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Impact of Ag_2S NPs on soil bacterial community – A terrestrial mesocosm approach

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ABSTRACT

Soils might be a final sink for Ag_2S nanoparticles (NPs). Still, there are limited data on their effects on soil bacterial communities (SBC). To bridge this gap, we investigated the effects of Ag_2S NPs (10 mg kg^{-1} soil) on the structure and function of SBC in a terrestrial indoor mesocosm, using a multi-species design. During 28 days of exposure, the SBC function-related parameters were analysed in terms of enzymatic activity, community level physiological profile, culture of functional bacterial groups [phosphorous-solubilizing bacteria (P-SB) and heterotrophic bacteria (HB)], and SBC structure was analysed by 16S rRNA gene-targeted denaturing gradient gel electrophoresis.

The SBC exposed to Ag_2S NPs showed a significative decrease of functional parameters, such as β-glucosidase activity and L-arginine consumption, and increase of the acid phosphatase activity. At the structural level, significantly lower richness and diversity were detected, but at later exposure times compared to the $AgNO_3$ treatment, likely because of a low dissolution rate of Ag_2S NPs. In fact, stronger effects were observed in soils spiked with $AgNO_3$, in both functional and structural parameters. Changes in SBC structure seem to negatively correlate with parameters related to phosphorous (acid phosphatase activity) and carbon cycling (abundance of HB, P-SB, and β-glucosidase activity). Our results indicate a significant effect of Ag_2S NPs on SBC, specifically on parameters related to carbon and phosphorous cycling, at doses as low as 10 mg kg^{-1} soil. These effects were only observed after 28 days, highlighting the importance of long-term exposure experiments for slowly dissolving NPs.

1. Introduction

Silver nanoparticles (AgNPs) are broadly used in many applications and products, due to their anti-microbial, anti-inflammatory, and anti-cancer properties (McGillicuddy et al., 2017). The global production of AgNP-based products has been estimated at 450 tons per year (McGillicuddy et al., 2017), whereas the market of AgNP-based antimicrobials has been predicted to grow from 0.79 billion dollars in 2014–2.54 billion dollars in 2022 (Pachapur et al., 2016). The increasing application and consumption of AgNPs-containing products results in AgNPs release into wastewater treatment plants (WWTPs) (European Commission Science for Environment Policy, 2017). In these plants, it has been shown that most of the AgNPs are captured and settled in the sludge (Kaegi et al., 2011), where the estimated AgNPs

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concentration ranges between 1 and 6 mg kg^{-1} (Gottschalk et al., 2009). During wastewater treatment, the majority of AgNPs (79% as estimated by Wang et al., 2016) are transformed to Ag sulfide NPs (Ag_2S NPs), which are then incorporated into biosolids. Additionally, this sulfidation process also was suggested to occur for silver in ionic form (Ag^+) as a means to reduce silver toxicity (Kent et al., 2014).

In many countries, these biosolids are amended to agricultural soils to improve soil fertility (Doolette et al., 2016). In the United Kingdom for example, of the 1.05 million tons of dry biosolids produced per year, 85% on average are applied on land (European Commission Science for Environment Policy, 2017). Agricultural soils are often considered a sink for Ag_2S NPs, characterized by a low dissolution rate of these Ag_2S in biosolids and most likely also in soils. For instance, Wang et al. (2016) demonstrated that the $\geq$ 87% of Ag_2S NPs from biosolids remained in this form at least up to 400 days after soil spiking. The dissolution rate of Ag_2S NPs depends on the degree of sulfidation (Reinsch et al., 2012) and a higher degree of sulfidation of silver results in lower dissolution and a lower toxic effect on soil organisms (Starnes et al., 2015; Wang et al., 2017) and microorganisms (Reinsch et al., 2012; Doolette et al., 2015). Indeed, the toxicity of AgNPs strongly depends on its speciation (Doolette et al., 2015), and insoluble Ag_2S NPs ($K_{sp} = 8 \times 10^{-51}$) are less toxic and less bioavailable compared to non-sulfidated AgNPs (Doolette et al., 2015).

Only a limited number of studies have thus far explored the toxic effects of Ag_2S NPs on terrestrial organisms. Wang et al. (2017) showed that Ag_2S NPs were bioavailable to some extent and caused toxic effects in *planta*. A substantial increase of Ag accumulation on leaves (3.8–5.8 μg Ag g^{-1} dry mass) was demonstrated when wheat (*Cucumis sativus*) and cucumber (*Triticum aestivum* L.) plants were exposed to 10 mg (Ag) L^{-1} as Ag_2S NPs for one week (Wang et al., 2017). However, Ag_2S NPs fate and toxicity to plants are still a matter of debate since different studies have used different plant species and different silver concentrations (Wang et al., 2017), obtaining conflicting results. A study by Starnes and their collaborators suggests that the exposure to Ag_2S NPs (at 10 mg Ag L^{-1}) resulted in a 20% increase in mortality rate of *Caenorhabditis elegans* (Starnes et al., 2015). However, this observed toxic effect is mainly due to the solubilization of Ag^+ (released from Ag_2S particles) in soils.

Although a few studies have investigated the stability (Reinsch et al., 2012), toxicity, fate (Schlich et al., 2018), and (bio)availability (Wang et al., 2016) of Ag_2S NPs in soils, the impact on soil bacterial communities (SBC) and microbiological processes is still unknown. SBC play a vital role in soil ecological functions and have been considered a sensitive target for assessing the impact of manufactured nanomaterials on terrestrial compartments (Doolette et al., 2016). Some microbial endpoints have been used to infer on these effects such as soil enzyme activities (Samarajeewa et al., 2017), biological nitrification, microbial respiration rates and organic matter decomposition (Samarajeewa et al., 2017), community level physiological profiling (Samarajeewa et al., 2017) and microbiome structure and diversity (Doolette et al., 2016). Currently, the effects of Ag_2S NPs on SBC are difficult to assess, since the few reported studies used different NP concentrations, exposure times, soil types, and were conducted at different scales (e.g., microcosms, mesocosms). Even so, most studies showed a stronger impact of the ionic silver when compared to AgNPs and Ag_2S NPs (Doolette et al., 2016). Although Ag_2S NPs were the least toxic form, impacts on the structure (Judy et al., 2015) and composition (Doolette et al., 2016) of SBC were confirmed. In terms of function-related parameters, a decrease in the abundance of the genes *nirK*, encoding nitrite reductase, and *amoA*, encoding ammonia oxidizing bacteria, was demonstrated as well as a reduction of the nitrification rate (Doolette et al., 2016). Studies with single microbial species also reported toxic effects, e.g. on *Escherichia coli* (*E. coli*) (Reinsch et al., 2012) and *Arthrobacter globiformis* (Schultz et al., 2018).

In a terrestrial ecosystem, the presence of different organisms might change the bioavailability of contaminants in soils, in some localized spots, as well as their toxicity to soil microorganisms. In fact, changes in oxygenation levels and soil pH might occur due to the organism's behaviour (burrowing, ingestion and egestion, among other activities) (El-Wakeil, 2015), promoting changes in microbial communities' abundance and structure. Thus, microbial responses to the contaminants can be altered and pose a risk for the terrestrial ecosystem functions.

Our study aims to investigate the effects of Ag_2S NPs on SBC at structural (Denaturing Gradient Gel Electrophoresis - DGGE) and functional (culture of functional bacterial groups, soil enzymatic activity, and Community Level Physiological Profiling - CLPP) levels, using a more realistic exposure scenario than has been reported previously, in order to obtain relevant information at the ecosystem level. Thus, indoor terrestrial mesocosms experiments were performed in which a 1000x predicted environmental concentration estimated for 2050 (10 μg kg^{-1} - sludge treated soils) (Giese et al., 2018) was tested in the presence of biota, simulating a worst-case scenario. Besides SBC, wheat plants (*Triticum aestivum* L.) and terrestrial organisms, such as isopods (*Porcellio scaber*), mealworms (*Tenebrio molitor*), and earthworms (*Lumbricus rubellus*) were also included in the mesocosms to simulate a real edaphic scenario. The impact of Ag_2S NPs on SBC was evaluated as a function of time (0, 14 and 28 days) and the impact was compared with that of the ionic Ag form ($AgNO_3$) and an unexposed control.

2. Material and methods

2.1. Silver exposure

The study was conducted with well defined sulfidized silver nanoparticles colloids (Ag_2S NPs) and $AgNO_3$ (Sigma-Aldrich, 99% purity, CAS 7761–88–8, Germany). $AgNO_3$ was used as a Ag control for comparing the toxicity of the NPs with the toxicity of the ionic Ag form. The polyvinylpyrrolidone (PVP) coated Ag_2S NPs colloids were synthesized and characterized by Applied Nanoparticles (Barcelona, Spain). These particles were used in this work as a model of sulphidized Ag_2S NPs, in which no degree of sulfidation was demonstrated. These particles were supplied in suspension with a stock concentration of 1320 ± 48 mg L^{-1}. These particles presented an average diameter of 20.4 ± 11.9 nm [mean (n = 3) ± standard deviation] (Fig. S1), a ζ-potential of − 23.8 ± 4.5 mV in miliQ water (16.12 mg Ag_2S NPs mL^{-1}, conductivity 0.174 mS cm^{-1}, pH 8.72). Additionally, Ag_2S NPs colloids, measured by ICP-MS (fully described in Supplemental materials S1), showed high stability in ultrapure water over-time, and a very low proportion of dissolved Ag during the 48 h (<0.1%, Table S1).

