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Microbial composition of Saharan dust plumes deposited as red rain in Granada (Southern Spain)

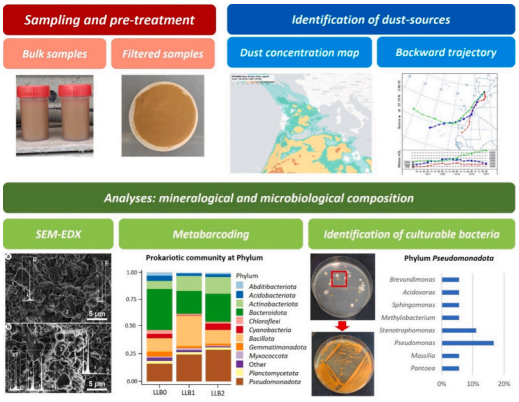
Azahara Navarro [a], Ana del Moral [b,*], Bettina Weber [c,d], Jens Weber [c], Alberto Molinero [a], Rafael Delgado [a], Jesús Párraga [a], Fernando Martínez-Checa [b]

[a] Department of Edaphology and Agricultural Chemistry, University of Granada, Granada, Spain
[b] Department of Microbiology, University of Granada, Granada, Spain
[c] Institute of Plant Sciences, University of Graz, Graz, Austria
[d] Multiphase Chemistry Department, Max Planck Institute for Chemistry, Mainz, Germany

HIGHLIGHTS

- Metabarcoding analysis was conducted on red rain samples collected in Granada.
- Metabarcoding revealed high abundance of the bacterial phyla *Bacillota* and *Pseudomonadota;* fungi were dominated by *Ascomycota* and in one sample by *Chytridiomycota*.
- Extremophilic microorganisms (*Peribacillus frigoritolerans* and *Bacillus halotolerans*) were isolated from the red rain samples.
- Nanobacteria were identified in SEM imagery of red rain samples.

GRAPHICAL ABSTRACT



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ABSTRACT

During durst storms, also biological material is transported from arid areas such as the Sahara Desert. In the present work, rain samples containing significant amounts of mineral dust have been collected in Granada during different red rain episodes. Biological features (bacteria, biofilm, pollen grain and fungal spore) as well as size-particle distribution and mineralogical composition were studied by SEM. Nanobacteria were observed for the first time in red rain samples. A preliminary metabarcoding analysis was performed on three red rain samples. Here, *Bacillota* made up 18 % and *Pseudomonadota* 23 % of the whole prokaryotic community. The fungal community was characterized by a high abundance of *Ascomycota* and, dependent on the origin, the presence of *Chytridiomycota*. By means of 16S rRNA sequencing, 18 cultivable microorganisms were identified. In general, members of the phyla *Pseudomonadota* and *Bacillota* made up the majority of taxa. Some species, such as *Peribacillus frigoritolerans* and *Bacillus halotolerans* were isolated during three different red rain episodes. Generally, red rain carries a wide variety of microorganisms, being their ecosystem and health effects largely unknown.

* Corresponding author.
E-mail address: admoral@ugr.es (A. del Moral).

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1. Introduction

Aerobiology is a branch of biology that studies organic particles, also known as bioaerosols, such as dispersal units, cells or fragments of bacteria, viruses, fungi, insects or pollen (Kellogg and Griffin, 2006). Airborne bioaerosols may account for up to 25 % of the atmospheric aerosol load (Carslaw, 2022; Diehl et al., 2006; Fröhlich-Nowoisky et al., 2016; Maki et al., 2022). They can also pose a threat to health because of their potential role as vectors for human, animal, and plant pathogens (Huffman et al., 2013), causing allergies and different diseases (Cao et al., 2014; He et al., 2016; Reinmuth-Selzle et al., 2017; Ren et al., 2014; Sadakane et al., 2022; Shiraiwa et al., 2017).

The atmosphere is the largest biome on Earth, but it probably remains the most obscure environment when it comes to microbial functioning (Péguilhan et al., 2023). Atmospheric bacteria are subjected to various environmental stressors, such as UV radiation, extreme temperatures, desiccation, oxygen deficit and lack of nutrients (Aguilera et al., 2018; Chen et al., 2023; Fröhlich-Nowoisky et al., 2016; Lin et al., 2022; Smith et al., 2011). Although the source of atmospheric bacteria is still not fully explained, their origin is likely linked to terrestrial vegetation, soils and bodies of water (Smets et al., 2016).

Arid regions, such as deserts, constitute the major source of mineral dust particles in the atmosphere (Ginoux et al., 2012), supplying large amounts of dust (Zender et al., 2004) that can favour the spread of microbial communities via natural atmospheric pathways (Federici et al., 2018; González-Toril et al., 2020; Gat et al., 2022). The Sahara Desert is the most important source of mineral dust in the world, which is subsequently transported southwards (60 %) and westwards to the Atlantic (25 %), eastwards to the Middle East (5 %) and northwards to Europe (10 %) (Goudie and Middleton, 2006; Shao et al., 2011).

