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Effects of increasing concentrations of unamended and gypsum modified bauxite residues on soil microbial community functions and structure – A mesocosm study

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A B S T R A C T

Bauxite residues (BR), commonly named red muds, are the saline-sodic waste produced during the extraction of alumina from bauxite. In this study, four kinds of BR were mixed at increasing concentrations with two soils in a mesocosm experiment. Unamended BR from Provence (PRO) and Guinea (GUI) bauxite were selected, and Modified Bauxite Residues from PRO and GUI (MBR-PRO and MBR-GUI) were obtained by gypsum application and repeated leaching, in order to reduce their pH, electrical conductivity (EC) and exchangeable sodium percentage (ESP). Several indicators of microbial community functions and structure (growth of culturable bacteria; enzymatic activities; C-sourced substrates degradation (Biolog®); bacteria and fungi PCR-RFLP fingerprints) were measured after 35 days of incubation. Results showed that PRO residue had stronger negative effects than GUI on all the tested indicators. Residues modified by gypsum addition (MBR-PRO, MBR-GUI) were equally or sometimes less harmful compared to unamended residues. Microbial activities (bacterial growth and enzyme activities) were more inhibited than the diversity of microbial functions (Biolog®), and the structure of bacterial and fungal communities was not affected by increasing concentrations of bauxite residues. EC and ESP were the main factors explaining the inhibition of microbial activities, although the origin of bauxite residue is of great importance too.

1. Introduction

Bauxite residues (BR), commonly named red muds, are a by-product of the Bayer process, used to extract alumina from bauxite ore (Di Carlo et al., 2019; Evans, 2016). Widely used, this process has the disadvantage of generating huge amounts of BR which are characterized by high pH and electrical conductivity (EC) (Gräfe et al., 2011), poor nutrient contents (e.g. N, P, K) (Di Carlo et al., 2019; Wong and Ho, 1993), low permeability and poor structure (Santini and Fey, 2016). The use of caustic soda during the Bayer process is the reason for the high pH and EC values of these residues, high EC being mainly due to high Na content (Gräfe et al., 2011). The mineral fraction in BR is

mostly dominated by Fe and Al oxides (Gräfe et al., 2011), although various concentrations of heavy metals and metalloids (As, Cr, V, Ni, Pb, among others) are also observed (Xue et al., 2016) which, according to Zou et al. (2018), may have harmful effects on the environment.

Although several valorisation paths have been investigated (for a review, see Klauber et al. (2011)), they are not sufficient to recycle all the BR generated, which has to be stocked in storage areas (Jones and Haynes, 2011). Thus, worldwide production of BR is increasing and is nowadays estimated at 120 million tonnes a year, in addition to the 3 billion tonnes previously produced and already stacked (Power et al., 2011). This raises concerns about their potential spread to the surrounding environment, by water leaching, fugitive dust, or worse

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scenarios such as the collapse of a dam, as happened in 2010 in Hungary (Gelencsér et al., 2011; Gräfe et al., 2011), all of which can contaminate soils. Thus, assessing the ecotoxicity of BR on living organisms is crucial to understand its effects on the environment, information that would be useful to implement management methods in case of major pollution, but also with the aim of BR remediation. Moreover, since each bauxite ore has its own mineralogical characteristics, as mentioned by Hackenberger et al. (2019), each BR can thus have specific ecotoxicological properties which have to be highlighted. To estimate their toxicity, BR have been investigated through laboratory bioassays on microorganisms (e.g. *Vibrio fischeri* bioluminescence test (Klebercz et al., 2012; Rékási et al., 2013)), plants (germination and growth inhibition (Courtney and Mullen, 2009; Rékási et al., 2013; Di Carlo et al., 2020)) and animals (e.g. Collembola (Rékási et al., 2013) earthworms (Hackenberger et al., 2019; Maddocks et al., 2005)). Other studies have focused on the effects of BR on soil biota, especially on soil microorganisms and their functions, considering field samples enriched in BR as well as laboratory mesocosms. On this point, numerous studies were performed with acidified or metal polluted soils since, under certain threshold concentrations, BR can improve the quality of those particular soils by pH neutralization or metal adsorption/precipitation effects (Garau et al., 2011, 2007). Microbial bioindicators have also been investigated in laboratory and field studies on unamended and remediated BR (without soils) (Klebercz et al., 2012; Rékási et al., 2013). One of the most used techniques to remediate BR is to reduce their harsh physico-chemical characteristics by amendment with gypsum, followed by leaching treatments. The removal of Na^+ is made possible by the intake of Ca^{2+} provided by gypsum application, by ion exchange processes on the BR particles (Gräfe and Klauber, 2011). The pH is neutralized by the reaction of Ca^{2+} with carbonate that precipitate calcite (Burke et al., 2013). Moreover, by reducing the Na content, gypsum addition is known to encourage aggregation and improve the structure of bauxite residues (Xue et al., 2016). By reducing pH, salinity and improving the structure of BR (Courtney et al., 2003; Courtney and Kirwan, 2012; Kirwan et al., 2013), gypsum amendment followed by repeated leaching could improve BR quality (Courtney et al., 2014b; Gräfe and Klauber, 2011) which could make this substrate less toxic toward microbiota when mixed with soils. However, to our knowledge, no studies have been carried out to assess the effect of such Modified Bauxite Residues (MBR) on soil microbial communities using mesocosms and increased MBR concentrations. In a same way, comparison with unamended BR was also not investigated.

Thus, the aim of this study was to determine and to compare the impacts of increasing concentrations of unamended BR and MBR on a large set of soil microbial indicators. We hypothesized here that microbial responses would be affected by 1) BR origin (bauxite source), 2) BR amendment and modification with gypsum and 3) BR and MBR increasing concentrations. To improve the robustness of this study, two soils were used and experimentally contaminated with BR and MBR in increasing concentrations (10–50% w/w) in mesocosms. Soil effect was not tested in our hypotheses. Indeed, each soil and its associated microbial community are unique, and we did not aim here to compare the responses between the two different soils. This mesocosm approach is widely used, for example in studies investigating the effects of heavy metal pollution (Nwuche and Ugoji, 2010) or of mine wastes (Valentín-Vargas et al., 2014) on soil biota. BR were here added at high concentrations to amplify the response of microbial community, in order to 1) better target the factors explaining the measured effects and 2) identify the functions that were the most affected by increasing BR concentrations. This kind of approach is an intermediate scale between lab bioassay on a single test organism and the field experiment and has the advantage to study a complex community of organisms in controlled conditions. Therefore, it gives a more accurate vision of the impact of a substance towards soil properties, and is much easier and economic than field approaches, which are thereafter necessary to test the results obtained at more realistic spatiotemporal scales (Kampichler

et al., 2001). To this end, enzymatic activities, growth of culturable bacteria, Community Level Physiological Profiles (CLPPs) and PCR-RFLP fingerprints were measured in soils, BR and BR-soil mixture mesocosms.

2. Materials and methods

2.1. Bauxite residues and soil sampling

Two unamended bauxite residues (BR) coming from distinct origins were used in this study. One is a by-product of alumina extraction from bauxite from Provence, France (PRO), and the second from Guinean bauxite (GUI). PRO was collected on a tailing located in Le Griffon (Vitrolles, France). Sludgy BR were disposed there from 1953 to 1968 and have been neither treated nor modified in any way since, except by natural weathering processes. GUI was directly collected from an operating alumina plant in Europe after mechanical dehydration of fresh bauxite residues by filter press. Aliquots of these unamended BR were modified with gypsum amendment prior mesocosm experiment to obtain Modified Bauxite Residues (MBR). This treatment consisted in an addition of gypsum (5% w/w for GUI and 10% w/w for PRO) and repeated leaching. Leaching was performed in 100 L plastic barrels, fitted with taps at their base, with 1 L of deionised water a day until the leachates reached a value of electrical conductivity of 4.5 mS cm^{-1} . As the leachate conductivity was not decreased enough (in prior tests) in PRO with 5% w/w gypsum amendment, the quantity of gypsum had to be increased to 10% w/w. This difference can be explained by the high sodium content (Table 1) in the PRO residue, as a result of its past management (no treatment or washing before its disposal) and maybe of its former production process. Indeed, high sodium content and exchangeable sodium percentage (ESP, Table 1) slow down the exchange processes initiated by the inputs of Ca^{2+} , so the gypsum quantity was increased. The resulting modified residues were respectively named MBR-PRO and MBR-GUI. Two local soils with different characteristics were used to assess the impact of bauxite residues on soil microbial communities: soil 1 (S1), a topsoil provided by the company Arnaud ($43^{\circ}31'18''\text{N}/5^{\circ}21'00''\text{E}$, Les Milles, France); soil 2 (S2), a grassland soil collected (0–5cm depth) at Les Pennes Mirabeau (Bouches-du-Rhône, France) ($43^{\circ}24'19.3''\text{N}/5^{\circ}18'01.7''\text{E}$). Bauxite residues and soils were sieved at 2 mm and stored in polypropylene barrels, at 4°C before use.

