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# Methane emission suppression in flooded soil from Amazonia

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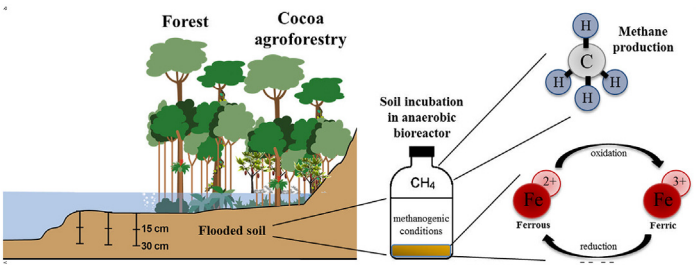
## HIGHLIGHTS

- High content of iron in Amazonia floodplain soil evidences less methane emission.
- Methanogenesis is suppressed by Fe(III) reduction in Amazonian floodplain soil.
- Fe(II) concentration was positively correlated to methane production in soil.
- *Methanobacterium* and *Desulfobulbus* were predominant in soil layer at 0–15 cm.
- '*Candidatus methanoperedens nitroreducens*' was ubiquitous at the soil layers.

## GRAPHICAL ABSTRACT

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## ABSTRACT

The coupling between ferrous iron and methane production has important global implications, with iron ions acting as electron acceptors for anaerobic oxidation of methane (AOM) and inhibitors of methanogenesis in different environments, including floodplain soils. In this sense, we analyzed the relationship between Fe(II) concentration and methane production in soil layers collected at 0–15 cm and 15–30 cm from flooded-forest and -agroforestry in Amazonian clear water floodplain incubated in anaerobic batch reactors using acetate, formate and glucose as organic sources. High throughput sequencing of archaeal and bacterial 16S rRNA genes was employed to assess the abundance and composition of the active methanogenic and methanotrophic microbial groups potentially involved in Fe(III)-dependent AOM in the soil used as inoculum. Positive correlation was revealed between Fe(II) concentration and methane production, with higher accumulation of Fe(II) in incubated soil layer collected at 0–15 cm in both forest and agroforestry sites for all the three organic sources. The accumulation of Fe(II) in the incubated soil evidenced the oxidation of Fe(III) potentially by *Methanobacterium*, *Desulfobulbus* and '*Candidatus methanoperedens nitroreducens*' living in anaerobic condition at this soil layer. The results point out to the microbial ferric iron reduction as an important

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potential pathway for anaerobic organic matter decomposition in Amazonian floodplain, evidencing methanogenesis suppression by Fe(III) reduction in flooded-forest and -agroforestry in Amazonian clear water river floodplain.



## 1. Introduction

Floodplains and other wetlands occupy approximately 800,000 km$^2$ in the lowland Amazon basin and include open water, flooded forest and floating macrophyte ecosystems (Melack and Hess, 2010). These lowland Amazon areas release significant amounts of carbon, about 1.2 ± 0.3 ton ha$^{-1}$ yr $^{-1}$ (Richey et al., 2002; Melack et al., 2004), derived from primary production on the floodplain and inputs of organic carbon from neighboring upland drainages (Melack et al., 2009; Ringeval et al., 2014; Pangala et al., 2017). This release represented approximately the net carbon accumulation of Amazon moist forest of ~1.1 ton ha$^{-1}$ yr$^{-1}$ (Meir et al., 1996).

Methane fluxes from the stream surface and soil are low compared to the carbon dioxide fluxes, but are important because methane is approximately 26 times more effective than carbon dioxide in absorbing infrared radiation that can significantly contribute to climate change (Solomon et al., 2007). Tropical soils can be one sources or sinks of methane, depending primarily on soil moisture, climatic conditions, land use and anthropogenic activities (Keller and Reiners, 1994; Steudler et al., 1996; Keller et al., 1990). When soils are flooded and became anoxic conditions, methane is produced by methanogenesis, while microbially-mediated aerobic and anaerobic oxidation of methane serve as the primary biological sink of this greenhouse gas (Agostinetto et al., 2002; Knittel and Boetius, 2009). In this sense, it is important to know the role of the soil microbial community in the regulation of the metabolic processes involved in the methane flux on soil under different land use systems.

Iron and manganese ions have been reported as electron acceptors in methane oxidation under anaerobic conditions. The first was shown to have an inhibitory effect on methanogenic activities in soils from flooded areas (Achtnich et al., 1995; Lueders and Friedrich, 2002; Knittel and Boetius, 2009; Ettwig et al., 2016; Vaksmaa et al., 2017). The anaerobic oxidation of methane (AOM) coupled to the reduction of oxidized metals was demonstrated in environmental samples and in freshwater enrichment culture by combining the reduction of Fe(III) by *Archaea* to the oxidation of methane (Ettwig et al., 2016; Scheller et al., 2016; Vaksmaa et al., 2017). The Fe(III) is thermodynamically more favorable than other electron acceptors studied for AOM, and they can reduce it to Fe(II) coupled to methane oxidation (Ettwig et al., 2016).

In flooded soils a dominant oxidation-reduction pair is composed by Fe(III) and Fe(II)forms. The alternation between these forms, ferric (Fe(III)) and ferrous (Fe(II)), is of great importance for the carbon and nutrient cycle, as ferric iron may serve as electron acceptor in microbial oxidation of organic compounds (Emsens et al., 2016). In shallow freshwater environments, which intercalate flood and dry periods, Fe(III) oxides generated during oxidation (dry period) allow Fe(III) reducers to divert the electron flow towards Fe(III) reduction and outcompete methanogenesis during the initial stages of anaerobiosis (flood period). This process depends on the temperature, the amount of organic matter and the presence of iron in soil (Lovley, 1991).