The Lufa 2.2 soil (LUFA-Speyer 2.2, LUFA Speyer, Speyer, Germany) was used as a natural and standard soil, because this soil is suggested as a reference soil for testing NPs toxicity (Geitner et al., 2020). This soil was spiked with Ag_2S NPs or $AgNO_3$ to a final concentration of 10 mg Ag kg^{-1} soil. For this, an Ag_2S NPs stock suspension and an $AgNO_3$ stock solution were prepared, using deionized water, with a final concentration of 1150 mg Ag L^{-1} and 1148 mg Ag L^{-1}, respectively. These stock solutions were added to the soil, to maximize homogeneity by mixing thoroughly, and the soil was brought to 55% of the water holding capacity (WHC) using de-ionised water. Spiked and watered soils were mixed manually which has been shown to result in a homogeneous exposure throughout replicates. Once prepared, soils were transferred to each replicate (column) and incubated for two days at 20 °C± 2, to achieve a chemical equilibrium stage of the soil. This is a common procedure for equilibrium in soil for metal exposure in ecotoxicological tests and it has also been tested for nanomaterials (see Neves et al., 2019). Indeed, silver transportation, speciation, and availability might change in soils due to silver exchange between the solid (soil) and liquid phase (pore water). Each mesocosm column was watered daily with artificial rainwater (16 mL per day; NaCl (0.01 mM), $(NH_4)_4 SO_4 \cdot H_2O$ (0.0053 mM), $NaNO_3$ (0.0059 mM), and $CaCl_2 \cdot H_2O$ (0.0039 mM); pH= 5.1) dripping/distributing the water volume over the whole corer surface by a syringe avoiding the edges. The soil pH was measured at

sampling times (day 0, 14 and 28); five grams of soil were shaken with 25 mL of a 0.01 M $CaCl_2$ solution, and pH measured after a 2 h settling period. Soil pH did not change with Ag_2S NPs or $AgNO_3$ exposure in time (Table S2).

2.2. Mesocosm setup

The terrestrial mesocosm was established in indoor conditions [(temperature 20 ± 2 °C and photoperiod 16 h (light):8 h (dark)] and three soil treatments were used: non-exposed soil, as the negative control (CT), soil spiked with Ag_2S NPs and soil spiked with $AgNO_3$ (as the ionic control, Ag^+). For each treatment, 10 replicates were made. Each mesocosm replicate (column = 20 cm long and 11 cm diameter) was filled with 2.6 kg of Lufa 2.2 soils: 1.3 kg (wet soil) of control or spiked soil (with Ag_2S NPs or $AgNO_3$) in the upper layer (1–8 cm), and 1.3 kg of non-treated soil in the bottom layer (8–16 cm). Total soil height for the two layers was 16 cm.

The bottom of the corer was closed with a PVP nylon mesh (1 mm) and a funnel was placed underneath with a 50 mL tube to collect leachate. To the upper layer of each replicate ten isopods (*Porcellio scaber*), ten mealworms (*Tenebrio molitor*), six earthworms (*Lumbricus rubellus*) and four plants (*Triticum aestivum* L.) were included. A destructive sampling approach was used at each sampling time. At day 0 (after 2 days of spiking equilibrium), 14 and 28 (after organisms were introduced), several replicates were removed from the design (three, three and four, respectively at each sampling time and from each treatment) and the 0–4 cm, 4–6 cm, 10–14 cm, and 14–16 cm mesocosm layers were destructively sampled for all invertebrates, plants, soil chemical analysis and SBC (Fig. 1).

During the experiment, organisms' mortality (isopods, mealworms, and earthworms) was recorded and was reported to be similar among treatments (data not shown).

2.3. Soil chemical analysis

The total soil Ag concentration was determined using an *aqua regia* digestion (3:1 HCl:HNO_3) using a microwave (Milestone) (US-EPA, 1996). Digests were diluted to an acid concentration of ca. 3% and measured using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (Perkin Elmer Nexion 350 D). Calibration procedures used known Ag concentrations matrix-matched in 3% HCl solutions, and only the correlation coefficient greater than 0.999 was considered. ^{103}Rh was applied in all samples as internal standard quality control. The ICP-MS instrument detection limit for Ag was 0.028 μg l^{-1} (mean blank + 3σ reagent blank, n = 22) and the instrument method had a precision of 0.28% (n = 22).

To analyse Ag concentration (total and soluble) in soil pore water, the procedure was adapted from Lahive et al. (2017). After sampling, the soil was moistened (to 100% of its water holding capacity) to achieve an extractable aqueous sample volume, and incubated overnight at 20 °C. The soils (35 g) were then transferred to inserts with 0.02 g glass wool and 0.45 μm PVDF filters, previously soaked with a Cu solution (0.1 M $CuSO_4$) to minimize Ag^+ adsorption (Cornelis et al., 2010), and the pore water was removed by 1 h centrifugation at 2000 g. Part of this pore water extract was sampled for total metal analysis and the remaining water was centrifuged in a Cu soaked 10 kDa ultra-filtration tube at 4000 g for 30 min to measure the soluble Ag concentration. Ag analyses were completed as described above by ICP-MS.

2.4. Culture-dependent analysis of SBC

To assess Ag_2S NPs effects on the culturable fraction of the SBC, Colony Forming Units (CFUs) were counted in Nutrient Agar (NA) (Merck, Darmstadt, Germany), which is a general purpose medium for heterotrophic bacteria, and in the National Botanical Research Institute's phosphate (NBRIP) medium (Nautiyal, 1999), which was designed to count bacteria able to solubilize phosphate (P-SB). Media were sterilized at 121 °C for 20 min and supplemented with 0.2 mg L^{-1} cycloheximide (95% purity, Acros organics, New Jersey) to prevent the growth of fungi.

From samples collected at 0 and 28 days, three grams of soil were suspended in 25 mL of phosphate saline buffer (PBS, pH = 7.4), in triplicate. Soil samples were shaken at 304 g with twenty sterile glass beads (4 mm) for 10 min at 20 °C, followed by fold serial dilutions (10^{-2} dilution) in PBS. From each suspension, 100 μL was plated on NA or NBRIP (three replicates, with four technical replicates per medium), and the plates were incubated at 20 °C, for three days. Colony counts were reported as % of the variation of Log_{10} [colony forming units (CFU) per gram of soil].

To assess the usefulness of the medium in the context of this study, the identity of a randomly selected colony from NBRIP was confirmed by 16S rRNA gene sequencing (data not shown).

2.5. Enzymatic activity

Dehydrogenase (DHA), β-glucosidase (βG), acid phosphatase (AP), and arylsulfatase (AS) activities were determined according to the methods described by Dick et al. (1996), with some modifications. The

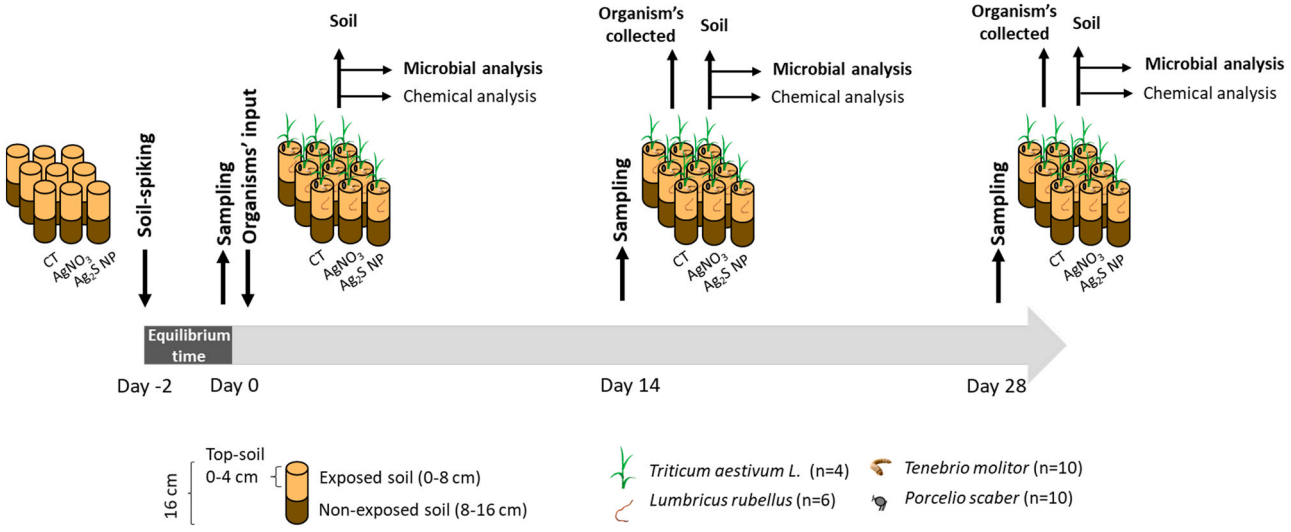

Fig. 1. Scheme of the temporal procedures during the 28 days of the mesocosm experiment.