2.2. Experimental protocol

Mesocosm experiments were conducted with the two soils and the four kinds of bauxite residues. Soils were mixed, on a dry weight basis (w/w), with five concentrations (10%, 20% 30%, 40% and 50%) of each residue (PRO, MBR-PRO, GUI, MBR-GUI) to a total mass of 50 g. The different mixtures were then incubated at their maximum water holding capacities (WHC) in plastic boxes, with their lid let slightly loosen to enable gas exchange with the atmosphere and maintain oxic conditions. Soils and residues (0% and 100% of residue respectively) alone were also prepared. For each modality, five replicates were performed and the mesocosms were incubated during 35 days in the dark (to avoid germination and growth of soil seed bank) at 18°C in a growth-chamber. During the course of the incubation, WHC was regularly checked and maintained constant with distilled water in order to not bring any additional element to the different mixtures that may interfere with certain physico-chemical variables. All microbial variables measurements were done after the incubation period.

2.3. Physico-chemical characterization

Soils and bauxite residues were characterized. The water holding capacity (WHC) was measured after water saturation of the samples and 48 h of incubation in a chamber with constant pressure of 1 bar. Samples were then oven-dried at 105°C during 24 h, and WHC was

Table 1
Physico-chemical characteristics and total element contents of soils and bauxite residues prior to incubation.

			Soils		Bauxite residues				
			Soil 1	Soil 2	PRO	MBR-PRO	GUI	MBR-GUI	
<i>Physico-chemical characteristics</i>	Clay (< 2 µm)	%	21.8	20.2	41.2	21.2	27.2	23.5	
	Silt (2–50 µm)	%	39.1	31.8	35.6	28.2	43.8	43.9	
	Sand (50–2000 µm)	%	39.1	48.0	23.2	50.6	29.0	32.6	
	C _{org}	g kg ⁻¹ DW	25.7	27.3	1.2	1.1	2.0	2.0	
	N _{tot}	g kg ⁻¹ DW	1.40	2.97	0.3	0.09	0.18	0.25	
	P ₂ O ₅	g kg ⁻¹ DW	ND	0.034	0.014	0.009	0.02	0.025	
	CEC	cmol ⁺ kg ⁻¹ DW	9.4	17.0	24.1	8.3	8.0	5.4	
	WHC	%	18.0	18.1	29.8	23.3	26.7	26.8	
	pH		8.9	9.1	10.7	9.1	8.8	8.6	
	EC	µS cm ⁻¹	112	61.3	1 800	1 400	1 300	900	
	ESP	%	3.8	1.2	94.1	33.5	26.9	8.9	
	Particle density	g mL ⁻¹	ND	2.2	3.2	3.1	3.2	3.2	
	<i>Major elements</i>	Fe	µg g ⁻¹	13 843	13 532	170 600	153 039	129 512	125 944
		Ca	µg g ⁻¹	139 838	139 777	20 272	29 671	13 792	13 921
Al		µg g ⁻¹	3 902	24 671	54 944	60 166	17 966	18 363	
Na		µg g ⁻¹	1 825	2 787	48 457	44 584	7 325	5 978	
Ti		µg g ⁻¹	1 162	1 795	20 175	19 939	14 176	13 859	
Mg		µg g ⁻¹	6 203	5 235	1 920	2 286	861	887	
K		µg g ⁻¹	6 748	5 364	550	496	513	499	
Mn		µg g ⁻¹	384	311	747	606	528	544	
<i>Trace elements</i>		Cr	µg g ⁻¹	41.3	117	796	847	2 258	2 402
		V	µg g ⁻¹	37.4	52.4	929	846	1 825	1 914

PRO: residue from Provence bauxite; MBR-PRO: gypsum modified residue from Provence bauxite; GUI: residue from Guinean bauxite; MBR-GUI: gypsum modified residue from Guinean bauxite.; ND: not determined.

expressed in % (Chamayou and Legros, 1989). Particle density was expressed in g mL⁻¹ and assessed by plunging a known mass of oven-dried (105 °C) sample in deionised water and measuring the displaced water volume (Baize, 2000). pH and electrical conductivity (EC) were measured in aqueous extract (1:10 w/v for 24 h) according to Santini et al. (2013). Some analyses were conducted by the *Laboratoire d'Analyses des Sols*, INRA (Arras, France): particle size analysis (ISO 11277), organic carbon (C_{org}) and total nitrogen content (N_{tot}) (NF ISO 10694 and NF ISO 13878), P Olsen (P₂O₅) (NF ISO 11263) and the cation exchange capacity (CEC) with Metson method (NF X 31–130). Total content of major and trace elements was assessed, respectively, by ICP-AES and ICP-MS, following dissolution with hydrofluoric acid (NF X 31–147). The same analytical apparatus was used for bioavailable element (major and trace). Bioavailable elements were extracted using an extraction solution (1:2 w/v for 2 h) composed of DTPA (5 mM), TEA (0.1 M) and CaCl₂ (0.01 M) according to Lindsay and Norvell (1978). Exchangeable Ca, Mg, K and Na were extracted with 1 M ammonium acetate pH 7 (1:20 w/v for 1 h) and analysed by ICP-AES (Helmke and Sparks, 1996). Exchangeable sodium percentage (ESP) was calculated as the content of Na⁺ as a percentage of total exchangeable cations (Li et al., 2019). pH and EC were measured in soils, BR and all the modalities and replicates of soil-BR mixtures. Exchangeable Ca, Mg, K and Na were measured on composite samples from the 5 replicates of each mesocosm modality.

2.4. Measurement of microbial variables

2.4.1. Growth of culturable bacteria

Growth of culturable bacteria was monitored by inoculating a sample suspension to a bacteria growth medium. Ten µL of a sample suspension (1:100 w/v in sterile pyrophosphate 2 mM) were inoculated in 190 µL of a rich, complete and unselective medium (Nutrient broth, composed of tryptone, meat extract and sodium chloride, Biokar Diagnostics, France) at 20 g L⁻¹, as recommended by the manufacturer. The increase in optical density (OD) was followed every 30 min at 620 nm with a microplate reader Infinite® M200 (Tecan, Switzerland). After 20 h of growth, mean value of the absorbance of the 5 replicates was calculated, then expressed as a percentage of the control

(soil not amended with red mud) from each mesocosm series. Results were used as a proxy of total bacterial biomass in samples.

2.4.2. Community Level Physiological Profiles (CLPPs)

CLPPs of the aerobic culturable heterotrophic microbial communities were determined with the Biolog EcoPlates™ (Biolog, USA) according to the protocol from Floch et al. (2011). Microplates used for this assay contained a different C source in each well (a total of 31 C sources and one control well without C) and a colourless tetrazolium salt as electron acceptor of bacterial respiration, which turns to purple formazan when the carbon substrate is metabolized by bacteria. Briefly, 1 g of mesocosm sample was suspended in 9 mL of a sterile sodium pyrophosphate solution (2 mM), thereafter one hundred-fold diluted in sterile NaCl (0.15 M). Following the recommendations of Calbrix et al. (2005), dilutions were adjusted to obtain the same concentration of microbial cells for each sample using results from growth of culturable bacteria (see 2.4.1). Microplates were incubated at 30 °C under a humid atmosphere, and kinetics were monitored by measuring the optical densities (OD) twice a day at 590 nm during 90 h with a microplate reader Infinite® M200 (Tecan, Switzerland). Well initial OD (at t₀) was subtracted from well OD at t_{90h} in order to get rid of substrates and soil-residue mixtures inherent colours. In the same way, the OD of the control well was subtracted from the 31 other wells OD. Growth in wells was considered positive when OD was above the arbitrary threshold of 0.1 (Floch et al., 2011). The positive values were used to calculate three indicators after 90 h of incubation. Average Well Colour Development (AWCD) was used to assess the average activity of the microbial communities by calculating the mean OD for the 31 C-sourced wells (Garland and Mills, 1991). Richness (S) corresponded to the number of positive well (OD > 0.1) of each sample. Shannon diversity index (H') was calculated using eqs. (1) and (2), where arguments are i: a positive well, S: total number of positive wells (S, richness), OD_{ni}: OD of an i positive well, OD_N: sum of the OD of the positive wells.

$$H' = - \sum_{i=1}^S OD_i \log_{10} OD_i \quad (1)$$

$$OD_i = \frac{OD_{n_i}}{OD_N} \quad (2)$$

2.4.3. Enzymatic activities

Three enzymatic activities were measured: β -glucosidase (β -Glu), acid (PhA) and alkaline (PhB) phosphomonoesterases. Enzymatic activities were measured by fluorometry using 4-methylumbelliferone as fluorochrome (Marx et al., 2001). The Modified Universal Buffer (MUB), composed of Tris, maleic acid, citric acid, boric acid and sodium hydroxide was used to perform the measurement of enzymatic activities (Frankenberger and Tabatabai, 1982).