Agroforestry systems in secondary tropical forest constitute a traditional production model in family farming in the Northeast region of Pará state, Brazil (Schwartz et al., 2015). In agroforestry systems, trees may contribute to the soil ecosystems, providing nutrients and carbon substrates, facilitating the synthesis of organic matter by the addition of biomass to the soil. Besides that, interactions between soil and plant may regulate the relative contribution of organic inputs to nutrient release through mineralization processes and synthesis of soil organic matter. All these factors will have impact on nutrient and soil carbon stocks, and consequently on the methanogenic activities in this tropical ecosystem (Barrios et al., 2012).

Because of the substantial effects that the iron present in soils from flooded areas may have on the inhibition or suppression of methanogenesis and AOM, like rice paddy soil (Achtnich et al., 1995; Jäckel and Schnell, 2000; Jäckel et al., 2005; Zhou et al., 2014; Peng et al., 2015), we hypothesized that microbial ferric iron reduction is an important pathway for anaerobic organic matter decomposition in flooded-forest and -agroforestry in Amazonian clear water river floodplain. For this purpose, soil samples from these areas were incubated in anaerobic reactors under controlled conditions for methane production. The reactors were monitored for the concentration of methane gas in the headspace by gas chromatography, and total iron, Fe(III) and Fe(II) content in soil were determined by colorimetric assay using two different reagents, to evaluate if the occurrence of iron in soil is related to the decrease of methane emission. Besides that, the composition of the active methanogenic and methanotrophic microbial groups potentially involved in Fe(III)-dependent AOM in the soil used as inoculum in anaerobic reactors was revealed by high-throughput sequencing of 16S rRNA genes amplified using cDNA as template.

## 2. Material and methods

### 2.1. Study site

The study was conducted in a cocoa agroforestry site, located at clear water floodplain in the lower Tocantins basin, Baião municipality, Pará state, Brazil (2°40′51″S and 49°39′05″W) (Fig. S1). This basin has a drainage area of 767,000 km$^2$, representing 11% of the Brazilian territory, and is the second largest in Brazil. The climate is tropical with two seasonal periods: dry season from August to November, and a rainy season from December to July, with annual average rainfall of 2533 mm (ANA, 2009).

### 2.2. Soil samples collection

Underwater soil cores (0–30 cm soil layer and 10.4 cm diameter) were collected from three sampling points during the flooded period (May 2018) using a universal hand core sediment sampler (Aquatic Research Instruments, ID, USA) in three sampling points from one forest site and one cocoa agroforestry site both located at clear water floodplain. Cores were sliced into two layers from 0 to 15 cm and from 15 to 30 cm depth, and the soil slices were homogenized per depth and sampling sites to obtain four composite soil samples. Samples were transported to the laboratory under ice and stored at 4 °C until incubation in batch reactors. For molecular

analysis, a subsample containing 3 g of soil was collected from each of two soil layers, immediately preserved in LifeGuard Soil Preservation Solution (Quiagen, Hilden, Germany) and transported to the laboratory under ice, and stored at −80 °C until further processing within 72 h after sampling.

### 2.3. Anaerobic batch reactors

The experiment was carried out with the four composite soil samples from the Amazonian clear water floodplain. The anaerobic batch reactors were prepared in duplicate in 0.5 L Schott Duran® flasks, where 0.23 L was medium and 8% (w/v) was soil as inoculum (20 g). The medium (Zinder et al., 1984) was prepared with the following composition (g $L^{-1}$): $NH_4Cl$ 0.5, $KH_2PO_4$ 0.4, $MgCl_2 \cdot 6H_2O$ 0.1, and $CaCl_2 \cdot 2H_2O$ 0.05; 1.0 mL of 0.1% resazurin as redox potential indicator; 10 mL $L^{-1}$ trace metal solution was added consisting of (g $L^{-1}$): nitrilotriacetic acid 4.5, $FeSO_4 \cdot 7H_2O$ 0.556, $MnSO_4 \cdot H_2O$ 0.086, $CoCl_2 \cdot 6H_2O$ 0.17, $ZnSO_4 \cdot 7H_2O$ 0.21, $H_3BO_3 \cdot NiCl_2$ 0.19, and $Na_2MoO_4 \cdot 2H_2O$ 0.02. Vitamins solution (2.5 mL) was used: biotin and folic acid at 0.002 g $L^{-1}$; thiamine, riboflavin, nicotinic acid, lipoic acid, 4-aminobenzoic acid, and calcium pantothenate at 0.005 g $L^{-1}$; pyridoxine 0.01 g $L^{-1}$, and vitamin $B_{12}$ 0.0001 g $L^{-1}$. A volume of 2.5 mL $NaHCO_3$ at final concentration of 10% was used as a buffer to maintain pH 7.0; and 2.5 mL $Na_2S \cdot 9H_2O$ at final concentration of 5% was used as reducing agent. The reactors were subjected to a $N_2$ atmosphere (99.99%) for 20 min after distribution of the solutions, and capped with butyl rubber stoppers, wrapped and kept at 2 °C higher than air temperature measured in field (28.86 °C to forest and 27.93 °C to agroforestry).

The following conditions were used for each soil sample, subjected to depletion of organic matter, in batch reactors: water control (230 mL of distilled water), medium control (222.5 mL of medium, 2.5 mL of vitamin solution, 2.5 mL of $Na_2S \cdot 9H_2O$ and 2.5 mL of $NaHCO_3$); reactors plus organic sources (217.5 mL of medium, 2.5 mL of vitamin solution, 2.5 mL of $Na_2S \cdot 9H_2O$, 2.5 mL of $NaHCO_3$, and 5 mL of organic sources: 1 M of glucose, sodium formate or sodium acetate; final concentration of 20 mM). The batch reactors were kept under $N_2$:$CO_2$ gas mixture atmosphere (70:30%) and monitored for the concentration of methane gas in the headspace by gas chromatography (GC, 2014 Shimadzu, Kyoto, Japan) using a flame ionization detector. Nitrogen was used as the carrier gas at 400 kPa head pressure and the oven and detector temperatures were set isothermally at 150 °C and 400 °C, respectively. The chromatography device was daily calibrated with seven certificated standards: 0.96, 1.84, 3.58, 11.0, 24.0, 102.0 and 1030.0 ppm. After seven days of the methane production, soil sample from each batch reactor was collected for Fe(II) extraction and determination. Just the methane concentration determined at the end of the monitoring was used as data in this study.