The Mediterranean area, in particular Spain and especially the city of Granada, located in the southeast of the Iberian Peninsula, is being increasingly affected by dust intrusions (Párraga et al., 2021). This phenomenon can be accompanied by a characteristic precipitation known as red rain, dust rain, bloody rain, coloured rain or muddy rain (Ávila and Peñuelas, 1999; Fiol et al., 2005).

Díaz-Hernández and Párraga (2008) described for the first time a type of giant quasi-spherical particles generated in the troposphere, which they called "iberulites" (50–200 μm diameter). Both morphological and compositional studies of iberulites were carried out, providing information on their genesis in the troposphere and highlighting their role as potential carriers of microorganisms (Párraga et al., 2021).

Airborne microbes attached to mineral particles can travel over long distances and then disperse into new locations, being able to colonize new environments and contributing to an increase in the bacterial diversity (Rahav et al., 2020; Varga, 2016). During the transport, airborne dust particles can protect bacterial cells from desiccation and favour their survival because of their high content in nutrients and atmospheric water (Amato et al., 2017).

A striking type of nano-sized particles (20–150 nm diameter) has been described as nanobacteria, being an order of magnitude smaller than bacteria (Psenner and Loferer, 1997; Uwins et al., 2000). Nanobacteria were defined for the first time as "stressed or resting forms of larger bacteria" (Morita, 1988). Afterwards, Folk (1993) also coined this term to describe them as "quasi-spherical small objects in rocks with coccoidal or rod shapes" (Folk, 1993). Nanobacteria have been detected in wastewater(Kim et al., 2004), animal (Breitschwerdt et al., 2001) and human blood (Martel et al., 2008). Besides, they have also been observed in the stratosphere (Sommer et al., 2004) and in meteorites (Benzerara et al., 2003). Recent studies show that they are involved in the carbonate and soil microstructure formation (Astafieva and Balaganskii, 2018).

Red rain has been extensively studied at the chemical and mineralogical level (Ávila et al., 1998, 2007; Rodríguez-Navarro et al., 2018), but little is known from a microbiological perspective (Itani and Smith, 2016). Most studies have focussed on describing the microbiota transported in airborne dust (Federici et al., 2018; Gat et al., 2022; Giongo et al., 2013; Meola et al., 2015; Petroselli et al., 2021; Sánchez De La Campa et al., 2013), whereas the microbiota in rad rain (wet deposition) have only rarely been studied (Itani and Smith, 2016; Peter et al., 2014; Péguilhan et al., 2023).

The aim of this work was to study the microbial communities contained in red rain, being scarcely studied so far. For that, red rain samples have been collected in the city of Granada (Southern Spain), an area frequently affected by dust intrusions, to analyse the microbial diversity, mineralogical composition and the possible relationship between mineral particles and microbes. We applied advanced methods (SEM-EDX, identification of the 16S rRNA gene, metabarcoding, etc.) to achieve this goal. This work shows that red rain acts as carrier of microorganisms and biological material. Based on this, we will be able to address other studies that may reveal microbial transport patterns and improve our knowledge about the atmospheric extreme life.

2. Materials and methods

2.1. Sampling site

This study was conducted in the metropolitan area of the city Granada (37° 08′ 59″ N, 03° 37′ 59″ W, 650 m.a.s.l.), located in the southeast of the Iberian Peninsula, around 50 km from the Mediterranean Sea. The climate is semi-arid to dry ombrotype and meso-Mediterranean thermotype with marked cycles of drought and precipitation every 5 to 10 years. The basin of Granada is often affected by Saharan dust intrusion events and red rain episodes, because of its proximity to North Africa (Valenzuela et al., 2012).

2.2. Collection of red rain

The passive collector of red rain was installed on the roof of the Faculty of Pharmacy (University of Granada) (Fig. 1), being a 10-metre-high building situated in the metropolitan area of Granada city (37° 11′43″ N, 3° 35′ 46″ W, 760 m.a.s.l.).

Samples were collected after 11 red rain episodes bound to nine dust events, which were recorded in February 2017 and from May 2021 to March 2022 (Table 1). For that purpose, we used a glass collector (17.5 cm diameter) previously sterilised in an autoclave (at 121 °C for 20 min) and placed over a two-metre mast on the rooftop terrace of this building. Samples were collected right after precipitation and then poured into 50 mL sterile polypropylene containers. The duration of sample collection was variable depending on weather conditions (between 5 and 24 h).