Briefly, 1 g of sample was suspended in 19 mL of MUB and 180 μ L were inoculated in a black 96 well microplate. Twenty microliters of substrate dissolved in dimethylsulfoxide (DMSO) were added. Substrates used were 4-methylumbelliferyl phosphate (MUF-P) at 2 mM for PhA and PhB activities (Drouillon and Merckx, 2005) and 4-methylumbelliferyl glucopyranoside (MUF-G) at 5 mM for β -Glu measurement (King, 1986). For β -Glu and PhA, MUB buffer was adjusted at pH 6, and at pH 11 for PhB. For controls, MUF substrate was replaced by 20 μ L of the appropriate MUB buffer. Calibration curves from 0 to 8 μ g mL⁻¹ of MUF were performed with soil suspensions according to Marx et al. (2001). Fluorescence was measured every 2 min during 3 h at an excitation wavelength of 325 nm and an emission wavelength of 450 nm, with a microplate reader Infinite® M200 (Tecan, Switzerland). Enzyme activities were expressed in mU (nanomole of MUF released per minute) and per gram of dry weight soil within each concentration modality (mU g DW⁻¹). Results were finally expressed as percentage of activity compared to the control soils.

2.4.4. PCR-RFLP fingerprints

Restriction fragment length polymorphism (RFLP) fingerprint tool was used to determine whether increasing concentration in red mud residues can have an influence on the genetic structure of microbial communities of the S2 soil (S1 soil was not tested because of DNA amplification failures). With this aim, HinfI and MboI restriction enzymes were used to investigate fungal and bacterial community structures, respectively. DNAs were extracted with ZymoBIOMICS™ DNA Mini Kit (Zymo Research, USA) according to the manufacturer recommendations. DNAs were thereafter stored at -20 °C prior to fingerprint analyses. For bacteria, 16S rRNA genes were PCR amplified using 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGYTACCTTGTACGACTT-3') primers. For fungi, the ITS region was amplified using ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4-R (5'-CAGACTT(G/A)TA(C/T)ATGGTCCAG-3') primers. DNA quantity was standardized in each PCR well by Nanodrop Spectrophotometer (Thermo Fischer, USA) prior to PCR amplification. PCR mix consisted of 12.5 μ L AccuStart™ II PCR ToughMix® (QuantaBio, USA), 1 μ L primers and 11.5 μ L DNA-nuclease free water mix (water and DNA fractions were determined by QuBit measurement). Amplification reactions were performed in a thermocycler with initial denaturation at 94 °C for 3 min, followed by 40 cycles of 30 s at 94 °C, 30 s at 55 °C, 2 min at 72 °C, followed by a final extension at 72 °C for 5 min. Then, enzymatic digestion was performed using the mix: 10 μ L of PCR product, 18 μ L of nuclease free water, 2 μ L of buffer and 1 μ L of enzyme, during 4 h at 37 °C. Restriction endonucleases used were MboI for bacteria and HinfI for fungi, according to the manufacturer recommendations for each (Thermo Scientific, USA). A 3% agarose gel, dissolved in Tris Borate EDTA (TBE) stained with ethidium bromide, was used to perform the electrophoresis gel migration. Gel image acquisition was performed under UV light using GelDoc™ XR (Biorad, USA). A binary matrix was built with the presence or absence of fragments generated by the enzyme digestion. The Dice similarity coefficient was used to construct dendrograms and to evaluate the similarity between mesocosm fingerprints, and microbial structures were considered the same when similarity was above an arbitrary threshold of 0.7. Analyses were made

with the XLSTAT, 2016 software (Addinsoft, France).

2.5. 2.5 Statistical analyses

First, the hypotheses of data normality (Shapiro-Wilk test) and variance homogeneity (Levene test) were tested prior to analyses. For variables that respected these two conditions, ANOVA were performed to test the concentration factor (within subject factor; 5 levels: 10%, 20%, 30%, 40%, 50%) and the bauxite residue factor (between subject factor; 4 levels: PRO, MBR-PRO, GUI, MBR-GUI), at a significance level of $P < 0.05$, to assess which factor had the strongest effect on the microbial variables. Then, a *post-hoc* test of pairwise multiple comparisons of Tukey (honestly significant difference, HSD) was performed on the concentration factor to assess whether its various levels were significantly different from each other, at a significance level of $P < 0.05$. A Dunnett *post-hoc* test was performed to verify whether the different concentrations were significantly lower (left-tailed test) or higher (right-tailed test) than the controls, at a significance level of $P < 0.05$. For variables that did not respect the two conditions, the same factors were tested but with appropriate analyses. The Welch ANOVA (adjusted F) and the *post-hoc* test of pairwise multiple comparisons of Tamhane's T2 were used, both at a significance level of $P < 0.05$. A Kruskal-Wallis test was performed on data to assess whether the different concentrations were different from the control, at a significance level of $P < 0.05$.

For each soil, a principal component analysis (PCA) was performed on microbial and physico-chemical (pH and EC) datasets. For the similarity matrix calculations, the Pearson's correlation coefficient was used. All these analyses were conducted using XLSTAT software version 2016 (Addinsoft™, France).

3. 3 Results

3.1. Physico-chemical analyses

Both S1 and S2 soils belonged to the loam textural class (USDA, United States Department of Agriculture) and their total organic carbon (C_{org}) content, WHC and pH were also similar. EC was superior in S1 (112 μ S cm⁻¹) than in S2 (61.3 μ S cm⁻¹), and CEC was higher in S2 (S1 soil: 9.4 cmol⁺ kg⁻¹ DW, S2 soil: 17.0 cmol⁺ kg⁻¹ DW). Texture of bauxite residues depended on the BR type and the gypsum amendment. GUI and PRO had a clay and clay-loam texture, respectively. Their respective MBR had both loam textures. EC, ESP, CEC and pH values in PRO residue were much higher than in GUI residue. For both residues, gypsum addition resulted in clearly decreased values of these variables (Table 1). Decrease of CEC in MBR was probably because of particle aggregation by Ca²⁺ and the loss of clay-like fraction. Overall, the four residues were characterized by very poor contents in C, N, P and a particle density around 3 g mL⁻¹ (Table 1).

Both soils originated from calcareous mineral parent materials, and thus had high amounts of total Ca (Table 1). For the other major elements (Na, Mg, K, Mn, Fe), contents were also similar for both soils, except for Al, which was considerably more abundant in S2 soil. Concentrations in metals and metalloids were low in both soils (Table 1). Compared to soils, residues were characterized by high contents in V and Cr (Table 1) and, to a lesser extent, Ni, Co, As, Sn, Sb and Pb (Supplementary data, Table S1). Sodium in both residues decreased with gypsum addition and leaching treatment (Table 1).

Bioavailable (DTPA-extractable) element concentrations were the highest in PRO, while concentrations in GUI were, most of the time, close to 0 (Table 2). Aluminium and Cr were the most abundant in PRO, followed by V. Bioavailable trace metal contents were lower in MBR-PRO than in PRO, suggesting an effect of the leaching performed during gypsum modification of PRO. For GUI and MBR-GUI, some values were equal or slightly higher in the modified residue, but, as content was low in both residues, this result could be caused by the uncertainty of the

Table 2
Bioavailable (DTPA extractable) trace metal elements in the four bauxite residues prior to incubation.