### 2.4. Iron extraction from soil

Iron was extracted from wet soil samples (0.5 g) collected from each batch reactor, by adding 25 mL of 1 M HCl which has been bubbled with $N_2$ for 1 h, in closed 50 mL centrifuge tube at 70 °C in a water bath in the dark for 1 h, avoiding major air contact (Porsch and Kappler, 2011). The suspension was vacuum filtered using 0.45 μm pore size cellulose ester membrane filters (MF-Millipore™, Darmstadt, Germany) and maintained in the dark at 4 °C in penicillin type flasks closed with gas proof butyl stopper and analyzed until one month after extraction.

### 2.5. Iron analysis

Total iron ($Fe_{total}$) and Fe(II) analysis were made based on the literature, by colorimetric methodology using two different colorimetric reagents: 1,10-phenanthroline and ferrozine (Sigma-Aldrich®, Darmstadt, Germany) in order to verify possible concentration differences between them on the quantification. Fe(III) concentration was determined by the difference between $Fe_{total}$ and Fe(II).

The extraction solution was used to determine the concentration of Fe(II) and $Fe_{total}$ by UV−Vis absorption spectrometry (HTX Multi-Mode Microplate Reader, Synergy™) with 1,10-phenanthroline, using an adapted methodology based on Tarafder and Thakur (2013) and Freitas et al. (2015), by controlling the final pH of the reaction with NaOH. All the reactions were performed in three technical replicates.

The concentration of Fe(II) and $Fe_{total}$ by UV−Vis absorption spectrometry (HTX Multi-Mode Microplate Reader, Synergy™) with ferrozine was done by using the extraction solution, based on the methodology of Lovley and Phillips (1986a) and Schnell et al. (1998). All the reactions were performed in three technical replicates.

### 2.6. Total carbon, dissolved organic carbon and air temperature

The total carbon ($C_{total}$) was determined directly from soil samples collected in field by dry combustion using the CHNS/O elemental analyzer (PerkinElmer, Waltham, MA, USA). The determination was performed using 5−7 mg of dry soil sieved in 0.15 mm mesh.

Dissolved organic carbon (DOC) is the fraction of organic substances that passes through a filter ranging in size from 0.22 to 0.7 μm (Danielsson, 1982). To determine DOC, 1 g of each soil sample collected in field was stirring with 100 mL of ultrapure water at 142 rpm during 24 h. The supernatant was centrifuged at 6000×*g*, filtered on glass fiber membranes (mesh <0.7 μm) previously calcined at 500 °C, and analyzed for DOC content on TOC-5000A analyzer (Shimadzu, Columbia, MD, USA) with non-dispersive infra-red after purging with 2 M HCl.

The air temperature was measured using digital thermometer (Incoterm®, Porto Alegre, Brazil).

### 2.7. Soil RNA isolation, cDNA synthesis and 16S rRNA gene amplicon sequencing

Total RNA was isolated from the 12 soil slices before homogenization for composite samples preparation (2 soil layers × 3 sampling points × 2 sampling sites) by using RNeasy PowerSoil Total RNA kit (Qiagen, Hilden, Germany). The RNA concentration and purity were assessed spectrophotometrically (Nanodrop ND-1000, Nanodrop Technologies, Inc., Wilmington, DE, USA) to determine absorbance at the following wavelengths: 230, 260, 280, and 320 nm. The concentration of isolated RNA was between 100 and 200 ng $\mu L^{-1}$. The 260/280 ratio was around 2.0. Complementary DNA (cDNA) was synthesized from a single stranded RNA isolated from soil samples using a QuantiNova Reverse Transcription kit (Qiagen, Hilden, Germany) with integrated removal of genomic DNA contamination. Amplicon libraries were prepared according to the 16S Metagenomic Sequencing Library Preparation (https://support.illumina.com), using the cDNA as template and specific primers for the 16S rRNA gene of total archaeal and bacterial communities. The primers SD-Arch-0349-aS-17 (5′-GYGCASCAGKCGMGAAW-3′) and SD-Arch-0519-aA-16 (5′-TACCGCGGCKGCTG-3′) (Klindworth et al., 2013) were used to obtain amplicons of approximately 185 bp from V3 region of the 16S rRNA gene for *Archaea*. A fragment of approximately 390 bp of the V4 region of the 16S rRNA gene of *Bacteria* was amplified using

the primers 515FB (5′-GTGYCAGCMGCCGCGGTAA-3′) (Parada et al., 2016) and 806RB (5′-GGACTACNVGGGTWTCTAA-3′) (Apprill et al., 2015). Twelve 16S rRNA amplicon sequencing libraries were prepared using the Illumina Nextera sample preparation kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Libraries were quantified using Qubit dsDNA HS kit on a Qubit 2.0 fluorometer (Life technologies, Carlsbad, CA, USA) and KAPA SYBR FAST qPCR Master mix, Illumina standards and primer premix (KAPA Biosystems, Wilmington, MA, USA) according to the Illumina suggested protocol. The resulting libraries were denatured with 10 μL NaOH, diluted to 8 pM in Illumina's HT1 buffer, and spiked with 1% PhiX (Illumina, San Diego, CA, USA). Equal concentration of libraries was used for the MiSeq Reagent v2 sequencing reagent kit (Illumina, San Diego, CA, USA). The equipment used for sequencing of the amplicons obtained for the *Archaea* and *Bacteria* 16S rRNA genes was a *MiSeq Personal Sequencing System Illumina* (Illumina, San Diego, CA, USA) operated in Rapid Run Mode to generate 2 × 250 bp paired-end reads.