DHA activity was evaluated by suspending soil (2.5 g) in a 3% triphenyl tetrazolium chloride (TTC; Sigma-Aldrich, 98%) solution (1 mL), followed by 24 h incubation at 37 °C, in darkness (Dick et al., 1996). The triphenyl formazan (TPF) produced was extracted with methanol. Then, soil samples were centrifuged (3000 rpm for 15 min) and the supernatant was measured photometrically at a wavelength of 546 nm. The results were expressed as μg of TPF g^{-1} 24 h^{-1}. The βG, AS and AP activities were measured using specific substrate solutions: 4-nitrophenyl β-D-glucopyranoside substrate (0.05 M) (Acros organics, 99%), 4-nitrophenyl sulfate (0.05 M) (Acros organics, 99%), and *p*-nitrophenyl phosphate (0.05 M) (Sigma-Aldrich), respectively (Dick et al., 1996; Tabatabai, 1994). The soil samples (0.5 g) were incubated at 37 °C for 1 h with 0.5 mL of substrate solution and 2 mL of the modified universal buffer (MUB) with pH 6.5 (βG) and pH 6.0 (AP); or acetate buffer (0.5 M; pH = 5.8) for AS activity (Tabatabai, 1994). The reaction was stopped with 0.5 M $CaCl_2$ (0.5 mL) and 0.5 M NaOH (2 mL) and the soil samples were centrifuged at 4562 g for 15 min. The supernatant was subsequently measured spectrophotometrically at wavelengths of 400 nm (βG) and 410 nm (AP, AS).

All enzymatic activities were measured in 96 wells microplates, in three replicates (with four technical replicates) using a microplate spectrophotometer (Thermo Scientific Multiskan Spectrum, USA).

2.6. Community level physiological profiling (CLPP)

Community-level physiological profiling (CLPP) was performed using 96-well Biolog® Ecoplates that contained 31 different carbon sources (Biolog, Hayward, CA, USA). The rate of utilization of these carbon sources was verified by the reduction of tetrazolium violet redox dye, which changed from colourless to purple as a function of microbial activity (Garland, 1997). Following the method by Samarajeewa et al. (2017), three grams of soil were collected and added to 27 mL of sterile water with 20 sterile glass beads. This soil suspension was shaken at 304 g for 10 min, at 20 °C and then left for 45 min. The suspension was diluted 100-fold to attain a cellular density of approximately 10^7 cell mL^{-1}. One hundred microliters of the suspension were then inoculated into each well, and plates incubated at 20 °C. The colour development was measured by reading optical density at a wavelength of 590 nm at 24 h intervals over 192 h of plates incubation, using a microplate spectrophotometer (Biolog, MicroStation TM, USA).

Measurements were calibrated against blank wells (water) (Garland, 1997), and the optical density values below 0.06 were set as 0. Optical density curves of CLPP were constructed for each technical replicate (n = 3). The total area under the curve (AUC) was calculated using the trapezoidal integration function, included in the trapz {pracma} package (version 2.2.2) in R program (version 3.5.1).

Additionally, the 31 carbon substrates from Biolog®Ecoplate were divided into six groups following the method of Sala et al. (2006): (1) carbohydrates, (2) carboxylic acids, (3) amines and amides, (4) amino acids, (5) polymers, and (6) phenolic acids. Thus, the substrate well averaged colour development (SAWCD) index was calculated based on optical density values obtained after 192 h of Biolog®Ecoplate incubation for each soil treatment.

2.7. PCR-DGGE

Soil (0.25 g) was collected from each corer, and the total DNA was extracted using the UltraClean® Power Soil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) following the instructions of the manufacturer.

The V3 region of the 16S rRNA gene was amplified using a nested PCR strategy. First, the 16S rRNA gene was amplified using the universal primers 27 F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The PCR mixture (25 μL) contained: nuclease-free water (16.25 μL), NZYTaq 2x Green Master Mix (6.25 μL; 2.5 mM $MgCl_2$; 200 mM dNTPs; 0.2 U/μL DNA polymerase) (NZYTech, Portugal), each primer (0.75 μL of a 10 mM solution), and a DNA sample (1 μL). The amplification conditions consisted of an initial denaturation step (94 °C for 3 min), followed by 30 cycles comprising the following steps: denaturation (94 °C for 1 min), annealing (52 °C for 1 min) and extension (72 °C for 2 min), and a final extension step (72 °C for 10 min). The second PCR was conducted as described above, using 1 μL of the first PCR product as template, and the universal primers [338f-GC (5'-GACTCCTACGGGAGGCAGCAG-3') and 518r (5'-ATTACCGCGGCTGCTGG-3')], (Muyzer et al., 1993), and the amplification conditions were: denaturation (94 °C for 5 min), annealing (52 °C for 30 s) and extension (72 °C for 30 s), and a final extension step (72 °C for 30 min). Positive and negative controls were included, consisting of identical reactions but using purified bacterial DNA from *Escherichia coli* ATCC 25922 and nuclease-free water as templates, respectively. Amplification was confirmed by electrophoresis (1.5% agarose).

PCR products were loaded into 8% (w/v) polyacrylamide (37.5:1, acrylamide:bisacrylamide) gels with denaturing gradient ranging from 35% to 62.5% [100% denaturant corresponded to 7 M urea and 40% (v/v) formamide]. A DGGE marker composed of 8 bands (Henriques et al., 2004) was included in the extremities of each gel. The electrophoresis was performed on a D-Code™ System (Bio-Rad, Hercules, CA, USA) with 1X TAE buffer (Sigma-Aldrich, Germany) at 60 °C in two steps, (1) for 15 min at 20 V and (2) for 16 h at 70 V. Gels were stained in a solution of ethidium bromide (0.5 μg mL^{-1}) for 5 min and rinsed in distilled water (20 min). Images were captured by the Molecular Imager® Gel Doc™ XR+ System (BioRad Laboratories, Hercules, California, USA).

DGGE patterns were analysed using Bionumerics Software (Applied Maths, Belgium).

2.8. Statistical analysis

All microbial endpoints [CFU counts, enzymatic activity, and CLPP] were analysed using Sigma plot V.12.5 (SysStat software Inc., CA, USA). Assumptions of homogeneity of variance and test for normality of distributions were verified using Levene's test and Shapiro-Wilk's test, respectively. When the normality test failed, a Kruskal-Wallis test followed by the appropriate post-hoc (Holm-Sidak) test was used. A level of p = 0.05 was considered to assume statistical significance. A two-way ANOVA analysis was conducted to assess the effect of the two factors (soil treatments vs. time of exposure). Additionally, the Tukey's HSD (honestly significant difference) and Dunnett's method test was applied for the post-hoc analysis, to obtain multiple comparisons between treatments or toward the respective control, respectively.

DGGE band matrix was analysed in PRIMER v6 software (Primer-E Ltd., Plymouth, UK). Band position and intensity was used to calculate the richness, the Shannon-Wiener diversity and Pielou's indexes. A one-way ANOVA followed by the Dunnett's method was used to discriminate differences in diversity indices. Further, two-dimensional Principal Coordinate Analysis (PCoA) was performed based on the Bray-Curtis similarity after the matrix was square root transformed. Additionally, a PCoA analysis was performed as described above for DGGE patterns of samples obtained after 28 days of exposure. Functional variables that presented a good correlation ($R \geq 0.6$ based on Pearson correlation) with SBC structure were represented as vectors in the PCoA plot.

Differences in SBC structure between treatments were evaluated through PERMANOVA based on 999 permutations.

3. Results

The effects of Ag_2S NPs on SBC were assessed in different layers (0–4 cm, 4–6 cm, 10–14 cm, and 14–16 cm) of the mesocosm corer. As expected, effects were clearer in the top-soil layer (0–4 cm), for which significant differences were observed between SBC subjected to different treatments [non-exposed soil - CT and soil exposed to Ag_2S NPs or $AgNO_3$] (Fig. S2A–D). Moreover, a significant decrease in Shannon-Wiener and richness indexes was observed for soils exposed to Ag_2S

NPs, in comparison to the CT, exclusively for top-soil samples (Table S1). Based on these results and given that all the main biota groups (that were part of the mesocosms) were also in this layer, subsequent analyses were focused on the top-soil layer (0–4 cm).

Soil analysis of the top 0–4 cm of the soil corer confirmed the accurate soil spiking procedures (Fig. 2A), with the control soil presenting a basal value of 0.051 mg Ag kg^{-1}, while the $AgNO_3$ and Ag_2S NPs spiked soils presenting 9.97 and 12.20 mg Ag kg^{-1}, respectively. At day 28, the spiked soils still presented similar concentrations, with average values of 10.7 ± 0.5 ($AgNO_3$ addition) and 13 ± 1 mg Ag kg^{-1} (Ag_2S addition). The control soil maintained a similar basal concentration (0.045 mg Ag kg^{-1}).

Porewater analysis from top-soil (Fig. 2B) showed similar average values for non-exposed soil [0.03 ± 0.008 μg Ag L^{-1}] and soil exposed to Ag_2S NPs [0.10 ± 0.08 μg Ag L^{-1}], at day 0. Soil spiked with $AgNO_3$ showed an initial Ag dissolution and presented an average value of 2.47 ± 1.64 μg Ag L^{-1}.