Residues	Bioavailable elements ($\mu\text{g g}^{-1}$)									
	Al	Cr ^a	V	Fe	Mo ^b	Ti ^b	Cd ^b	Co ^b	Ce ^b	Cu ^b
PRO	15.4	14.2	8.0	2.2	1.3	0.4	< LOD	< LOD	0.7	0.6
MBR-PRO	1.2	< LOD	1.4	1.4	< LOD	< LOD	< LOD	< LOD	< LOD	0.3
GUI	1.7	< LOD	1.9	3.1	< LOD	1.2	1.2	1.2	0.4	< LOD
MBR-GUI	2.4	< LOD	2.0	4.4	< LOD	< LOD	< LOD	< LOD	0.4	< LOD

PRO: residue from Provence bauxite; MBR-PRO: gypsum modified residue from Provence bauxite; GUI: residue from Guinean bauxite; MBR-GUI: gypsum modified residue from Guinean bauxite.

< LOD: under limit of detection.

^a LOD = $1 \mu\text{g g}^{-1}$

^b LOD = $0.2 \mu\text{g g}^{-1}$

measurement. Bioavailable As, Sb, Ni, Pb, Zn and Mn concentrations were all under the detection limit in the four bauxite residues (data not shown).

3.2. pH, electrical conductivity (EC) and exchangeable sodium percentage (ESP)

pH in soil-residue mixtures was not affected in the same way by each residue. From a concentration of 10% PRO, the pH increased about 1 unit in both soils, then slowly rose to reach the pH of PRO alone (*i.e.* modality 100%, pH = 10.8). For the other residues, pH of the soils alone and of the mixtures at different concentrations were quite similar (Fig. 1).

EC increased linearly in both soils with residue concentrations. Highest conductivity values were observed within PRO mixtures, PRO having the highest intrinsic EC value ($1800 \mu\text{S cm}^{-1}$ on average). In contrast, the lowest values were observed within MBR-GUI mixtures, corresponding to residue inputs with the lowest initial EC ($900 \mu\text{S cm}^{-1}$ on average).

Exchangeable sodium percentage (ESP, %) was calculated as the proportion of exchangeable Na compared to exchangeable Ca, K and Mg. Exchangeable Ca was similar in both soils and exhibited strong values in the two Modified Bauxite Residues (MBR-PRO and MBR-GUI), which is a consequence of gypsum addition (Supplementary Data, Table S2). Surprisingly, the exchangeable Ca content was also high in the GUI residue and could be a consequence of an industrial process prior stacking which we are not aware of. PRO residue exhibited the highest content in exchangeable Na. It was strongly decreased by gypsum amendment (-71% between PRO and MBR-PRO exchangeable Na content, Table 1). In the case of the GUI residue, the decrease in exchangeable Na following gypsum addition was similar (-67%). In mesocosms, the ESP was gradually increased with increasing concentrations of MBR-PRO, GUI, MBR-GUI residues in the two soils (Fig. 1). The maximal ESP values were observed in 50% BR-soil mixtures, reaching about 25%, 17% and 8% for MBR-PRO, GUI and MBR-GUI respectively, in both soils. The ESP measured in PRO-soil mesocosms did not exactly exhibited the same response pattern between the two soils. In the soil 1, ESP values increased gradually, as for the other residues, reaching a maximum of 21% in the 50% modality. In the soil 2, for an unknown reason, the ESP increased strongly to reach a maximum value of 75% in the 50% modality of PRO-Soil 2 mixture. ESP and EC were significantly and positively correlated, and correlation values obtained with the different mesocosms were the same in both soils (0.70 and 0.71, Supplementary Data, Table S3).

3.3. Concentration effects on microbial variables

3.3.1. Growth of culturable bacteria

Growth of culturable bacteria did not have the same response patterns in the two soils (Supplementary Data, Fig. S1). In S1 soil, only the

PRO residue significantly reduced bacterial growth, and only for the highest concentrations (*i.e.* 40 and 50%). In S2 soil, more significant effects were observed with regard to the residue used. Depending on the residue concentration, all the residues exhibited significant bacterial growth inhibition. PRO and GUI unamended residues showed higher inhibitory values compared to their respective modified residue. ANOVA revealed that concentration was the main factor affecting growth of culturable bacteria in both soils (Supplementary data, Table S4).

3.3.2. Community Level Physiological Profiles (CLPPs, Biolog EcoPlates™)

Considering AWCD_{90h}, ANOVA revealed that both concentration and residue type had significant effects on both soils, although the concentration factor had a greater inhibitory effect (Supplementary data, Table S4). In the S1 soil, increasing concentrations of unamended BR PRO and GUI had the strongest inhibitory effect. MBR were less inhibitory and MBR-GUI exhibited the lowest inhibition levels. In S2 soil, the four BR showed a significant inhibition effect at similar levels, and the MBR were more detrimental compared to Soil 1 (Fig. 2).

Shannon Index (H'_{90h}) (Fig. 2) was used to measure microbial diversity in Biolog EcoPlates™ assays. In the S1 soil, only PRO at increasing concentrations decreased significantly H'_{90h} , even if H'_{90h} was not significantly different ($P < 0.05$) from the control. MBR residues did not have negative effects on H'_{90h} . Response patterns partially differed in S2 soil. MBR-PRO showed a stronger decreasing effect than PRO. For GUI, responses observed were the same as in the S1 soil, *i.e.* significant decreasing effect on H'_{90h} by the unamended GUI residue (between concentrations), and no effect of its modified residue (MBR-GUI). ANOVA showed that the concentration factor had a stronger inhibitory effect on H'_{90h} than residue type factor in both soils (Supplementary data, Table S4).

Finally, richness (S) followed almost exactly the same response patterns as the H'_{90h} (data not shown) in the different BR-soil mixtures.

3.3.3. Enzymatic activities

Results of ANOVA (Supplementary data, Table S4) showed that, in both soils, all enzyme activities were significantly affected by the residue type, the residue concentration and the interaction of the two, except for PhB. Response patterns differed according to enzymes, residues and soils (Fig. 3).

In all mixtures, β -glucosidase was always significantly inhibited compared to the control modality, and this inhibition was strongest in the S1 soil. Except for MBR-PRO, increasing concentrations of residues affected β -Glu activity. In the S2 soil, only PRO showed this concentration inhibition pattern, whereas null or converse inhibition effects with increasing concentrations were observed for the other residues.

PhA activity globally showed similar response patterns to β -Glu regarding the residues tested, their concentrations and the soils used. The main difference observed was when GUI residue was tested with