### 2.8. Data preprocessing and taxonomic classification of 16S rRNA amplicon sequence

Each of the 12 libraries of archaeal and bacterial 16S rRNA genes generated about 10 and 20 megabytes in size, respectively. Filtered merged paired reads after chimera removal accounted for 2500 and 20,000 for *Archaea* and *Bacteria*, respectively. All 16S rRNA gene sequence reads were processed and analyzed using QIIME v.1.9.1 software (Caporaso et al., 2010a). Briefly, fastq files with forward and reverse reads were merged using UPARSE algorithm (Edgar, 2013). Sequences that did not merged with this algorithm were merged using VSEARCH v.2.10.4, defining 2 bp as the minimum length for overlap. The merged reads were further preprocessed by (*i*) trimming of bad quality reads, (*ii*) removal of artificial sequences such as primers and adapters, (*iii*) disposing short length reads lesser than 100 bp (*Archaea*) and 200 pb (*Bacteria*), and (*iv*) removal of ambiguous sequences. Then, USEARCH (Edgar, 2010) was employed to remove chimeras in the preprocessed reads. After chimera removal, the preprocessed reads were aligned using PyNAST (Caporaso et al., 2010b) with SILVA database (https://www.arb-silva.de) and sorted with >97% similarity into operational taxonomic units (OTUs) using closed reference OTU picking approach.

### 2.9. Data analysis of 16S rRNA amplicon sequence

In order to reveal the active methanogenic and methanotrophic microbial groups potentially involved in Fe(III)-dependent AOM inhabiting the soil used as inoculum in the anaerobic batch reactors, the number of 16S amplicon sequences in each library was normalized using the OTU table (2500 sequences for *Archaea* and 20,000 sequences for *Bacteria*) and the rarefaction method in QIIME v.1.9.1. The OTU tables obtained for the 16S rRNA gene sequences of *Archaea* and *Bacteria* were used separately to filter the methanogenic and methanotrophic microbial groups potentially involved in Fe(III)-dependent AOM using the 'filter_taxa_from_OTU_table.py' command in QIIME v.1.9.1. The sequences were recovered from the filtered OTU table using the Pear v.0.9.11 package. Relative abundances of active methanogenic and methanotrophic groups were estimated by dividing the number of sequences classified as the methanogenic and methanotrophic groups by the total number of sequences classified as methanogens and methanotrophs per sample, respectively.

### 2.10. Statistical analysis

*Post hoc* analysis using Tukey's HSD test with a significance level of 0.05 was used to determine the meaningfulness of the differences between the two soil layers (0–15 cm and 15–30 cm depths) for methane concentration in the reactor headspace and Fe(II) concentration in the incubated soil within forest and agroforestry sites. The same statistical test was used to determine separately the significance of the differences between the two soil layers and sampling sites for total carbon and DOC in the soil used as inoculum based on samples collected in field and air temperature in the moment of the soil sampling. Spearman's rank correlation coefficients were calculated followed by Bonferroni correction to explore the relationship between the methane concentration in the reactor headspace and Fe(II) concentration in the incubated soil by using 'multtest' package in R version 3.3.3 (R Core Team, 2017).

## 3. Results and discussion

The definition of biogeochemical drivers of the methane production in natural and anthropic flood ecosystems is important in order to predict methane emissions from these environments to the atmosphere. Previous studies showed that methanogenesis, and consequently the methane emission, can be inhibited or suppressed by the addition of Fe(III) in soil under anoxic conditions, like rice paddy soil (Achtnich et al., 1995; Jäckel and Schnell, 2000; Jäckel et al., 2005; Zhou et al., 2014; Peng et al., 2015).

Based on these facts, we analyzed the potential of methane production by soil from Amazonian clear water floodplain incubated in anaerobic batch reactors. Anaerobic oxidation of methane (AOM) coupled to the reduction of iron oxides is the key process explaining the high concentrations of dissolved Fe(II) at depth in the flooded soil (Sivan et al., 2016). The methane concentration in the reactor headspace was compared with Fe(II) concentration in the incubated soil (Figs. 1 and 2, Table S1), and Spearman's rank correlation revealed positive correlation between methane and Fe(II) (Table 1).

Methane production and Fe(II) concentration was higher at 0–15 cm than 15–30 cm soil layer in both forest and agroforestry sites (Figs. 1 and 2, Table 1 – Tukey's HSD test, and Tables S1 and S2). Frenzel et al. (1999) and Riedinger et al. (2014) also showed higher methane production and Fe(II) concentration at superficial than deeper layers in soil. Besides that, it is believed that Fe(III) is not available in deeper soil layers to microbial reduction, being restricted to the superficial sediments, typical characteristic in freshwater environments (Lovley and Phillips, 1986b). In this study, acetate, formate and glucose were used as organic sources in the anaerobic reactors, with higher accumulation of Fe(II) in incubated surface soils (Table S1). In anaerobic rice paddy soils, acetate is a major fermentation product (Yoshida, 1975), and the disappearance of added acetate and the production of $^{14}CO_2$ from [2–$^{14}$C] acetate are associated with the accumulation of Fe(II) (Kamura et al., 1963).

Total carbon and iron content in soil are important factors to drive the methanogenesis process in flooded soils (Lovley, 1991). Allochthonous organic matter input occurs in predominance of respiration relative to photosynthesis (Gagne-Maynard et al., 2017; Martins and Probst, 1991). In the rainy season, the allochthonous dissolved organic matter sourced from the terrestrial ecosystem is the origin from the carbon in the aquatic systems by surface and groundwater (Aitkenhead-Peterson et al., 2002).