After 28 days of exposure, a similar Ag content in porewater samples was verified for CT and Ag_2S NPs, although a slight increase in Ag dissolution was detected when compared with day 0, presenting an average concentration of 0.33 ± 0.08 μg Ag L^{-1} and 0.67 ± 0.02 μg Ag L^{-1}, respectively. Soils spiked with $AgNO_3$ revealed a significant (Tukey test: $F = 35.474$; $p < 0.001$) free Ag^+ concentration, when compared with other treatments, presenting an average value of 1.74 ± 0.22 μg Ag L^{-1}.

3.1. Effects on culturable bacteria

The abundance of heterotrophic bacteria and of bacteria able to solubilize phosphate was determined using a culture-based method (Fig. 3A and B).

The Ag_2S NPs treatment did not significantly affect the CFUs counts, in either culture medium, although, a slight reduction was observed after 28 days of exposure [less 3.4% and 5.9% of Log_{10}(CFU.g^{-1} soil) for NA and NBRIP culture media, respectively]. In contrast, the $AgNO_3$ treatment resulted in a significant (Dunnett's Method: $df = 0.1$; $q = 6.363$; $p < 0.001$) reduction of 10% of the CFUs counted in NA medium after 28 days (Fig. 3A). For P-SB an even stronger impact of $AgNO_3$ was observed (Fig. 3B), corresponding to a significant reduction of 71.3% (Dunnett's Method: $df = 0.66$; $q = 10.7$; $p < 0.001$) and 91.8% of Log_{10} (CFU.g^{-1} soil) (Dunnett's Method: $df = 0.918$; $q = 14.691$; $p < 0.001$), at sampling day 0 and 28 after the beginning of the mesocosm trial, respectively.

3.2. Treatment effects on enzymatic activity

The effects of Ag_2S NPs treatment on soil enzymatic activities (DHA, βG, AP, and AS) were assessed over time and are represented in Fig. 4 (A–D).

The DHA activity (Fig. 4A) showed the greatest sensitivity to $AgNO_3$ treatment resulting in significant activity decrease in the three sampling times. For this enzyme, an activity reduction of 72.8% was observed after 28 days of exposure (Tukey HSD: CT vs. $AgNO_3$: $p < 0.001$). No significant effect from Ag_2S NPs exposure was observed (Tukey HSD: CT vs. Ag_2S NPs: $p = 0.852$). Accordingly, significant differences between $AgNO_3$ and Ag_2S NPs treatments were observed (Tukey HSD: $AgNO_3$ vs. Ag_2S NPs: $p < 0.001$).

A time-dependent effect was verified for βG activity (Fig. 4B). Thus, at an earlier time of exposure, the $AgNO_3$ treatment resulted in a significant (Tukey HSD: CT vs. $AgNO_3$: $p = 0.020$) activity reduction ($AgNO_3$ 0 day vs. CT 0 day: 75.3%). Despite an apparent recovery after 14 days for the $AgNO_3$ exposure, a significant reduction on βG activity was again observed after 28 days, but now for both treatments [Tukey HSD: (CT > $AgNO_3$: $p < 0.001$); (CT > Ag_2S NPs: $p = 0.001$)].

Moreover, the level of inhibition on βG activity observed for soils exposed to Ag_2S NPs was lower than for soils exposed to $AgNO_3$ (Tukey HSD: $AgNO_3$ < Ag_2S NPs: $p = 0.019$).

After day 28, AP activity (Fig. 4C) significantly increased in soils exposed to Ag_2S NPs (Tukey HSD: CT < Ag_2S NPs: $p = 0.007$), while no significant effect was observed for soils exposed to $AgNO_3$.

The activity of AS significantly decreased after 14 days in soils spiked with $AgNO_3$ (Tukey HSD: CT > $AgNO_3$: $p = 0.047$), recovering to levels similar to the control at day 28 (Fig. 4D). No significant effects were observed on the AS activity from Ag_2S NPs exposure.

3.3. Effects on carbon source consumption

The Ag_2S NPs effects on carbon substrate utilization were assessed using the Biolog® Ecoplate and are shown in Fig. 5(A–C).

The analysis of AUC (Fig. 5A) revealed an over-time significant increase on substrate consumption for non-exposed soils (AUC: CT 0 days < CT 14 days; Tukey HSD: $F = 554.985$, $p < 0.001$) until 14 days, and a slight decrease between 14 and 28 days of incubation (AUC: CT 14 days > CT 28 days; Tukey HSD: $F = 554.985$, $p = 0.915$).

Time-dependent effects on AUC calculated for soils exposed to silver were observed. A significant inhibitory effect of Ag_2S NPs on L-arginine consumption (Fig. 5B) was observed after 28 days ($p < 0.05$, Table S2). On the other hand, after 14 days the $AgNO_3$ treatment caused a significative reduction ($p < 0.05$, Table S2) of the consumption of the following substrates: tween 80, L-erythritol, D-glucosaminic acid and L-

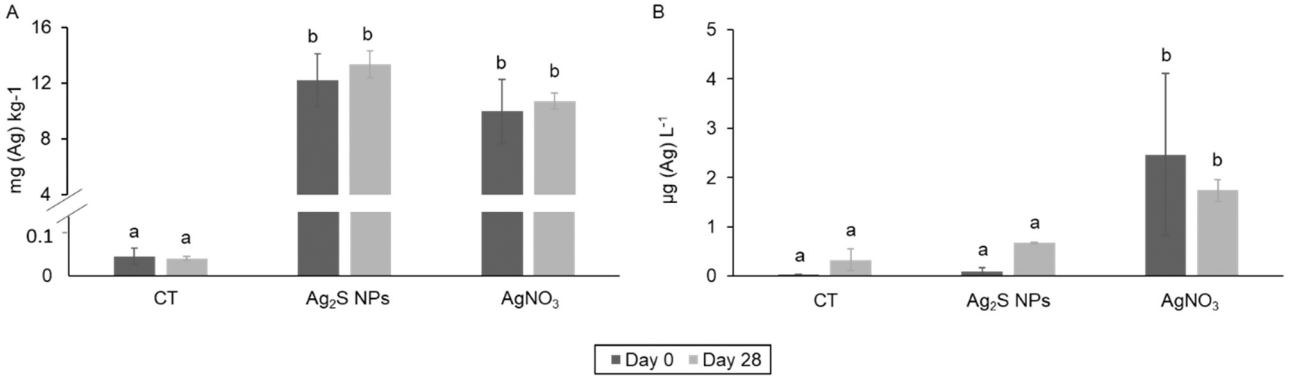

Fig. 2. Silver content in soils (A) and silver dissolution into soil porewater (B), after 0 (2 days after soil spiking) and 28 days of exposure. The data was expressed as mg (Ag) kg^{-1} of soil (A) and as μg (Ag) L^{-1} of porewater [average (n = 3) ± standard deviation]. The treatments included: the non-exposed soil (CT), soil exposed to Ag_2S NPs and soil exposed to $AgNO_3$. Different letters (^{a,b}) indicate statistical significance ($p < 0.05$) between soil treatments and the time of exposure, using the two-way ANOVA (Tukey post-hoc test, $p < 0.05$).

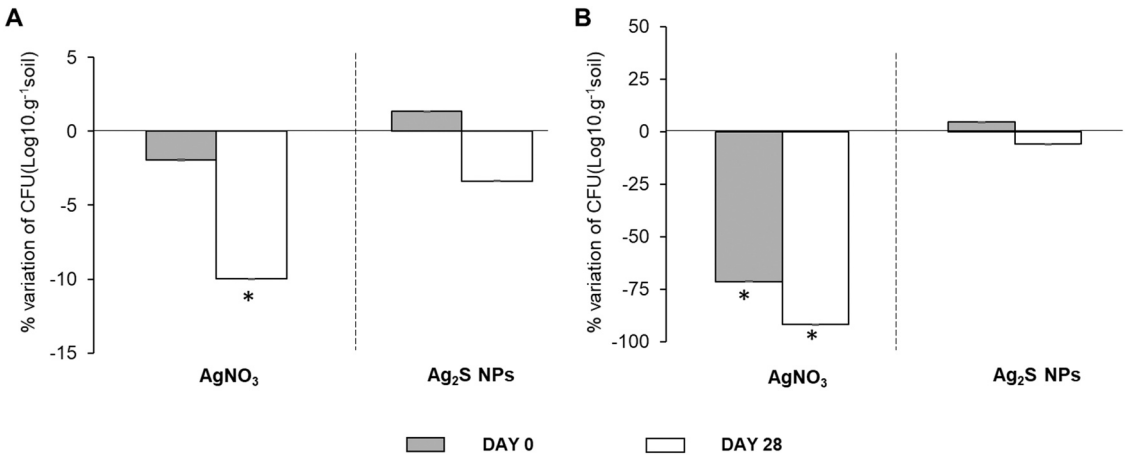

Fig. 3. Soil colony forming units (CFUs) measured in Lufa 2.2 soil, sampled at day 0 and 28 from the mesocosm experiment, previously spiked with $AgNO_3$ and Ag_2S NPs. CFUs are presented as % of variation on CFUs (Log10 g^{-1} soil) (± standard deviation) from the respective control (non-exposed soil), n = 3. Heterotrophic bacteria in nutrient agar (NA) medium (A), and P-solubilizing bacteria in National Botanical Research Institute Phosphate (NBRIP) medium (B). The grey bars represent the soil samples collected at the beginning of the mesocosm soil experiment (day 0), and the white bars represent samples after 28 days. Asterisks (*) indicate significant differences using two-away ANOVA (Dunnett's post-hoc test), $p < 0.05$) relative to the respective control.