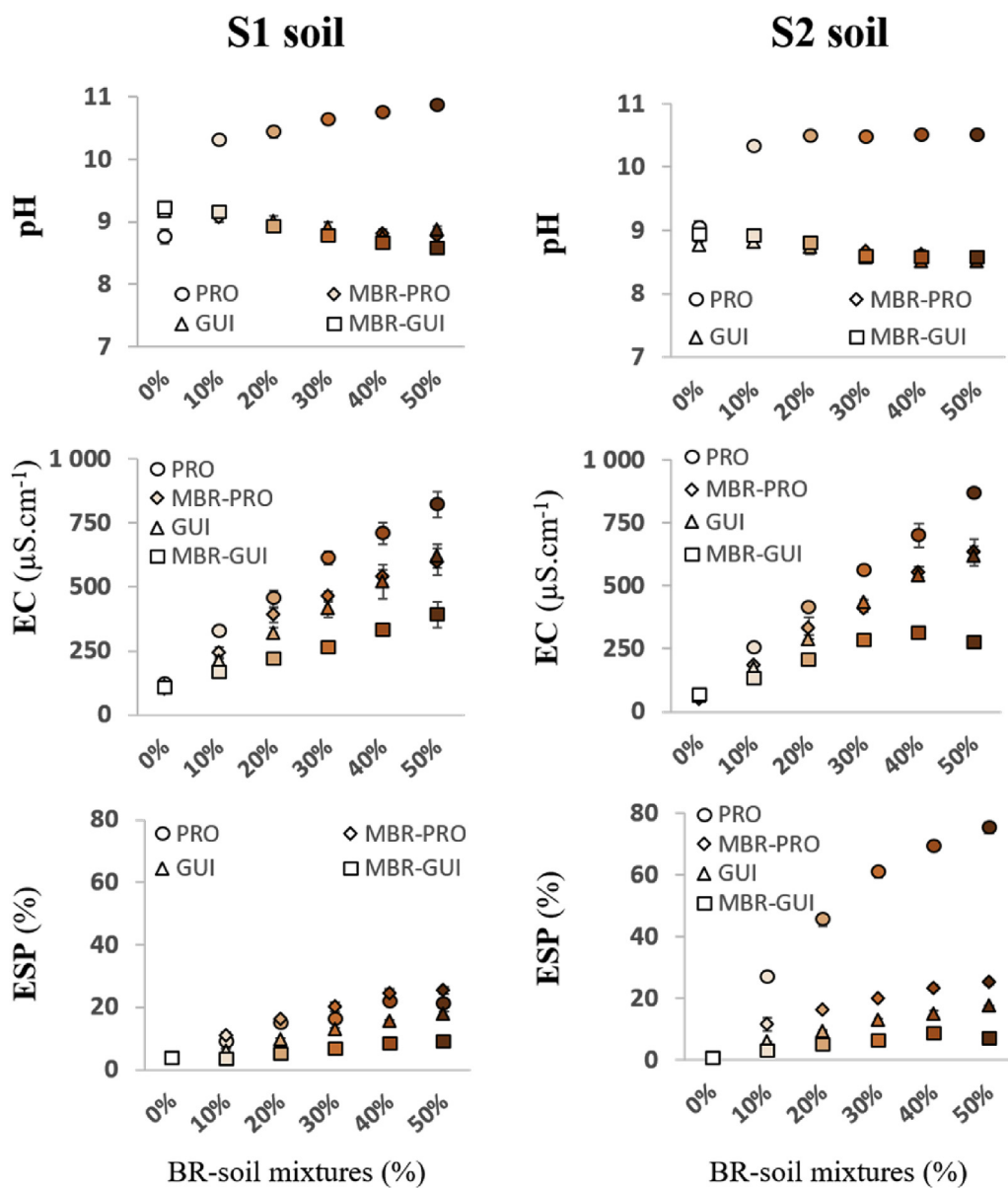


Fig. 1. Influence of bauxite residues at different concentrations on pH, electrical conductivity (EC, $\mu\text{S cm}^{-1}$) and exchangeable sodium percentage (ESP, %) in bauxite residues-soil mesocosms after 35 days of incubation. For pH and EC, markers are means ($n = 5$) \pm their standard deviations. For ESP, markers are means ($n = 3$) from analytical repetitions, with their relative standard deviation (rsd). PRO: residue from Provence bauxite; MBR-PRO: gypsum modified residue from Provence bauxite; GUI: residue from Guinean bauxite; MBR-GUI: gypsum modified residue from Guinean bauxite. \square : 0%, \square : 10%, \square : 20%, \square : 30%, \square : 40%, \square : 50% bauxite residue concentration in BR-soil mixtures.

the S2 soil. PhA activity of S2 soil amended with this residue were not affected compared to control, except at the highest concentrations (40% and 50%). Conversely, MBR-GUI was far more inhibitory than GUI resulting in about 80% inhibition. PhA was also the only enzyme to be totally inhibited, as observed in S1 soil-20% PRO.

The alkaline phosphatase (PhB) presented quite different response patterns from the other enzyme activities. Only the unamended BR from Provence bauxite (PRO) showed significant inhibitions of this enzyme activity in both soils. For all the other mixtures, no significant inhibitions were observed between control soils and bauxite residues amended soils. Furthermore, trends in increased PhB activities were observed in some mesocosms enriched in MBR residues, especially MBR-PRO in S1 soil and MBR-GUI in S2 soil at all the concentrations tested.

3.3.4. RFLP fingerprints

PRO, MBR-PRO and MBR-GUI at the two concentrations did not significantly alter the structures of the bacterial communities (MboI primers) in the S2 soil (Fig. 4). There was no cluster differentiation within these three residues. For GUI, two clusters were identified, but no clear trend emerged as results of 10% mixtures and control samples

are mixed.

With regard to fungal communities, two clusters were identified in PRO amended soils (Fig. 4). However, the distribution of the different samples in these clusters did not enable us to conclude on any structuration factor. MBR-PRO at 10% and 20% concentrations did not alter the structure of fungal communities, as almost all samples were clustered within the same group. For GUI amended soil samples, responses of fungal communities were similar to those of bacterial communities. Two clusters were identified as well, one with mostly 10% and 20% mixtures samples, and the other with samples from control and 10% concentration. Two clusters were identified in MBR-GUI mixtures, differentiating between control samples and residue-soil mixtures. Control samples had a higher richness in RFLP bands (data not shown).

3.4. Correlations and multivariate analyses

Correlation analyses were performed on biological variables, EC, ESP and pH (Supplementary Data, Table S3). Results showed that EC and ESP were the main factor influencing the biological variables in both soils. A positive and significant correlation was observed between EC and ESP in both soils. When significant, higher negative correlation

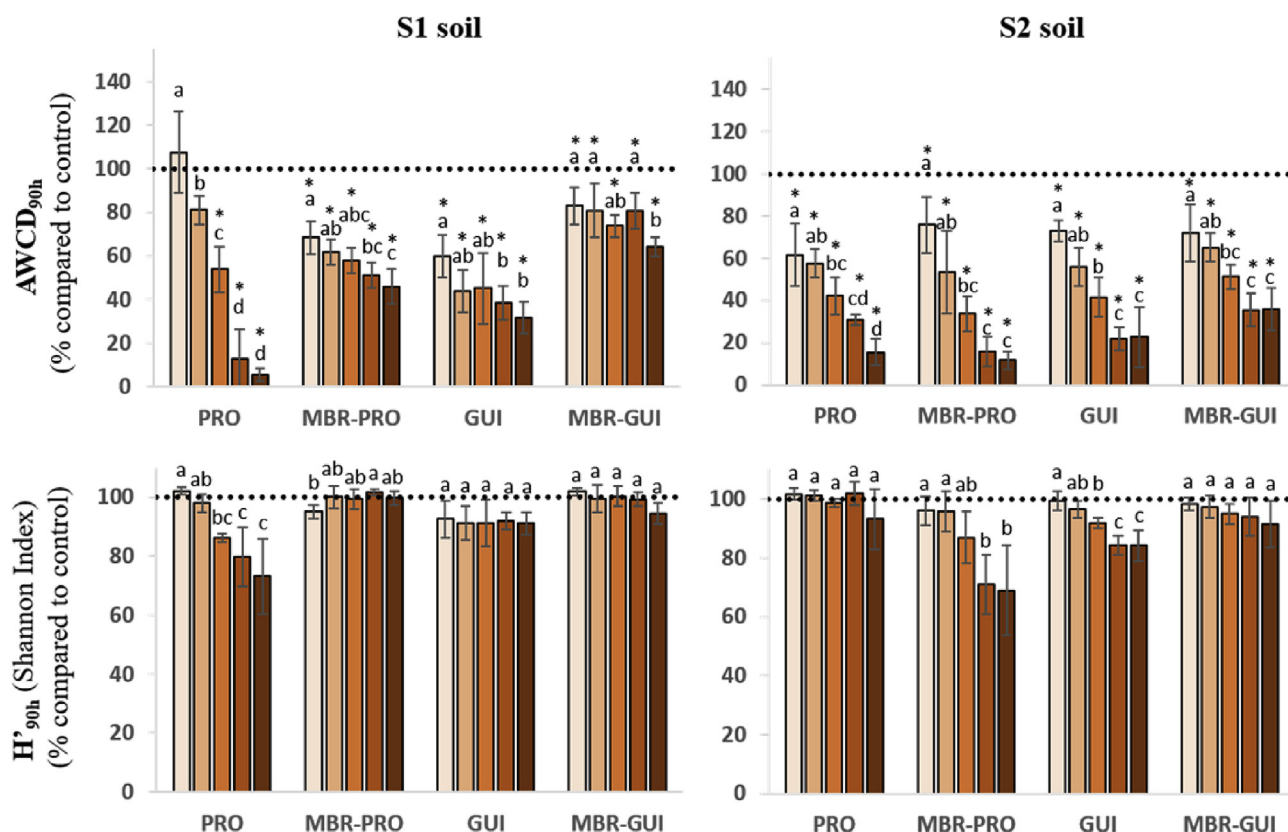


Fig. 2. Influence of bauxite residues at different concentrations on the Average Well Colour Development ($AWCD_{90h}$; Biolog Ecoplates™) and Shannon Index (H'_{90h}) expressed as a percentage of the $AWCD_{90h}$ or H'_{90h} in the uncontaminated control soils. Bars are means ($n = 5$) \pm their standard deviations. ■: 10%, ■: 20%, ■: 30%, ■: 40%, ■: 50% bauxite residue concentration in BR-soil mixtures. Bars within a same residue modality with the same letter do not differ significantly from each other at $P < 0.05$ (Welch ANOVA, post-hoc test Tamhane T2). The dotted horizontal line depicts the $AWCD_{90h}$ or H'_{90h} in the uncontaminated soils. Bars with * indicate that values are significantly different compared to controls at $P < 0.05$ (Kruskal-Wallis test). PRO: residue from Provence bauxite; MBR-PRO: gypsum modified residue from Provence bauxite; GUI: residue from Guinean bauxite; MBR-GUI: gypsum modified residue from Guinean bauxite. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

values were always observed between biological variables and EC rather than with pH. Principal component analyses (PCAs) were performed on the two soil data sets (Fig. 5). For both soils, the opposition of EC with most biological variables was representative of the F1 axes of the PCAs. Factorial maps showed clear inhibition gradients among variables following residue concentrations (Fig. 5). In S1 soil, PRO mixtures with increasing BR concentrations were those where microbial variables were the more strongly impacted by EC, while other residues were more aggregated close to the origin of the F1 axis, and so less impacted by EC. Considering S2 soil mesocosms, the inhibition gradient from 0 to 50% BR concentration was observable and marked for the four kinds of residue along the F1 axis.