The carbon cycling and stock in soil, and the dissolved organic carbon (DOC) concentrations, are controlled by a number of biotic and abiotic factors. The biotic factors of the DOC concentrations are related with microorganisms and vegetation cover, whereas abiotic

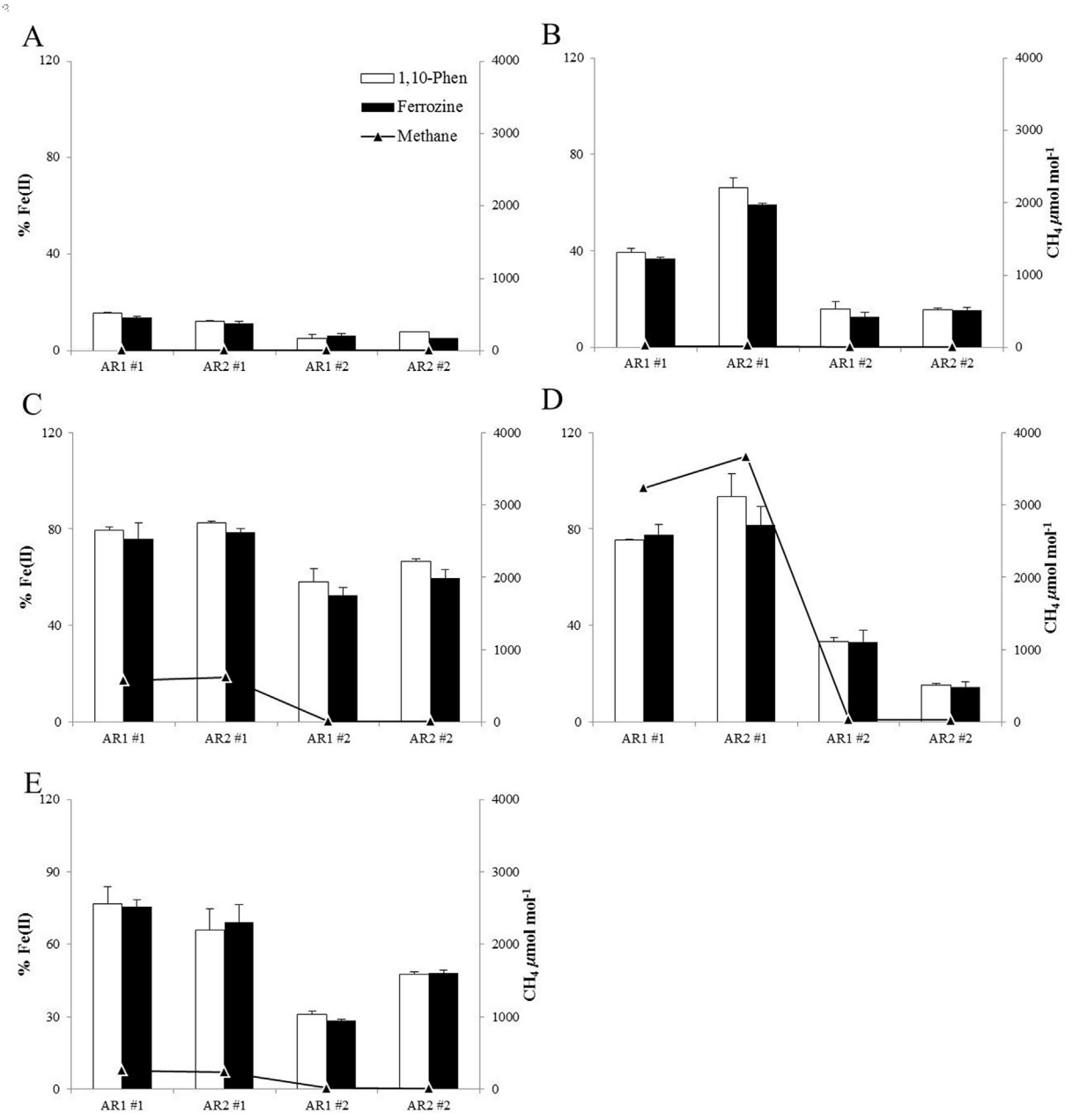


**Fig. 1.** Fe(II) and methane concentrations from incubated forest-soil samples: (A) water control, (B) medium control, (C) medium + acetate, (D) medium + formate, and (E) medium + glucose. On the X axis: AR = anaerobic reactor; #1 = soil layer 1 (0–15 cm); #2 = soil layer 2 (15–30 cm). 1,10-Phen = 1,10-Phenanthroline. Methane concentration was determined at the end of the monitoring, and iron analysis was conducted until a month after the extraction.

variables are soil texture, pH, rainfall seasonality, relief (Marques et al., 2012), and concentrations of iron and aluminum in the soil (Neu et al., 2017). In this study, total carbon percentage and DOC concentration were significantly higher at 0–15 cm than 15–30 cm soil layer ($P < 0.05$) in both forest and agroforestry sites (Table 2). The DOC concentrations are generally high in surface soils, as a result of initial enrichment of through fall and leaching of decomposing organic matter; soil solution concentrations typically decrease with soil depth as a result of mineral sorption, organic carbon remineralization (McClain et al., 1997) and Fe(III) concentration (Neu et al., 2016). Decreases in DOC concentration in depth is an indicative of the effective adsorption and complexation processes between carbon and Fe(III) (Lovley, 1991).

The Fe(III) percentage was significantly lower at 0–15 cm than 15–30 cm soil layer ($P < 0.05$) in forest and agroforestry sites (Table 2, Figs. S2 and S3, Table S2). Higher concentrations of iron and acidic pH with organic matter availability form stable precipitates (McDowell and Wood, 1984; McKnight et al., 1992; Moore et al., 1992; Nelson et al., 1993; Kalbitz et al., 2000) and high affinity (Gu et al., 1994). These reactions are responsible for removing the organic substances from the soil solution, resulting in low carbon availability (Neu et al., 2017). Taken together, the results point out to the microbial ferric iron reduction as an important potential pathway for anaerobic organic matter decomposition at 0–15 cm soil layer.