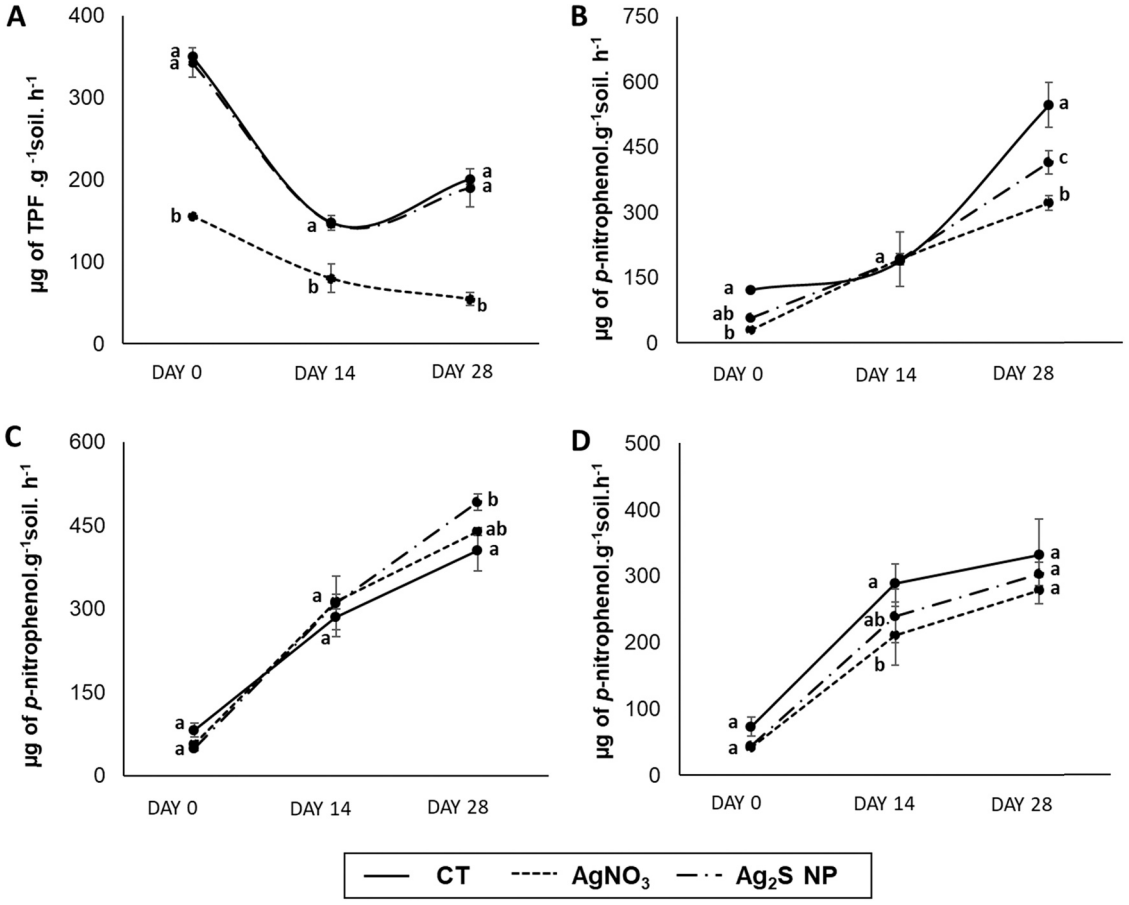

Fig. 4. Soil enzymatic activity measured in Lufa 2.2 soil, sampled at days 0, 14 and 28 from the mesocosm experiment, previously spiked with $AgNO_3$ and Ag_2S NPs. Also, the non-exposed soil was included in this experiment (CT). The data represent the mean (n = 3) of enzymatic activity (± standard deviation) of dehydrogenase (A), β-glucosidase (B), acid phosphatase (C) and arylsulfatase (D) activities. Different letters (a, b) indicate a significant difference using two-away ANOVA (Tukey post-hoc test, $p < 0.05$), among treatments.

serine. At day 28 $AgNO_3$ treatment resulted in a significant decrease of the consumption of pyruvic acid methyl ester, β-methyl-D-glucoside, and L-arginine ($p < 0.05$, Table S4).

In terms of SAWCD analysis, a significant effect was observed between sampling times but not between treatments versus control. Considering exposure time, SAWCD results were in accordance with AUC, with the pattern of substrate utilization being 0 days > 14 days > 28 days of exposure, for phenolic acid and amides/amines classes. For

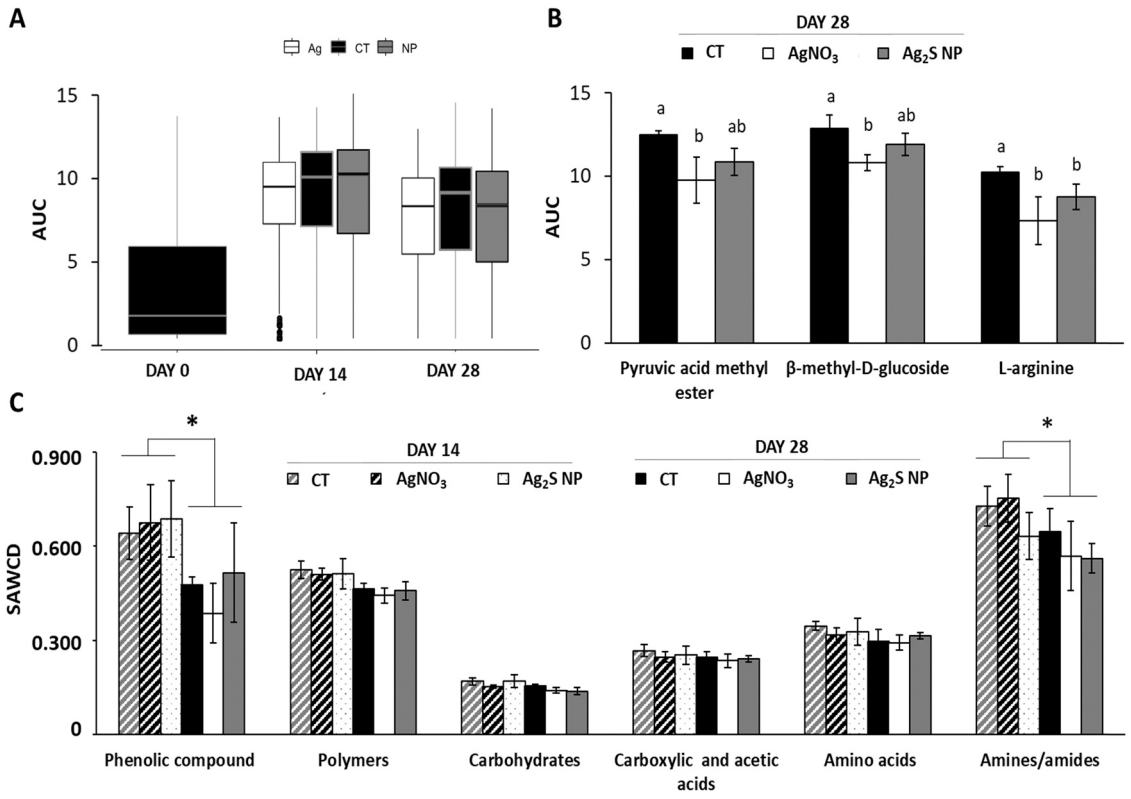

Fig. 5. Community level and physiological profile (CLPP) measured in Lufa 2.2 soil, sampled at days 0, 14 and 28 from the mesocosm experiment, previously spiked with $AgNO_3$ and Ag_2S NPs. For further comparison the non-exposed soil (CT) was included. The area under the curve (AUC) was calculated based on a trapezoidal integration function (A) over time; (B) after day 28, the Pyruvic acid methyl ester, β-methyl-D-glucoside, and L-arginine consumption; and (C) substrate average well colour development (SAWCD) after 14 and 28 days of exposure. The data represent the mean (n = 3) of AUC (± standard deviation). Letters ($^{a,\ b}$) represent significant differences using two-away ANOVA (Tukey post-hoc test, $p < 0.05$) between treatments.

the other classes (Fig. 5C) a significant difference over time was not observed.

3.4. Effects on SBC structure and diversity

The impact of Ag_2S NPs and $AgNO_3$ on the SBC structure was assessed over time and results are presented in Fig. 6 (A–B).

The cluster analysis (Fig. 6A) showed a temporal effect among soil treatments, in which the Ag_2S NPs treatment was completely separated from the other treatments only at day 28. The soil treated with $AgNO_3$ conversely revealed a strong impact on SBC from the experiment start onwards.

The first two axis of the principal coordinate analysis (PCoA) explained 49.6% of the total variation in the data: PCO1 axis (32.1%) and PCO2 axis (17.5%) (Fig. 6B). Along the PCO1 axis, samples tend to cluster as a function of exposure time while along the PCO2 axis separation seems to be influenced by treatment. At 28 days of exposure, a clear separation between soil exposed to Ag_2S NPs from the other treatments was observed.

