CTS rate (0, 2.5, 5, 10, 20 ton/ha) and description of some of their principal functionalities (a). Classified connectors phyla, all connectors lowest taxonomic classification in each CTS rate (0, 2.5, 5, 10, 20 ton/ha), and description of some of their principal functionalities (b). (c) class; (o) order; (f) family; (g) genus.

that Crenarchaeota was classified as a highly connected taxon only in the microbial network with a low CTS rate (5 ton/ha). Previous studies have shown that the diversity and abundance of organisms belonging to Crenarchaeota are higher in lower pH (Lehtovirta et al., 2009; Mendes et al., 2012). In our samples, the pH increased with an increased CTS rate (Miranda et al., 2018a). Also, Sandaa et al. (1999) showed previously that heavy metals are toxic to the growth and survival of some archaeal groups, which could explain the classification of Crenarchaeota as hub only in soils with low pH and CTS rate.

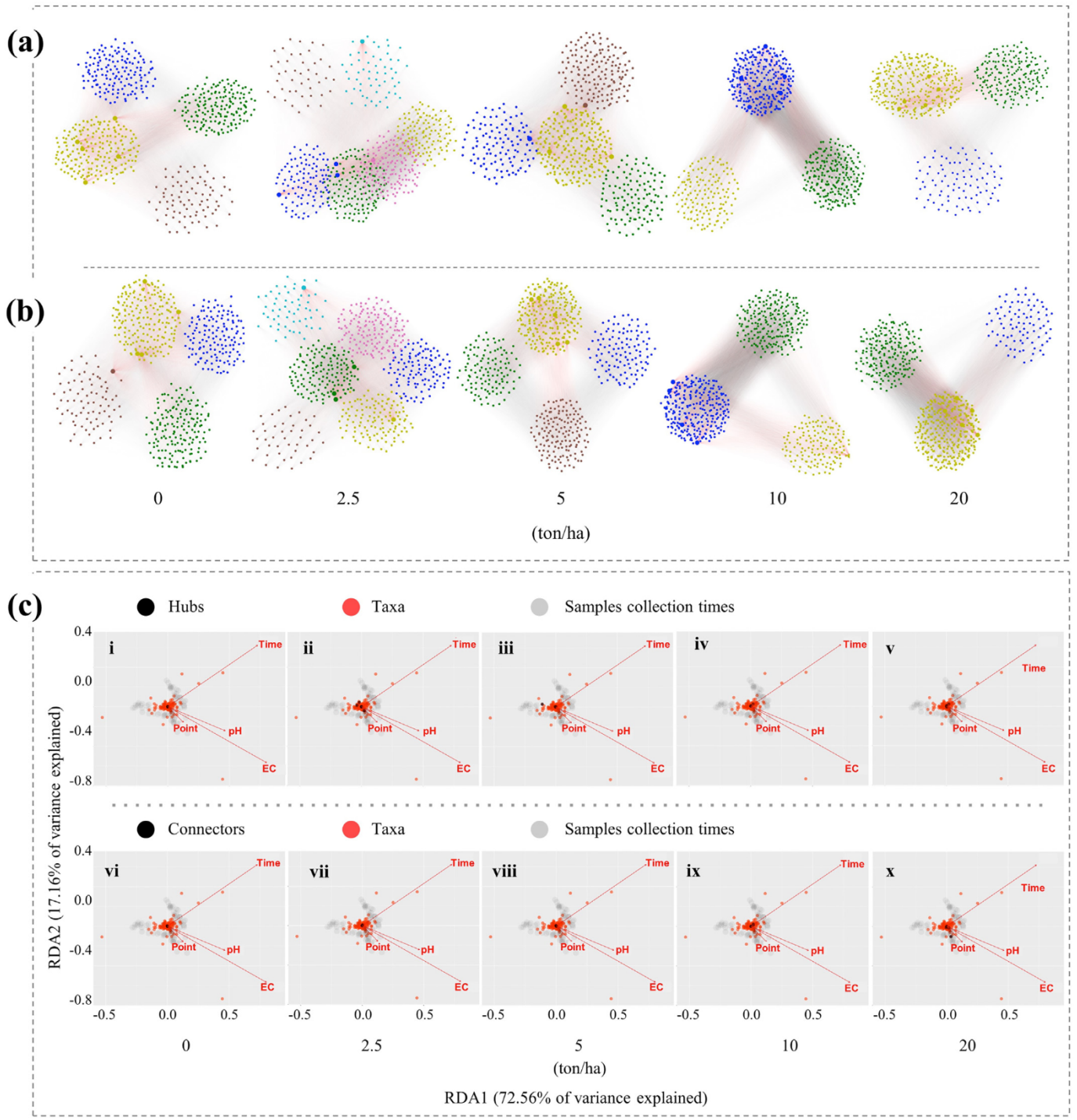


**Fig. 4.** Soil microbial network separated in highly connected subcommunities (modules) for each CTS rate, where dots represent nodes (taxon), enlarged nodes represent the identified hubs, and node colors represent the module (a). Highlighted interactions in red which depart from hubs show their interaction with other nodes. Same microbial network presented in (a), however, enlarged nodes represents the identified connectors taxa (b). Module color represents the same module within the same CTS rate; however, it does not represent the same module between different CTS rates. 0; 2.5; 5; 10; 20 are CTS rates in ton/ha. Redundancy analysis diagram (RDA) of correlation between significant physicochemical properties and taxa at genera level (c). First component (RDA1) explained 72.56% of the variables variance and second component (RDA2) explained 17.16% of the variable variance. Set of classified hubs (i – v) and connectors (vi – x) in each CTS rate are highlighted by black dots. **Time:** sampling time in days; **pH:** soil pH; **Point:** localization of soil sampling site in the experimental block; **EC:** electric conductivity.

The hub and connector genera within the microbial community shifted from metabolic functional traits to microorganisms with abilities to degrade toxic compounds, revealing a soil perturbation with the application of CTS on community organization. As shown by Karimi et al. (2019), which investigated bacterial co-occurrence networks in a variety of land uses including forests, grasslands, crops, and vineyards, the identified hubs belonged to Bacteroidetes and Actinobacteria, which shifted from taxa that tend to grow in high organic matter conditions in less impacted soils (forests) to taxa that can grow in adverse environments such as agricultural soils.

In our study, despite the increased number of nodes and links from unamended soil to the most CTS rates, we observed a decrease in