The concentration of Fe(III) in sediments frequently exceeds that

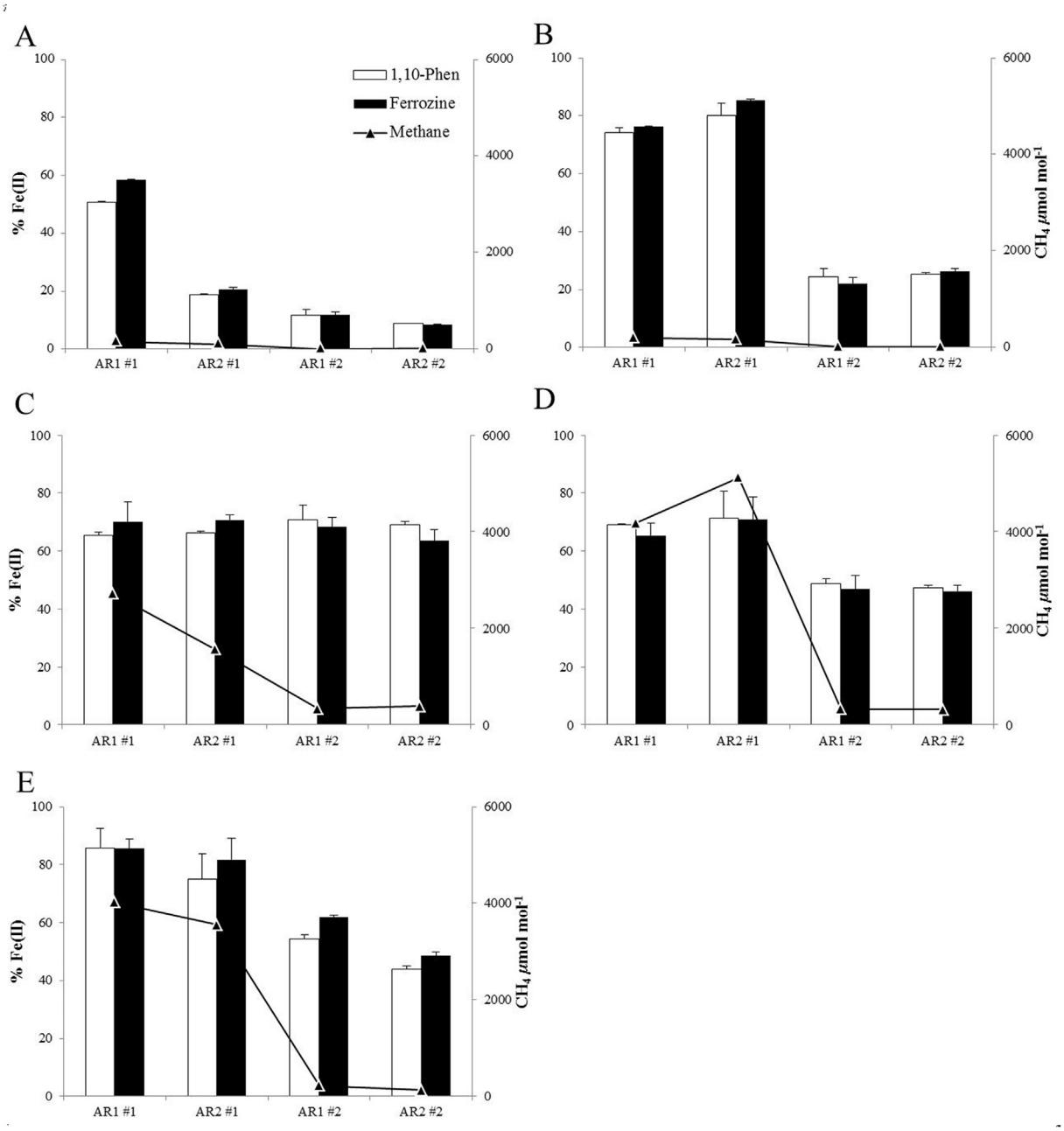


**Fig. 2.** Fe(II) and methane concentrations from incubated agroforestry-soil samples: (A) water control, (B) medium control, (C) medium + acetate, (D) medium + formate, and (E) medium + glucose. On the X axis: AR = anaerobic reactor; #1 = soil layer 1 (0–15 cm); #2 = soil layer 2 (15–30 cm). 1,10-Phen = 1,10-Phenanthroline. Methane concentration was determined at the end of the monitoring, and iron analysis was conducted until a month after the extraction.

of other electron acceptors such as oxygen, nitrate, and sulfate, and thus there is the potential for significant nutrient release from organic matter mineralization with Fe(III) as the electron acceptor (Lovley and Phillips, 1986a).

Despite the most microorganisms that are able to couple the oxidation of methane to the reduction of environmentally relevant oxidized metals species is still far from known (Ettwig et al., 2016), the accumulation of Fe(II) can be an evidence of the oxidation of Fe(III) by methanogenic and methanotrophic microorganisms living in anaerobic condition.

Although *in situ* temperature is commonly lower than the optimum temperature for methanogenesis in flooded soils (Zeikus and Winfrey, 1976), different temperatures may select different iron-reducing microorganisms (Aromokeye et al., 2018). This shows that, in addition to the chemical factors discussed above, temperature is also an important factor able to affect the iron-mediated AOM in the studied environment.