The Permanova analysis confirmed a significant spatial separation between soil treatments (CT, $AgNO_3$ and Ag_2S NPs) according to the time of exposure (day 0, 14, 28) (PERMANOVA: $F = 4.76$; $P = 0.001$), except for non-exposed soils that did not show a significant difference, in spatial separation, between day 14 and 28 (PERMANOVA: CT14 vs. CT28: $t = 1.93$; $P = 0.052$).

In terms of diversity indices, a significant decrease in richness and Shannon-Wiener diversity was induced by exposure to Ag_2S NPs after 28 days ($p < 0.05$; Table 1). On the other hand, significant effects of $AgNO_3$ were only observed in richness after 48 h of soil spiking (Table 1), which corresponds to the day 0 of mesocosm experiment (Fig. 1), suggesting binding of the Ag^+ to soil and thus lowered bioavailability.

3.5. Relationship between structure and functions of SBC

Due to late-term effects of Ag_2S NPs on SBC, at structural level, the PCoA based on relative band intensity from DGGE profiles (Fig. 6) was performed only considering the 28 days of exposure (Fig. 7). Moreover, different functional parameters were imposed as a vector, to obtain the correlation between both levels (structural and functional). Thus, the association between SBC structure and functional variables was assessed for samples collected at 28 days of exposure and results are shown in Fig. 7.

As explained above, a significant difference among treatments was revealed. The two main PCoA axes (Fig. 7) explained 65.1% of the total variation [PCO1 axis (42.5%) and PCO2 axis (22.6%)]. The PCO1 axis separated soil samples according to treatment. The highest negative correlation with axis 1 was determined for AP activity (Pearson correlation: $R = -0.79$, $p = 0.023$), consistent with a significantly increased AP activity in soils exposed to Ag_2S NPs in comparison to other treatments (Fig. 4C). On the other hand, the axis 2 separated the soils exposed to $AgNO_3$ from the other treatments, and the highest negative correlations with this axis were observed for βG, heterotrophic bacteria and P-SB (Pearson correlation: $R = -0.67$, $p = 0.144$; $R = -0.74$, $p = 0.796$; $R = -0.87$, $p = 0.864$, respectively). The other functional variable assessed correlated with both axes, namely L-arginine (PCO1: $R = 0.35$ and PCO2: $R = -0.50$; $p = 0.949$).

4. Discussion

Currently, there is a lack of research data indicating the potential effects of Ag_2S NPs on SBC, under realistic scenarios. There is also insufficient knowledge on the effects of Ag_2S NPs compared to those of Ag ionic forms. To address these knowledge gaps, we conducted an

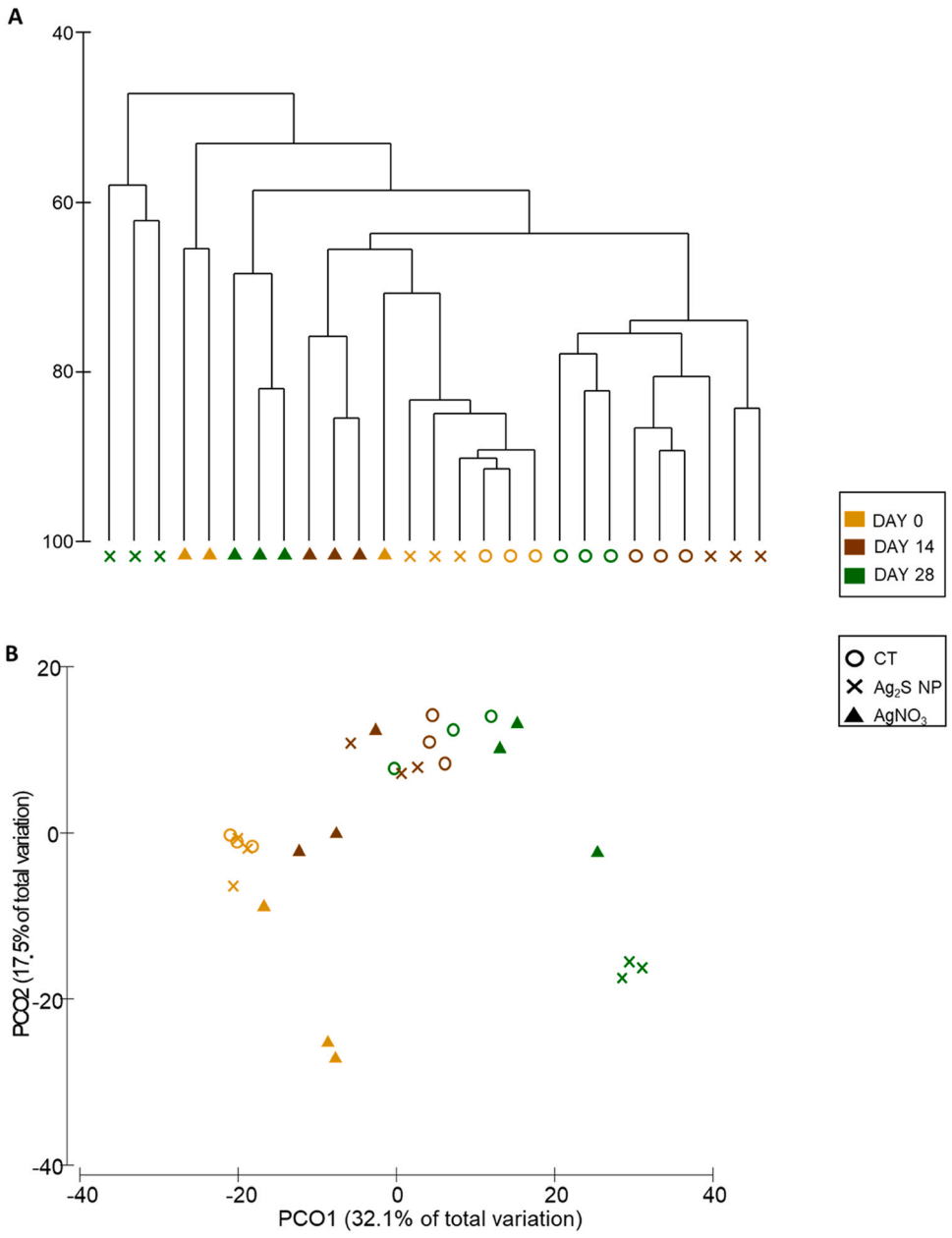

Fig. 6. Denaturing gradient gel electrophoresis (DGGE) analysis of the V3 region of the 16S rRNA gene amplified from SBC from non-exposed soil (CT), soil exposed to Ag_2S NPs and $AgNO_3$, after 0, 14 and 28 days of exposure. Cluster analysis (A) and Principal component analysis (PCoA) (B) were constructed based on Bray-Curtis similarity after square root transformation of DGGE relative abundance data (n = 3).

Table 1
Diversity indexes from SBC structure analysis were analysed over time and considering the following soil treatments: CT (non-exposed soil), and spiked soil with $AgNO_3$ and Ag_2S NPs.

Diversity indexes				
Soil Treatment	**Sampling time (day)**	**S**	**J'**	**H'**
CT	**0**	44 ± 1^{a}	0.922 ± 0.003^{a}	3.483 ± 0.022^{a}
$AgNO_3$	**0**	41 ± 2^{a}	0.897 ± 0.006^{a}	$\mathbf{3.321 \pm 0.051^{b}}$
Ag_2S NPs	**0**	44 ± 1^{a}	0.919 ± 0.004^{a}	3.469 ± 0.030^{a}
CT	**14**	48 ± 1^{a}	0.911 ± 0.006^{a}	3.527 ± 0.038^{a}
$AgNO_3$	**14**	$\mathbf{45 \pm 1^{ab}}$	0.903 ± 0.005^{a}	3.431 ± 0.020^{a}
Ag_2S NPs	**14**	$\mathbf{44 \pm 2^{b}}$	0.898 ± 0.013^{a}	3.403 ± 0.076^{a}
CT	**28**	45 ± 1^{a}	0.888 ± 0.028^{a}	3.380 ± 0.091^{a}
$AgNO_3$	**28**	43 ± 1^{a}	0.911 ± 0.014^{a}	3.428 ± 0.070^{a}
Ag_2S NPs	**28**	$\mathbf{37 \pm 2^{b}}$	0.885 ± 0.018^{a}	$\mathbf{3.193 \pm 0.042^{b}}$

The diversity indexes included: the richness (S), Pielou (J') and Shannon-Wiener index (H'). The data represent the mean of each diversity index (n = 3 ± standard deviation). Different letters (a, b) indicate statistical difference using two-away ANOVA (Tukey post-hoc test, $p < 0.05$) among treatments and within exposure time.