module number among the CTS gradient, which might demonstrate a loss of ecological types. The network module is a group of nodes highly connected within and can be considered as functional units in the microbial communities differing in microbial functions between them (Toju et al., 2018). The number of modules in a network has been considered another topological feature having a direct relationship with network complexity (Karimi et al., 2019) and is indicative of more ecological types, which are able to re-associate with new partners depending on the environmental conditions, and resistance of a network to disturbance and loss of individual species (Wood et al., 2017). Although there was an increase in organic matter and other nutrients in the treated soils at 5 ton/ha level, the treatment enriched the soil and favored the establishment of new ecological microbial types. Yet, the decrease in module number at the higher CTS rate suggests that those nutrients started to inhibit and compromise the microbial functional diversity. Those results reveal a community simplification and biodiversity loss, which were observed in Miranda et al. (2018b) that analyzed bacterial relative abundance and composition. Similarly, Price et al. (2021) observed that the community complexity and stability decreased by the annual application of high rates (28 and 42 ton/ha) of alkaline stabilized biosolids, suggesting that the application of higher rates of biosolids led to a more adapted and less cooperative microbiome. Furthermore, it is known that pH has a strong influence on bacterial community and drives the changes in soil chemical properties, such as nutrient availability and metal solubility (Lauber et al., 2009). Ling et al. (2016) showed that soil organic and chemical fertilization had a distinct effect on soil pH, which was identified as a key variable to impact the microbial community modularity. Since pH alteration with the application of CTS was observed, it may have contributed to observed modularity differences.

The permanent application of CTS has changed the soil physicochemical properties such as pH, EC, Cr, TOC, P, and Ca (Miranda et al., 2018a). Therefore, the RDA showed almost no relationship between the significant physicochemical properties and identified hubs and connectors, which might be showing the establishment of a selected bacterial group with metabolic abilities to survive in different physicochemical properties environments. Although Miranda et al. (2018b) showed that some of the less abundant phyla were influenced positively (e.g. GAL15, NKB19, GN02, OP3, and WS3) or negatively (e.g. AD3, Tenericutes, and Verrumicrobiota) mainly due to Cr concentration and soil pH, the most abundant phyla were not influenced by the CTS over time. This lack of influence supports the weak effect of the significant physicochemical parameters on the identified key microbial members since most of them belonged to the most abundant bacterial phyla such as Acidobacteria, Actinobacteria, Chloroflexi, Firmicutes, and Proteobacteria.