Methanogens and methanotrophs microorganisms are able to oxidize methane anaerobically dependent of Fe(III) reduction. This is associated with the reduction of methane emission in the presence of Fe(III) (Bond and Lovley, 2002; van Bodegom et al., 2004; Peng et al., 2015; Ettwig et al., 2016; Mohanty et al., 2017; Yan et al., 2018), once iron is an important electron acceptor in AOM by *Bacteria* and *Archaea* inhabiting tropical soil (Mohanty et al., 2017). In the incubated soil were detected active methanogenic and methanotrophic microbial groups potentially involved in Fe(III)-

**Table 1**
Statistical analysis. Tukey's HSD test and Spearman rank correlation for methane ($CH_4$) concentration in the reactor headspace and Fe(II) concentration in the incubated forest- and agroforestry-soil samples.

| Tukey's HSD test | | | | | | |
|---|---|---|---|---|---|---|
| | Forest | | | Agroforestry | | |
| | 1,10-Phen | Ferrozine | $CH_4$ | 1,10-Phen | Ferrozine | $CH_4$ |
| WC | **ns** | * | **ns** | **ns** | **ns** | **ns** |
| MC | **ns** | **ns** | ** | ** | * | * |
| MA | **ns** | **ns** | ** | * | **ns** | **ns** |
| MF | * | * | ** | ** | * | * |
| MG | **ns** | **ns** | ** | **ns** | **ns** | ** |

| Spearman's rank correlation | | | | |
|---|---|---|---|---|
| | 1,10-Phen *vs.* $CH_4$ | | Ferrozine *vs.* $CH_4$ | |
| | Estimate | P. value | Estimate | P. value |
| WC | 0.833 | * | 0.833 | * |
| MC | 0.953 | ** | 0.976 | *** |
| MA | 0.119 | **ns** | 0.762 | * |
| MF | 0.810 | * | 0.810 | * |
| MG | 0.905 | ** | 0.976 | *** |

1,10-Phen = 1,10-Phenanthroline, WC = water control, MC = medium control, MA = medium + acetate, MF = medium + formate, MG = medium + glucose.
Tukey's HSD test was performed to compare separately Fe(II) concentration in the incubated soil and methane ($CH_4$) concentration on the headspace (for both anaerobic reactors) between the two soil layers for forest and agroforestry. *$P < 0.05$; **$P < 0.005$; ns, not significantly different ($P > 0.05$).
Levels for the Spearman's rank coefficients are indicated at the * $P < 0.05$; **$P < 0.005$; ***P < 0.0005; ns, not significantly different ($P > 0.05$ level).

**Table 2**
Concentration of Fe(III) (average percentage of water control anaerobic reactor) in the incubated soil, total carbon, dissolved organic carbon in the samples collected in field, and air temperature (°C) in forest and agroforestry field.

| | Forest | | Agroforestry | |
|---|---|---|---|---|
| | Soil layer 1 | Soil layer 2 | Soil layer 1 | Soil layer 2 |
| **Fe(III) (%)** | 86.63bA ± 1.80 | 93.80 aA ± 1.26 | 62.91bB ± 20.17 | 89.76 aA ± 1.81 |
| **$C_{total}$ (%)** | 1.49 aA ± 0.70 | 0.42 aA ± 0.03 | 1.15 aA ± 0.85 | 0.73 aA ± 0.32 |
| **DOC (μM)** | 602.5 aA ± 155.8 | 335.8bB ± 40.8 | 322.5 aB ± 64.17 | 358.33 aB ± 119.17 |
| **Air temperature** | 26.86 aA ± 0.29 | | 25.93bA ± 0.06 | |

Soil layer 1 = 0–15 cm, Soil layer 2 = 15–30 cm, $C_{total}$ = total carbon, DOC = dissolved organic carbon. Values with the same lower-case letters did not reveal significant differences ($P < 0.05$) between the two soil layers. Values with the same upper-case letters did not reveal significant differences ($P < 0.05$) between forest and agroforestry.

dependent AOM belonging to the *Bacteria* domain: *Methylococcus* and *Desulfobulbus*, and *Archaea* domain: *Methanobacterium, Methanosarcina*, *Methanosphaera* and '*Candidatus Methanoperedens nitroreducens*' (Table 3).

Among these methanogenic microbial groups, *Methanobacterium* was predominant in soil layer at 0–15 cm from both forest and agroforestry sites. *Desulfobulbus*, a methanotrophic microbial group, also revealed higher abundance in the soil layer at 0–15 cm from both sampling sites compared to the soil layer at 15–30 cm. In turn, *Methyloccocus* showed the same abundance pattern in forest soil revealed for *Desulfobulbus*. High abundance was accounted for '*Candidatus methanoperedens nitroreducens*' in both sampling sites and soil layers investigated. Ettwig et al. (2016) presented the *Archaea Methanosarcinales* in a relationship with '*Candidatus Methanoperedens nitroreducens*' that couples the reduction of environmentally relevant particulate forms of iron to the oxidation of methane, filling one of the remaining gaps in the AOM. Both microbial groups present predominance over other microbial groups identified in this study. The results show couple higher concentration of Fe(III) to the predominance of '*Candidatus methanoperedens nitroreducens*' and *Methanosarcina* in the soil layer at 15–30 cm in forest and agroforestry sites (Tables 2 and 3).

Inhibition of methane production under Fe(III) reduction, and consequently the increase of Fe(II) concentration in soil, may occurs

**Table 3**
Relative abundance (%) of active methanogenic and methanotrophic microbial groups potentially involved in Fe(III)-dependent AOM detected in the soil samples used as inoculum in the anaerobic reactors.

| Microbial Groups | Forest | | Agroforestry | |
|---|---|---|---|---|
| | Soil layer 1 | Soil layer 2 | Soil layer 1 | Soil layer 2 |
| **Methanogenic** | | | | |
| *Methanobacterium* | 75.4 ± 18.82 | – | 78.0 ± 24.00 | 10.61 ± 6.74 |
| *Methanosarcina* | 24.6 ± 6.25 | 100.0 ± 1.15 | 22.0 ± 8.62 | – |
| *Methanosphaera* | – | – | – | 89.4 ± 139.15 |
| **Methanotrophic** | | | | |
| *Methylococcus* | 21.1 ± 4.00 | – | 1.2 ± 0.58 | 1.4 ± 0.58 |
| *Desulfobulbus* | 15.8 ± 3.00 | – | 19.3 ± 6.11 | 2.1 ± 1.00 |
| *'Candidatus Methanoperedens nitroreducens'* | 63.2 ± 8.89 | 100.0 ± 57.35 | 79.5 ± 19.29 | 96.5 ± 45.06 |