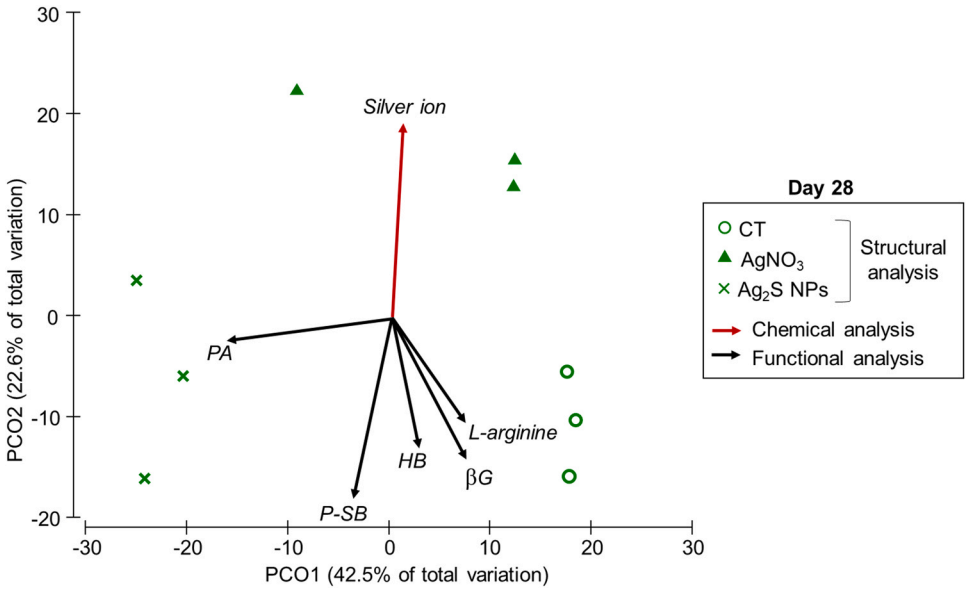

Fig. 7. Principal coordinates analysis (PCoA) ordinations based on Bray-Curtis similarity showing the spatial distribution of SBC structures among the different soil treatments: non-exposed soil (CT) and exposed soil to Ag_2S NPs or $AgNO_3$ (n = 3), after 28 days of exposure. The vectors represent a functional variable (Black: enzymatic activity and CLPP) or a chemical variable (Red: silver ion presence (Fig. 2) in soil pore-water) that presented a correlation ≥ 0.6 with SBC structure: AP - Acid Phosphatase; βG - β-Glucosidase; HB - Heterotrophic bacteria; P-SB - Phosphatase-Solubilizing Bacteria.

indoor mesocosm experiment to evaluate the impact of Ag_2S NPs on SBC during 28 days, at a nominal exposure concentration of 10 mg Ag kg^{-1} soil. Structure and function of $AgNO_3$– and Ag_2S NPs-exposed SBC were compared to those of communities in the control soil and related to the content/dissolution of Ag^+ in the soil pore water over time.

4.1. Effects of silver on SBC function

Colony bacterial counts are considered a suitable indicator for measuring Ag_2S NPs effects on SBC, as they usually present a fast response to metal contamination (Vasileiadis et al., 2015). Our study demonstrated that Ag_2S NPs did not significantly affect counts of heterotrophic bacteria and P-SB, albeit after 28 days of exposure a slight reduction was observed. Prior studies demonstrated that Ag_2S NPs are sparingly soluble in soils over a long period (> 1 year) (Wang et al., 2016), which may justify our results. However, the toxic effect of Ag_2S NPs on specific bacterial groups was detected in previous studies and related to their sulfidation degree (Schultz et al., 2018). For instance, a lower degree of sulfidation of Ag_2S NPs was reported to result in faster dissolution and release of Ag^+, thus enhancing the toxic effects of Ag_2S NPs on SBC (Wang et al., 2017; Schlich et al., 2018). A similar trend was also documented for *Escherichia coli* (Reinsch et al., 2012); *Arthrobacter globiformis* and *Pseudomonas putida* (Schultz et al., 2018). The study by Schultz et al. (2018) confirmed that the $AgNO_3$ treatment caused a higher toxic effect on bacterial counts (*Arthrobacter globiformis* and *Pseudomonas putida*) when compared to exposures to several NPs: Ag_2S (36 nm), Ag-PVP (49 nm) or Ag citrate (58 nm). These distinct toxic effects on SBC might be related with different free Ag^+ concentrations, in which a higher free Ag^+ concentrations resulting from $AgNO_3$ was documented that arising from AgNPs dissolution in soil. In accordance, our study demonstrated this distinct toxic effect, considering the silver form present, on bacterial counts. The effect of $AgNO_3$ on P-SB may indicate an impairment of phosphorous solubilization in soils, with consequences for crop production and soil fertility (Richardson and Simpson, 2011).

To assess the impact of nanomaterials on soil functions it is essential to evaluate the enzymatic activities related to nutrient cycling (Samarajeewa et al., 2017). DHA is one of the most sensitive enzymes to metal contamination (Samarajeewa et al., 2017). In our study, only the $AgNO_3$ treatment caused a strong inhibition of DHA activity over time, a result once again probably related with distinct free Ag^+ concentrations. The investigation conducted by Shin et al. (2012), found a high sensitivity of DHA in soils exposed to citrate-coated AgNPs at different concentrations (0, 1, 10, 100, and 1000 μg g^{-1}). Also, McGee et al. (2017), using a lower Ag concentration (1 mg kg^{-1}soil) and a non-sulfidized form of silver [AgNPs (20–30 nm and 0.5–1 μm)], observed a similar sensitivity of DHA. Although these studies demonstrated a significant impact of AgNPs on DHA activity, our study used the AgNPs in sulfidized form (Ag_2S NPs), which presents distinct physical and chemical properties. Consequently, the Ag^+ dissolution is also different; for instance, Ag_2S NPs were shown to be more stable than AgNPs (Wang et al., 2016). Additionally, Ag_2S NPs particles can be aggregated in soils, resulting in increased particle sizes (micro-particles) (Liu et al., 2019), with less surface area for oxidative dissolution and consequently decreased toxicity (Liu et al., 2019). The study by Liu et al. (2019) reported that hetero-aggregation, tested by combining the exposure of TiO_2 and AgNPs in soils, exhibit a higher toxic effect than the homo-aggregation of each of the single TiO_2 or Ag NPs. Although this was discussed based on the size of aggregates, the joint toxicity of a binary combination of metals cannot be disregarded.

Besides, DHA is only expressed in intact cells and does not accumulate extracellularly in the soils (Dick, 1996), so its activity has been related with microbial biomass, respiratory activity, microbial oxidative activity, and microbial viability (Dick, 1996; McGee et al., 2017; Samarajeewa et al., 2017). Accordingly, in our study the lower abundance of heterotrophic bacteria (NA medium) in $AgNO_3$-exposed soils may explain at least in part the decreased DHA activity.

βG activity has been also considered as an indicator of soil quality (Turner et al., 2002), related to carbon cycling in soils. Thus, the hydrolysis products resulting from βG activity are an essential energy source for microorganisms in soils (Dick, 1996). In accordance, studies from Kim et al. (2018) showed that exposure to silver-graphene oxide (0.1–1 mg g^{-1} soil) decreased the βG activity up to 80% in soils. Similarly, Samarajeewa et al. (2017) showed a significant decrease of this enzyme's activity upon exposure to 49 mg of AgNPs per kg of soil. In our study, a decrease on βG activity was observed for both treatments after 28 days of exposure, with approximately 56% and 33% decrease for the $AgNO_3$ and Ag_2S NPs treatments, respectively. Ag^+ dissolution did not explain the effect of Ag_2S NPs, and some specific-particle effects might occur, as suggested Starnes et al. (2015). In fact, the ratio between the silver total content in soil (Fig. 2A) and the dissolved/dissociated Ag^+ in the pore water (Fig. 2B) showed the highest value for Ag_2S NPs (19.8) than for $AgNO_3$ (6.15), suggesting a specific-particle effect for the Ag_2S NPs treatment. Although previous studies reported a decrease of AP

activity in soil exposed to AgNPs, regardless of the concentrations tested (Colman et al., 2013; Samarajeewa et al., 2017), our study showed a significant increase in this enzymatic activity, after 28 days of exposure to 10 mg kg^{-1} of Ag_2S NPs. Although our study did not evaluate the root biomass, we hypothesize that root growth and proliferation in soils may contribute to the increased AP activity in our experiment. On the other hand, the presence of different invertebrates possibly contributes to the complexation of the Ag_2S NPs in soils (e.g. ligation with organic matter or with clay), and therefore, protected/stimulate the activity of extracellular enzymes (Shin et al., 2012).

Although study from Vasileiadis et al. (2018) demonstrated that the AS enzyme is more sensitive to Ag^+ exposure (from $AgNO_3$ or acetate, respectively), our results corroborate this AS sensitivity to the $AgNO_3$ treatment but only after 14 days. After this period this enzymatic activity recovered, becoming similar to that in the non-exposed soil. This time-dependent effect might have been directly related to a rapid flux of free Ag^+ from $AgNO_3$ in the first days (short period) or by directly affecting the sulfatase producer microorganisms (Vasileiadis et al., 2018) followed by a gradually stronger adsorption of Ag^+ to organic S groups or even a reduction to metallic Ag^0 making Ag^+ less available (Settimio et al., 2014).