4. Discussion

The aim of this study was to assess the effects of several kinds of bauxite residues (BR) on a set of variables related to the microbial functioning of soils. Residues modified with gypsum (MBR) were also considered. This amendment is increasingly used to improve the harsh characteristics of bauxite residues but, depending the variables considered, data are scarce or non-existent about the potential ecotoxicological effects of these MBR on soil microbiota.

The residues tested during our study were generated after alumina extraction of two bauxite ores from different geographical locations (Guinea, GUI and France, PRO). PRO residue had higher EC, pH, ESP, Al total content, bioavailable metal content and clay-likes values, while GUI residue was more enriched in total Cr and V. According to Hackenberger et al. (2019), differences in BR quality can originate both

from bauxite origin and Bayer process efficiency, and this quality may affect BR ecotoxicological effects. With few exceptions, this is what we observed during our study, PRO having, in most cases, more inhibitory effects on microbial functions and activities. This kind of ecotoxicity modulation due to BR origin was also mentioned by Pagano et al. (2005) and Hackenberger et al. (2019), but on animal bioindicators.

In most cases, increasing concentrations of BR (unamended or modified) were associated with inhibition of microbial enzyme activities and average growth rates in Biolog® Ecoplates™ ($AWCD$). This is consistent with a previous study by Ujaczki et al. (2015) conducted with unmodified BR and unpolluted soil and where microbial variables (bacterial counts and *Vibrio fischeri* bioluminescence inhibition) and other test organisms were used to assess the ecotoxicity of BR in BR-soil mixtures. A concentration above 5% was identified by these authors as detrimental to the soil microbial variables they used and was consistent with our results. Considering enzymatic activities, their depletion may be explained at the microbial community and cell levels, but also by direct mechanistic effects of bauxite residue properties on enzyme catalytic properties. Indeed, the microbial growth and the synthesis of soil enzymes by the microbial cells might be low under high saline conditions, especially Na contents. This effect can be aggravated if some metals and metalloids are present in soils because salinity can make them more available and more toxic towards both microbial cells and catalytic sites of enzymes (Raiesi and Sadeghi, 2019). Chowdhury et al. (2011) also mentioned that increased stress levels, such as salinity, can down-regulate enzyme expression as well as modifying the whole microbial community functioning. Also, a well-known effect is that high Na content can makes enzymes less soluble and thus less active. Finally,

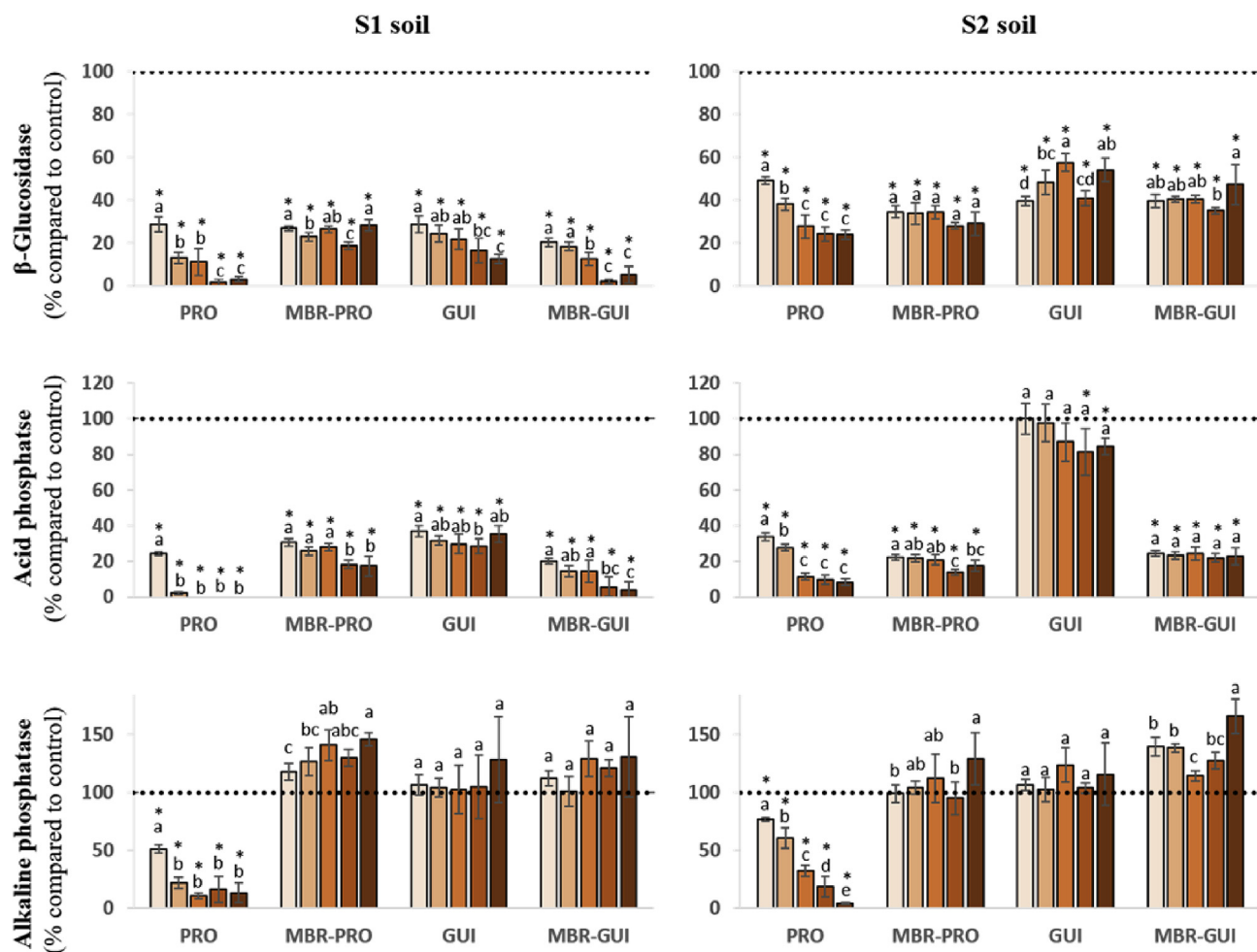


Fig. 3. Influence of bauxite residues at different concentrations on β -glucosidase (β -Glu), acid phosphomonoesterase (PhA) and alkaline phosphomonoesterase (PhB) activities expressed as a percentage of enzyme activities in the uncontaminated soils. Bars are means ($n = 5$) \pm their standard deviations. ■: 10%, ■: 20%, ■: 30%, ■: 40%, ■: 50% bauxite residue concentration in BR-soil mixtures. Bars within a same residue modality with the same letter do not differ significantly from each other at $P < 0.05$ (ANOVA, *post-hoc* test Tukey HSD). The dotted horizontal lines depict the enzyme activity in the uncontaminated control soils. Bars with * are significantly different compared to controls at $P < 0.05$ (Dunnett test). PRO: residue from Provence bauxite; MBR-PRO: gypsum modified residue from Provence bauxite; GUI: residue from Guinean bauxite; MBR-GUI: gypsum modified residue from Guinean bauxite.

a last aspect which might explain the decreased enzymatic activities is related with the enrichment of soils in Al and Fe oxyhydroxides from bauxite residues; an enrichment that is concomitant with salinity/Na levels in mesocosms. Indeed, Al and Fe oxyhydroxides can interact with enzymes by adsorbing, a phenomenon that is pH-dependent. According to [Clausi et al. \(2009\)](#) and to [Quiquampoix and Burns \(2007\)](#) such adsorption processes may alter/denature enzyme structures or occlude catalytic sites and thus decrease enzyme activities.