The negative relationship of genera hubs belonging to Chloroflexi, Planctomycetes, and Proteobacteria (*Rhodoplanes* genus) and the positive relationship of a genus hub belonging to Firmicutes with significant physicochemical variables in soil with the lowest level (2.5 and 5 ton/ha), show that the sampling time and location, and pH influenced more these taxa. Similarly, an unidentified genus connector belonging to the uncultivated mb2424 family of Acidobacteria was found in the highest CTS rate. Acidobacteria is a diverse and ubiquitous phylum and is one of the most abundant taxa found in soils being detected through cultivation-independent-based approaches such as 16S rRNA gene sequencing analysis. These bacteria can actively interact with the plant rhizosphere and act as plant growth-promoters (Kielak et al., 2016).

Furthermore, the presence of different plant species, i.e. cowpea and maize, cultivated during the soil sampling period (0, 45, 75, 150, and 180 days) modified the soil physicochemical properties. As shown by Miranda et al. (2018a, 2018b), the rhizosphere and the plant physiological state influenced the bacterial groups showing different patterns at the class level. Plants have a certain selective pattern on soil microbes and bacterial communities adapt to plant type and also change over time. In a study with lima bean, Sousa et al. (2020) showed that the abundance of Proteobacteria and Actinobacteria increased while Firmicutes decreased during plant cultivation. They suggest that these shifts might be related to root exudates secretion which could increase microbial activity in the rhizosphere (Sousa et al., 2020).

Finally, our network analysis showed that the bacterial community changed after the application of CTS and it agrees with Miranda et al. (2018a). The influence of CTS on the microbial community was due to its characteristics, which altered the soil physicochemical properties and promoted the growth of selected metabolically adapted groups that reflected on what we found through topological network analysis. Thus, applying the knowledge about the complex network, developed across multiple expertise, to the analysis of microbiome data and associated with relevant physicochemical variables and multivariate statistics techniques offer an opportunity to identify key microbes such as hubs and connectors. The keystone taxa represent nodes that are associated with many others and have important roles in shaping network structure, while the removal of this node may have a large impact on the community (Mendes et al., 2018; Rossmann et al., 2020). Recent studies reported a turnover of putative keystone species as environmental conditions change (Lu et al., 2013; Lupatini et al., 2014). In our study, the CTS application changed the soil chemical properties with a possible side effect on keystone taxa and hubs. Further analysis of the keystone species could help to understand their role in structuring the microbial community and the response towards the CTS application. Together, our study showed that the network approach might reveal central targets to quickly develop the understanding of microbe-microbe relationships and enable better future management of microbiome for biocontrol, ecosystem preservation, and sustainable improvement of agroecosystems.

## 5. Conclusions

This study provides an insight into how CTS application affected soil microbial community structure and dynamics by applying complex network principles to microbial co-occurrence patterns. It was shown that different CTS rates appeared to generate a distinct microbial community that fitted their functional traits to each soil physicochemical condition. Permanent application of CTS changed the microbial network structure, distribution of modules, identified hub and connector genera, and their distribution within modules. However, it is known that the interpretation of microbial networks solely is insufficient to assess the effect of the CTS on the soil microbiome. Further investigations are needed to circumvent existing methodological limitations and laboratory analysis is required to integrate and validate the patterns and potential identified key microbes revealed by the network analysis. Therefore, network topology analysis coupled with statistical techniques is the first step to predict the outcome of community alterations and effects of perturbations. This approach provides information of potential important taxa to community structure and functioning, which will help us to assess them to manipulate and manage the soil microbial community and apply to functional optimization of sustainable agricultural management and natural ecosystems preservation and recovery.

## Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

## CRediT authorship contribution statement

**Caroline Kie Ishimoto:** Conceptualization, Methodology, Formal analysis, Writing – review & editing. **Alexandre Hild Aono:** Conceptualization, Methodology, Formal analysis, Writing – review & editing.

**James Shiniti Nagai:** Conceptualization, Methodology, Formal analysis, Writing – review & editing. **Hério Sousa:** Conceptualization, Methodology, Formal analysis, Writing – review & editing. **Ana Roberta Lima Miranda:** Methodology, Project administration, Investigation, Visualization, Writing – review & editing. **Vania Maria Maciel Melo:** Methodology, Project administration, Investigation, Visualization, Writing – review & editing. **Lucas William Mendes:** Methodology, Project administration, Investigation, Visualization. **Fabio Fernando de Araujo:** Methodology, Project administration, Investigation, Visualization, Writing – review & editing. **Wanderley José de Melo:** Methodology, Project administration, Investigation, Visualization, Writing – review & editing. **Reginaldo Massanobu Kuroshu:** Software, Resources, Formal analysis, Writing – review & editing. **Elisa Esposito:** Conceptualization, Methodology, Formal analysis, Writing – review & editing. **Ademir Sergio Ferreira Araujo:** Methodology, Project administration, Investigation, Visualization, Writing – review & editing.

## Declaration of competing interest

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

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.147945.

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