Some fluctuation in soil enzymatic activity was observed, even in non-exposed soil (CT), over time. The SBC is dynamic, and the soil conditions (e.g. daily watering) and the invertebrates and plants (root growth) presence in our experiment might influence this fluctuation. Soil water content influences the SBC composition, resulting in an impact on oxygen concentrations and nutrient availability. The watering might reduce soil oxygen levels and promote anaerobic microorganisms (facultative and obligate) growth, whilst decreasing the total microbial activity (Sylvia et al., 2005). On the contrary, the presence of invertebrates can promote soil aeration (El-Wakeil, 2015), which indirectly influences the proliferation of different bacteria/fungi, ultimately resulting in a structural and composition change of the SBC. Moreover, these organisms have a crucial role in the decomposition of the organic matter. Soil and organic matter are also ingested, digested and excreted by invertebrates, producing a substrate that is more accessible to SBC but that may also contain Ag (as free Ag^+, and/or Ag_2S depending on the treatment plus some or all of the range of speciation forms noted above), inducing different microbial mobilizations. Indirectly, this can induce bacterial community adjustments, in terms of abundance and composition, according to the bacteria nutrient/metabolic requirements. Besides, plants and associated microorganisms alter the physicochemical properties of the root-soil interface, such as pH and redox conditions (Liu et al., 2019).

The physiological profiles were used to assess the impact of both Ag forms on bacteria consortia. The significant reduction in L-arginine consumption (amino acid class) for both silver forms, indicates a potential effect on carbon and nitrogen cycles that are closely related to plant fertility success. L-arginine plays a key role in nitrogen distribution and recycling in plants (Slocum, 2005), and consequently, might be related to the plant homeostasis. The study by Yang et al. (2018) demonstrated that AgNPs decreased the arginine and histidine content in wheat (*Triticum aestivum L.*), during 4 months in 2000 mg kg^{-1} amended soil. On the other hand, the decrease of L-arginine consumption might be partially explained by the decreased bacterial counts observed in our results, at least for $AgNO_3$, because heterotrophic bacteria are able to catabolize the soil arginine, involved with ammonification processes (nitrogen and carbon cycles) (Alef and Kleiner, 1986). Other studies reported a significant decrease of general carbon substrate utilization (AWCD) by the microbial community from soils exposed to AgNPs (uncoated, 20 nm) (Sillen et al., 2015) or decreased utilization of specific substrates such as amino acids and carbohydrates (Kumar et al., 2011), for soils exposed to metal NPs. Our results did not indicate a significant impairment of SBC activity and the effects attenuation may be related to the presence of different soil invertebrates and plants in this mesocosms approach. Plants may change the availability of metals on soils and may release exudates that can directly change the soil conditions (pH, for instance) (Liu et al., 2019), affecting the toxicity of $AgNO_3$ and Ag_2S NPs on SBC. Also, invertebrates may alter soil conditions, essentially through their behaviour in soils (Tourinho et al., 2015), contributing to the microbial composition/abundance (decreasing, for instance) changes, as a result of the inhibition of oxygenation level.

4.2. Effects of silver on SBC structure and diversity

The effects of Ag_2S NPs on the structure, composition, and diversity of SBC were previously assessed in only a limited number of studies (Judy et al., 2015; Doolette et al., 2016). The present study is a considerable contribution given its higher tier approach, using an indoor mesocosm including several species of invertebrates and a plant species making them more representative of field conditions. Furthermore, our study revealed a distinct impact of Ag_2S NPs and $AgNO_3$ on the SBC structure, in a time-dependent manner. During the exposure, a faster increase of Ag^+ concentration in the soil pore water from the $AgNO_3$ treatment was observed and might be responsible for an early structural change, as previously explained. Although Ag^+ dissolution from Ag_2S NPs was detected in very low amounts at 28 days of exposure, this Ag^+ dissolution wasn't enough to explain the latter's effects on SBC structure.

The impact of Ag_2S NPs on SBC structure has been already evaluated on natural soils after 28 days using metagenomics (Doolette et al., 2016) and phospholipid fatty acids (PLFAs) analysis (Judy et al., 2015). In this regard, Doolette et al. (2016) observed that the impact of Ag_2S NPs (152 nm) on the number of operational taxonomic units (OTUs) was lower compared to non-sulfidized AgNPs (44 nm) and $AgNO_3$, when using the toxicity threshold hazardous concentrations (HC20) approach [5.9 (Ag_2S NPs); 1.4 (AgNPs) and 1.4 ($AgNO_3$) mg Ag kg^{-1}]. On the other hand, the study by Judy et al. (2015) demonstrated that microbial biomass (inferred from PFLA analysis) decreased when Ag_2S NPs (30.1 nm) was applied in sandy loam soils (pH=6.8), at 1 and 100 mg kg^{-1}soil, however, the silver in sulfidized form was less toxic compared to the other silver NP (PVP-Ag - 20.6 mm) and ionic silver ($AgNO_3$). Although Ag toxicity is strongly dependent on soil characteristics (e.g. pH, soil type), nanomaterial characteristics (e.g. size, coating), and the specific microbial methodologies (Samarajeewa et al., 2017; Vasileiadis et al., 2018), both studies showed a similar toxicity pattern (Ag_2S NPs < AgNPs < $AgNO_3$) on microbial structure.

Moreover, a reduction of the alpha diversity of the bacterial community exposed to AgNPs has already been reported (Samarajeewa et al., 2017). Regardless of silver form, our study revealed a significant reduction in diversity and richness, after 14 days of exposure. In fact, the free Ag^+ concentration might explain the $AgNO_3$ impact on these indexes, as above mentioned. In our experiment, the negative impact of Ag_2S NPs on diversity indexes may result from the Ag^+ released (from these particles), or the accumulation of sulfidized particles in the plant tissues (Wang et al., 2017). In fact, this accumulation (at 10 mg kg^{-1}) reduces plant growth (Wang et al., 2017), changing the interaction between root exudates and microbes, which can result in an indirect change in the abundance of microorganisms in the soil. After day 28, the effect of $AgNO_3$ on SBC diversity and richness was gradually attenuated to values similar to the control, suggesting SBC resilience to this silver form or/and a decrease in Ag bioavailability. This pattern has been observed before for invertebrate species, where Ag^+ from $AgNO_3$ decreased significantly over time in porewater, reflecting a decrease of Ag bioavailability. Due to the increased silver adsorption to soils, the free Ag^+ concentration decreases in less than 14 days (Settimio et al., 2014).

4.3. Environmental relevance and integrative analysis between structure and functional levels of SBC

Shifts of SBC structure may lead to a change in the biological

function, albeit the functional redundancy reported for SBC (Vasileiadis et al., 2015). The relationship between the SBC structure and soil function is not easy to predict and is poorly documented. Our results demonstrated that Ag_2S NPs change the SBC structure and this structural change may affect, for instance, the phosphorous cycle, resulting in an increased AP activity. Also, the P-SB counts showed a good correlation ($R>0.6$) with the SBC structure from soils exposed to Ag_2S NPs. In accordance with these findings, the study by Fanin et al. (2015) showed a significant correlation (Mantel test) between CLPPs and SBC structure from different fertilization levels (N and P input) in undisturbed tropical forests.

Few studies are available that relate the N cycle with the SBC structure exposed to metal contamination (Kumar et al., 2014; Doolette et al., 2016), and other nutrient cycles are even less explored. Nevertheless, the effects of AgNPs (at 10 and 100 mg kg^{-1}) on SBC structure might impact the carbon cycle, through a decrease in microbial biomass (carbon) (Kumar et al., 2014), soil enzymatic activity (e.g. β-glucosidase) and/or soil microbial respiration.

5. Conclusions

This study provided evidence that Ag_2S NPs affect the SBC and that these effects become more pronounced over-time. Ag_2S NPs effects on the SBC are expected to potentially imbalance soil functions, namely those related to the carbon (L-arginine consumption and β-glucosidase activity) and phosphorus cycles (P-SB and PA). The structure of SBC and diversity indexes were significantly impacted by Ag_2S NPs, after 28 days of exposure.

Function and structure of SBC were also impacted by $AgNO_3$, predominantly at earlier times of exposure due to the initial high concentration of free Ag^+, with the expected decrease of Ag bioavailability over time, as a result of binding to lignin and clay particles, being a likely cause for the weakening of effects.

This study emphasizes the usefulness of the terrestrial mesocosm approach to evaluate the effects of sulfidation state of AgNPs on microbial parameters. Moreover, the observed temporal effects (28-days) from both ionic Ag and Ag_2S NPs highlight the importance of long-term exposure experiments under realistic environmental conditions.

CRediT authorship contribution statement

S. Peixoto: Methodology, Formal analysis, Investigation, Writing - original draft, Visualization. **Z. Khodaparast:** Methodology, Investigation. **G. Cornelis:** Methodology, Validation, Writing - review & editing. **E. Lahive**: Methodology, Validation, Writing - review & editing. **A. Green Etxabe:** Methodology, Writing - review & editing. **M. Baccaro:** Methodology, Writing - review & editing. **A.G. Papadiamantis:** Validation, Formal analysis, Investigation. **S.F. Gonçalves:** Methodology, Formal analysis. **I. Lynch:** Validation, Investigation, Writing - review & editing. **M. Busquets-Fite:** Methodology, Formal analysis, Investigation. **V. Puntes:** Validation, Investigation. **S. Loureiro:** Conceptualization, Validation, Investigation, Resources, Writing - review & editing, Supervision, Funding acquisition. **I. Henriques:** Conceptualization, Validation, Formal analysis, Investigation, Writing - review & editing, Visualization, Supervision, Funding acquisition.

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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2020.111405.

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