Our results showed that, rather than pH, EC was the principal factor influencing the inhibition of biological variables. A positive and significant correlation was observed between EC and ESP, which is consistent with previous studies concerning bauxite residues. Indeed, the high content of Na (soluble or in the solid phases) is the main ion explaining the high values of EC in bauxite residues ([Gräfe et al., 2011](#)). In BR research, high Na content and EC were identified as main factors of toxicity for microorganisms and plants ([Klebercz et al., 2012](#); [Ruyters et al., 2011](#)). They were also identified as crucial obstacles to the establishment of microbial communities in BR ([Gräfe and Klauber, 2011](#); [Jones et al., 2012](#); [Santini et al., 2015](#)). Indeed, salinity has been reported to reduce microbial biomass ([Dandapath et al., 2017](#)) and enzyme activities ([Frankenberger and Bingham, 1982](#); [Rietz and Haynes, 2003](#)), and to alter the functional structure and diversity of microbial communities ([Guo et al., 2019](#)) in soils. In further studies, it would be

very interesting to leach/desorb Na cations from the bauxite residue to the range present in soils, add such residue to a soil, and thus to examine microbial activity of such mixture. Such experiment could verify the hypothesis that EC and ESP are the main factors that affect soil microbial activity. However, many studies reported that in BR, salinity was not the sole factor explaining the ecotoxicity. The presence of trace elements in high concentrations has also been linked to toxicity in BR ([Fuller and Richardson, 1986](#); [Milačić et al., 2012](#); [Mišík et al., 2014](#)). Total concentrations of metals and metalloids cannot be used as a proxy of metal toxicity in soils, since their toxicity is related to multiple physical and chemical parameters, such as pH, redox potential (Eh), salinity or the presence of organic and mineral ligands ([Violante et al., 2010](#)). For example, alkaline pH results in the immobilization of most of metals. This was highlighted by previous studies in acidic and/or metal polluted soils, where the application of alkaline bauxite residues led to a reduction of the mobility and bioavailability of toxic metals ([Garau et al., 2011, 2007](#); [Lombi et al., 2002](#)). However, this cannot be generalized to every element, as the mobility of certain elements, such as metalloids (e.g. As, Sb) is enhanced in alkaline conditions ([Al-Abed et al., 2007](#); [Lehoux et al., 2013](#); [Mišík et al., 2014](#)). In soils, metals and metalloids, when bioavailable and present in high concentrations, are known to inhibit several microbial essential functions, such as respiration or enzyme activities ([Geiger et al., 1999](#); [Chen et al., 2018](#);

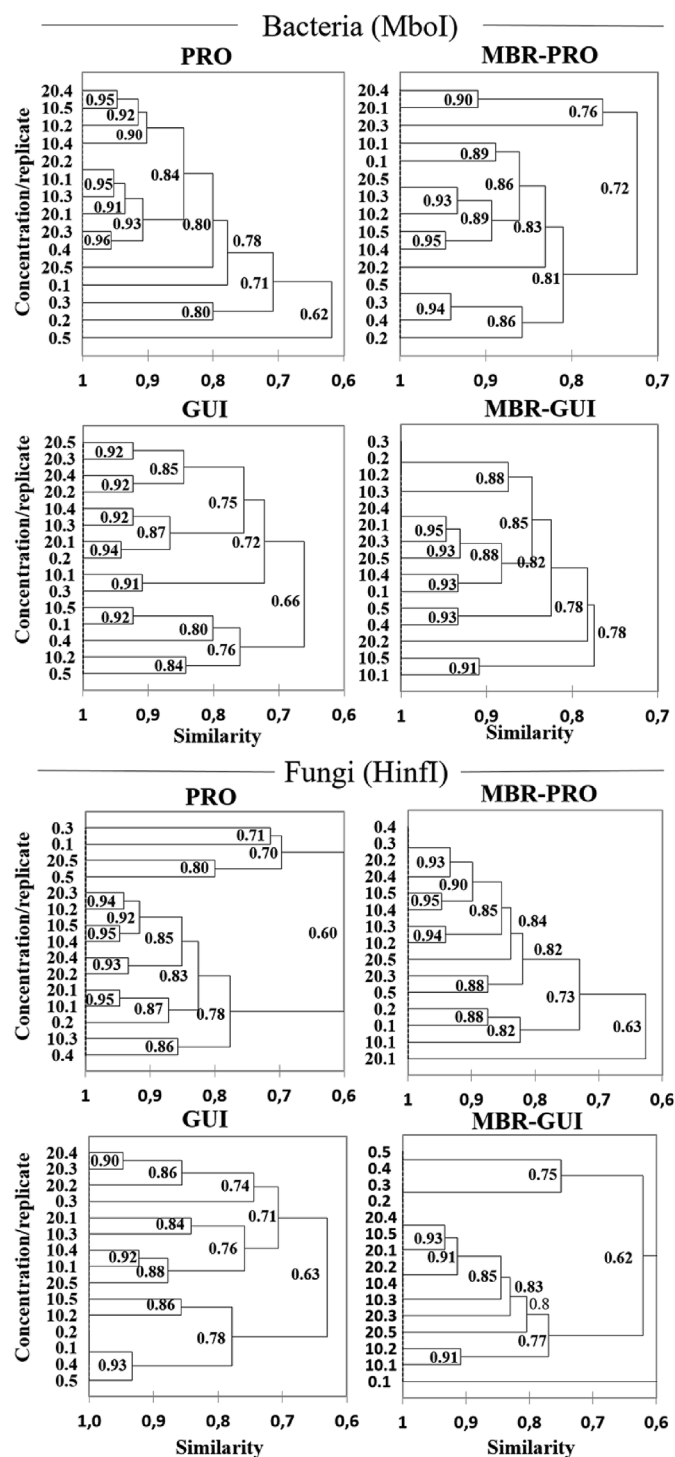


Fig. 4. Influence of bauxite residues at different concentrations on cluster analysis of PCR-RFLP fingerprints showing similarities among mesocosms. Each dendrogram stands for 3 concentrations of residue (0, 10 and 20%) mixed only with the S2 soil. Two restriction endonucleases were used: MboI for bacteria, on the top, and Hinfi for fungi, on the bottom. Y axis: the first number depicts concentration modality and the second number depicts replicate (e.g. 10.3 means 10% residue and mesocosm replicate n°3) (n = 5). PRO: residue from Provence bauxite; MBR-PRO: gypsum modified residue from Provence bauxite; GUI: residue from Guinean bauxite; MBR-GUI: gypsum modified residue from Guinean bauxite.

Chu, 2018). Moreover, in BR research, Al (Fuller and Richardson, 1986) and V (Mišik et al., 2014) were previously identified as toxic on higher plants in laboratory studies. However, in our study, the results for

DTPA-extractable elements were low or not detectable for every metal and metalloids considered, which suggests that they were not a main source of toxicity. Nevertheless, the BR assessed here exhibited high values of total content for elements such as Al, Co, Cr, Ni, V (among others), and that could be a problem if their mobility was modified by changes in physical and/or chemical conditions (e.g. pH, Eh, organic ligand inputs), as it was highlighted by Anton et al. (2012).

Gypsum (CaSO₄·2H₂O) amendment efficiently reduced some physico-chemical characteristics such as EC and pH of the BR assessed in our study, although to a more limited extent than observed in the literature (Courtney and Kirwan, 2012; Courtney and Timpson, 2005; Wong and Ho, 1993). The gypsum-leaching treatment has been widely studied and used in greenhouse and field experiments for its capacity to reduce the Na content (soluble or in solid phases) of BR by bringing Ca²⁺ to replace Na⁺ on the exchange complexes (Lehoux et al., 2013). By this mechanism, it causes the decrease of several physico-chemical characteristics such as pH, ESP and EC (Gräfe and Klauber, 2011; Burke et al., 2013), diminishes soluble ion contents (Li et al., 2019) and improves the structure by diminishing the clay-like fraction (Wong and Ho, 1991; Courtney and Harrington, 2012). This amelioration has beneficial effects on microbial communities, and gypsum application was found to enhance enzymatic activities (Courtney et al., 2014a) and the establishment of bacterial communities (Schmalenberger et al., 2013) during the remediation of BR tailings. Moreover, gypsum addition was recommended by Lehoux et al. (2013) as an emergency measure to reduce and buffer pH in BR contaminated soils after the Ajka (Hungary) spill. In our study, MBR-PRO and MBR-GUI showed equal and sometimes lower inhibiting effects on all the measured microbial variables compared to their respective unamended residues (except for PhB), which suggests an improvement of the conditions for microbial communities. However, even if physico-chemical characteristics of modified residues were improved, microbial activities still remained significantly lower than in the control soils, except for PhB and H'90h. This result showed that in the short term, modified residues mixed with soils conserve inhibitory effects on some microbial variables.