Soil layer 1 = 0–15 cm, Soil layer 2 = 15–30 cm.

due to the competition for common substrates, such as acetate and hydrogen, and electron donating between Fe(III)-reducing microorganisms and those methanogens. Fe(III) reducers are capable of use these substrates in concentrations much below that one metabolized by methanogens, maintaining their concentration in low levels, disadvantaging methanogenesis (Lovley and Phillips, 1986a, 1987; Lovley, 1987; Achtnich et al., 1995; Roden and Wetzel, 1996, 2003; Frenzel et al., 1999; Jäckel and Schnell, 2000; Bond and Lovley, 2002; Furukawa and Inubushi, 2002; Lovley et al., 2004; van Bodegom et al., 2004; Jäckel et al., 2005; Küsel et al., 2008; Teh et al., 2008; Huang et al., 2009; Amos et al., 2012; Zhou et al., 2014; Egger et al., 2015). Active microorganisms detected in the incubated soil in this study, such as *Methanobacterium*, *Methanosarcina,* and *Methanosphaera* may be using Fe(III) as an electron acceptor as preference for methanogenesis. Besides methane, studies show that the concentration of extractable nitrate ($NO_3^-$) decreased in soil under water, leading to the increase of Fe(II) concentration (Hall et al., 2013).

The increase of $CO_2$ and, in part, the accumulation of dissolved Fe(II) as a response to the addition of Fe(III), suggests that the active soil microbial community revealed in this study is able to perform the AOM with iron reduction, as also observed by Egger et al. (2015). In addition, the presence of Fe(III) oxides in anoxic environments may allow the community of Fe(III)-reducing microorganisms to predominate in comparison to those methanogenic (Frenzel et al., 1999).

It is known that the climate change related to the increase in the frequency and intensity of rainfall events, that is, soil moisture, is capable of affect the redox reactions that may control the production and emission greenhouse gas fluxes (Hall et al., 2013). Besides that, DOC concentration and electron acceptors concentration controls the redox processes (reduction-oxidation) by electron transfer by microorganisms, changing the emissions of greenhouse gas. Biogeochemical processes of greenhouse gas emission require the understanding of diverse factors that are integrated, since microbial composition to soil chemistry (Li, 2007).

Finally, we can consider that all the reduction reactions in the submerged soil are related with the decomposition of soil organic matter. It can be associated with Fe(II) formation when the soil is rich in iron oxide biologically active (Inubushi et al., 1984). Previous studies calculated that 1 mol of methane production can be reduced with the presence of 4 mol of Fe(III). In this sense methane production, and consequently its emission, can decrease in soil with high amounts of iron (Inubushi et al., 1984; Furukawa and Inubushi, 2002), since less methane production is related with high Fe(III) concentration, suggesting that microbial Fe(III) reduction can be a dominant pathway of the anaerobic carbon metabolism on soil (Roden and Wetzel, 1996). The Brazilian Amazonia soil is almost 50% ferralsols with pedogenic development, which indicates the high presence of iron oxide (Quesada et al., 2011), which may contribute to a lower methane emission compared to another flooded regions around the world, as rice paddy soil, for example.

While flooded areas worldwide contributes significantly with methane emission, and the addition of ferric iron in the soil can be used to the suppression of this greenhouse gas (Achtnich et al., 1995; Frenzel et al., 1999; Jäckel and Schnell, 2000; Jäckel et al., 2005; Teh et al., 2008; Huang et al., 2009; Zhou et al., 2014), the Amazonian floodplain emits less methane due to the high amount of iron present naturally in this soil, a characteristic that is not always taken into account with regard to methane emissions by the Amazon region.

## 4. Conclusions

Taken together, our findings show methanogenesis suppression by Fe(III) reduction in Amazonian flooded-forest and -agroforestry clear water river floodplain. The accumulation of Fe(II) in the incubated soil and the microbial community analysis evidence the reduction of Fe(III) potentially by *Methanobacterium*, *Desulfobulbus* and '*Candidatus methanoperedens nitroreducens*' living in anaerobic condition at the 0–15 cm soil layer. The high concentration of iron in the Amazonian soil may have a long-term effect on the decrease of methane emissions once the reduced iron is reoxidized continuously in the oxic/anoxic transition zones and in the soil surface after flooding. Therefore, our findings evidence that Fe(III) reduction in the studied soil naturally suppresses methanogenesis and consequently decrease methane emissions from these periodically flooded areas in Amazon. Based on that, we accepted the hypothesis that microbial ferric iron reduction is an important pathway for anaerobic organic matter decomposition in flooded-forest and -agroforestry in Amazonian clear water river floodplain. Also, the naturally predominance of a soil with high content of iron in Amazonia floodplain shows us the importance of ferric iron reduction and its relation with microorganism's metabolism in the decrease of methane emission to the atmosphere, an important greenhouse gas.

## Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Declaration of competing interest

None.

## CRediT authorship contribution statement

**Gabriele V.M. Gabriel:** Validation, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Luciana C. Oliveira:** Conceptualization, Writing - review & editing, Supervision, Project administration. **Dayane J. Barros:** Investigation. **Marília S. Bento:** Formal analysis, Investigation. **Vania Neu:** Conceptualization, Writing - review & editing. **Rogério H. Toppa:** Visualization. **Janaina B. Carmo:** Resources. **Acacio A. Navarrete:** Conceptualization, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.126263.

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