No significant trend was observed when assessing the structure of microbial communities in our experiment with unamended or modified BR. This is inconsistent with previous field studies, where significant modifications in microbial structure were found during the rehabilitation process in BR tailings (Banning et al., 2011; Garau et al., 2007). In contrast, with regards to functional diversity, bauxite residues at different concentrations almost always showed strong inhibitory effects on the average growth rates of microbial communities in Biolog Eco-plates™ (AWCD_{90h}). However, this did not imply that the functional diversity of these communities was systematically modified by BR inputs. In many of cases, it could be observed that functional diversity indicators (i.e. functional richness, S, and Shannon index, H') were not altered, and this was especially true with gypsum modified BR. This was also consistent with molecular structure indicators, since poor variations in DNA-fingerprints were observed. Finally, our various data indicate that a loss of activity, including enzyme activity, cannot be systematically associated with a modification of the functional diversity or of the molecular structure of the microbial community. Long-term research with more effective technologies such as pyrosequencing are necessary to confirm this statement and observe the tolerance and potential shifts of an autochthonous microbial community impacted by BR contamination. Also, with regards to microbial metabolic diversity, molecular tools based on RNA analysis would be useful to obtain complementary data about the impact of bauxite residues on microbial metabolic pathways.

5. Conclusion

This study showed the inhibiting effect of increasing concentrations of two bauxite residues on microbial community functions and

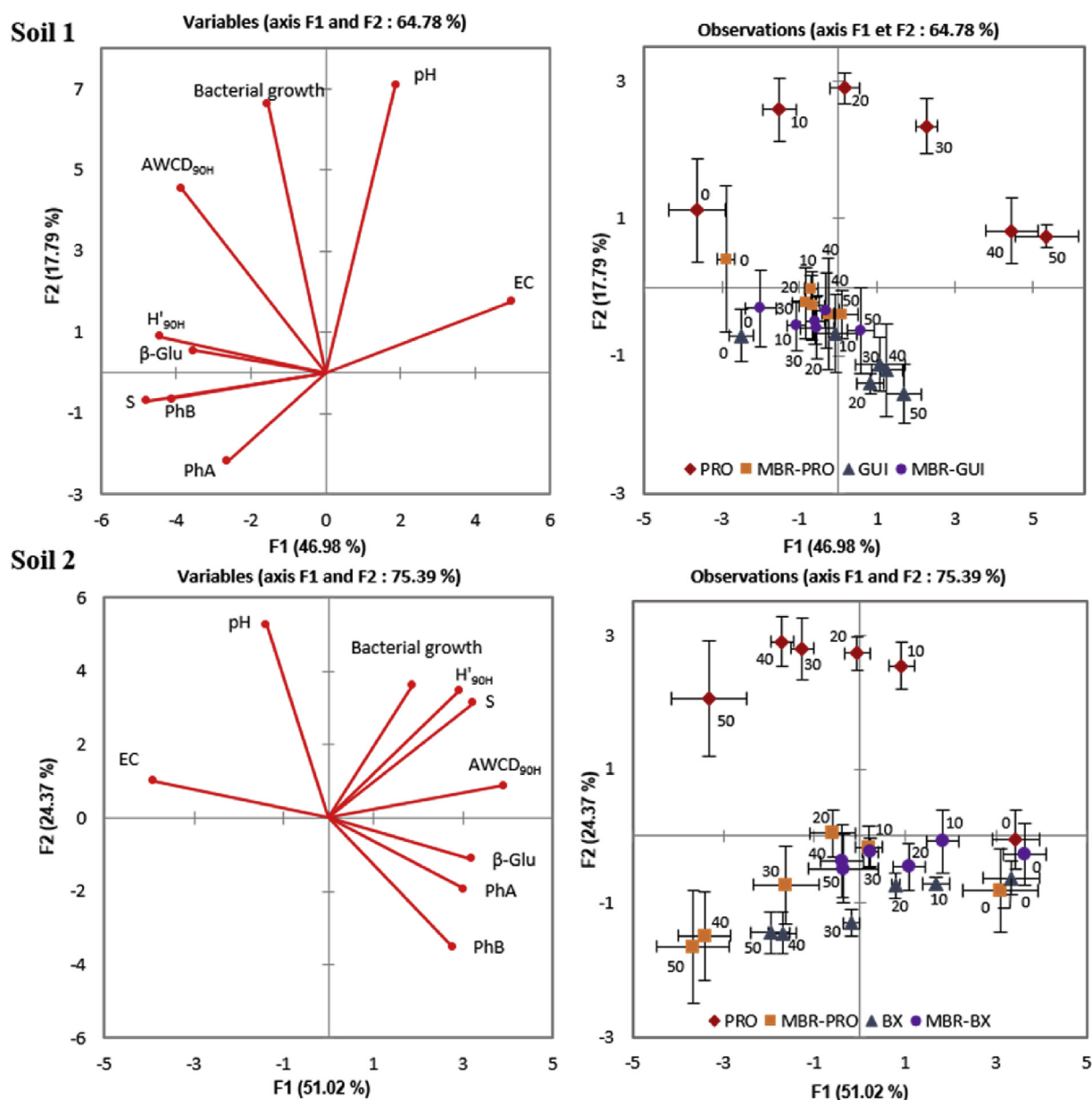


Fig. 5. Principal component analysis (PCA) of the biotic and physico-chemical variables accounting for Soil 1: 66.8% and Soil 2: 69.8% of total variance. For each point, value refers to bauxite residue concentration ($n = 5$) with their standard deviations (brackets). Diamonds: PRO, residue from Provence bauxite; Squares: MBR-PRO, gypsum modified residue from Provence bauxite; Triangles: GUI, residue from Guinean bauxite; Circles: MBR-GUI, gypsum modified residue from Guinean bauxite. β -Glu: β -glucosidase; PhA: acid phosphomonoesterase; PhB: alkaline phosphomonoesterase; AWCD_{90h}: Average Well Colour Development after 90h of incubation (Biolog EcoPlates™); S: richness (Biolog EcoPlates™); H'_{90h}: Shannon diversity index after 90h of incubation (Biolog EcoPlates™), EC: electrical conductivity. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

structure. EC and ESP appeared as the main factor explaining the inhibition impact on microbial variables, but further studies are needed to clearly assess the influence of trace metals and soluble ions. The two tested unamended residues, differing in the origin of the bauxite ores, showed different inhibition impacts on microbial variables, indicating the importance of the mineralogical composition and origin of the BR (bauxite ore and the efficiency of processes used to extract alumina). Gypsum amendment and repeated leaching with water successfully improved the physico-chemical characteristics of their respective initial unamended residues. The modified bauxite residues had an equal or, more interestingly, lesser inhibiting effect on microbial variables than the unamended BR. However, the microbial activities were always significantly lower compared to controls, except for PhB. Results from this work also showed that if the activities of microbial communities

were clearly impacted by BR, their diversity of functions and structure were not or were weakly altered, although we anticipate that more effective technologies, such as metabarcoding, are necessary to confirm this last trend.

CRediT authorship contribution statement

Camille Fourier: Conceptualization, Data curation, Methodology, Formal analysis, Resources, Supervision, Validation, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Mathieu Luglia:** Conceptualization, Methodology, Investigation, Validation, Visualization, Formal analysis, Resources, Writing - review & editing, Supervision. **Pierre Hennebert:** Conceptualization, Investigation, Methodology, Writing - review & editing. **Julie Foulon:**

Methodology, Formal analysis, Investigation, Validation, Visualization, Resources, Writing - review & editing. **Jean-Paul Ambrosi:** Formal analysis, Methodology, Resources, Validation, Writing - review & editing. **Bernard Angeletti:** Resources, Formal analysis, Methodology. **Catherine Keller:** Formal analysis, Methodology, Visualization, Resources, Writing - review & editing. **Stéven Criquet:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Validation, Visualization, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

None.

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