Additionally, we collected two control samples to study the culturable microorganisms in rain and air without a dust event intrusion (Supplementary Table 1). The rain sample was collected according to the procedure described above. The air sample was taken with an air-collector which was previously sterilised. Removable parts were autoclaved (121 °C, 20 min) and non-removable parts were rinsed off with 70 % ethanol. The duration of the air sample collection was 4 h.

The occurrence of red rain episodes was also studied and analysed by using dust concentration maps and backward trajectory models (Section 2.3). Additionally, we confirmed these episodes by the visualisation of brownish-reddish dust drops on the collector along with a characteristic haze.

Samples were immediately filtered for culturing and scanning electron microscopy after their collection, taking an aliquot of 50 mL for each analysis. Likewise, an aliquot of 50 mL was pre-treated with glutaraldehyde and kept in dark at −80 °C for metabarcoding analysis, until further processing.

2.3. Identification of dust sources and characterisation of the events

Records of African dust intrusions over the southeast of the Iberian

Peninsula were obtained (Ministry for the Ecological Transition and Demographic Challenge, 2021, 2022) to determine the dust events registered at the sampling site. Dust surface concentration maps were acquired using the MONARCH model (https://dust.aemet.es/products/daily-dust-products) (Klose et al., 2021; Pérez et al., 2011) to ascertain the source and analyse the distribution of each event.

The Hybrid Single Particle Lagrangian Integrated Trajectory model (HYSPLIT) (https://www.ready.noaa.gov/HYSPLIT.php) (Draxler and Rolph, 2015) was used to obtain 120-hour backward trajectories with the aim of determining the source region of the air masses bound to each dust intrusion. To that end, we used the vertical velocity model, and the altitude was set to 750, 1500 and 2500 m.a.g.l. respectively. After that, backward trajectories were sorted into three groups: 1) Northwest Africa (NW) 2) Northeast Africa (NE) 3) Non-African (NA) (Table 1).

Mean PM10 concentration registered during the sample collection period bound to each dust event was estimated using data from the following stations: *Granada Congresos* (Granada; 37°09′56″N, 3°36′00″W), *Granada Norte* (Granada; 37°11′44″N, 3°36′51″W) and *Ciudad Deportiva* (Armilla; 37°08′08″N, 3°37′09″W). The stations belong to the "Red de Vigilancia y Control de la Calidad del Aire de Andalucía (REDIAM)" and were chosen because of their proximity to the sampling site (Junta de Andalucía, 2021, 2022).

Table 1
Description of red rain samples.

Sample	Sampling date	Period of the event	Dust-source provenance[a]	PM10 (μg/m^3)
LLB0	22 Feb 2017	16–28 Feb 2017	NE	58.2
LLB1	22 May 2021	22 May 2021	NW	27.2
LLB2	17 June 2021	08–19 June 2021	NW	29.0
LLB3	22 Dec 2021	18–23 Dec 2021	NW	52.3
LLB4	27 Dec 2021	18–23 Dec 2021	NW	47.8
LLB5	06 Jan 2022	04–06 Jan 2022	NA	40.4
LLB9	28 Jan 2022	27–28 Jan 2022	NE	36.4
LLB11	14 Feb 2022	11–13 Feb 2022	NA	48.5
LLB13	24 Feb 2022	23 Feb 01 Mar 2022	NE	30.9
LLB14	04 Mar 2022	23 Feb 01 Mar 2022	NE	23.6
LLB15	15 Mar 2022	14–17 Mar 2022	NE	23.9

[a] Classification of samples into three groups according to the backward trajectory and dust concentration map of each dust event: 1) Northwest Africa (NW) 2) Northeast Africa (NE) 3) Non-African (NA).

2.4. Microscopy analyses

The study of mineral particles and biological material was performed by scanning electron microscopy using a GEMINI instrument (FESEM; CARL ZEISS, Jena, Germany) equipped with EDX-OXFORD10 (CIC, Universidad de Granada). To facilitate the observation of bacteria, liquid samples containing solid particles were first treated with 2.5 % glutaraldehyde in 0.1 M cacodylate buffer and with 1 % osmium tetroxide. Samples were subsequently filtered through a Millipore filter (0.2 μm pore size) to retain bacteria and dust particles. This filter was dehydrated with alcohol, dried using the critical point method and finally coated with gold to study the biological material and with carbon in case of mineral particles (Kuo, 2014). Measurements of maximum particle diameter were manually made on the images, using a ruler (250–300 particles per image). The agglomerated and stratified aspect is due to the preparation technique.

2.5. Metabarcoding analyses

For metabarcoding analysis, three samples (LLB0, LLB1, and LLB2) were used (Table 1). Samples were first filtered on PVC membranes with a pore size of 0.2 μm. Previously, the filtration apparatus was rinsed with

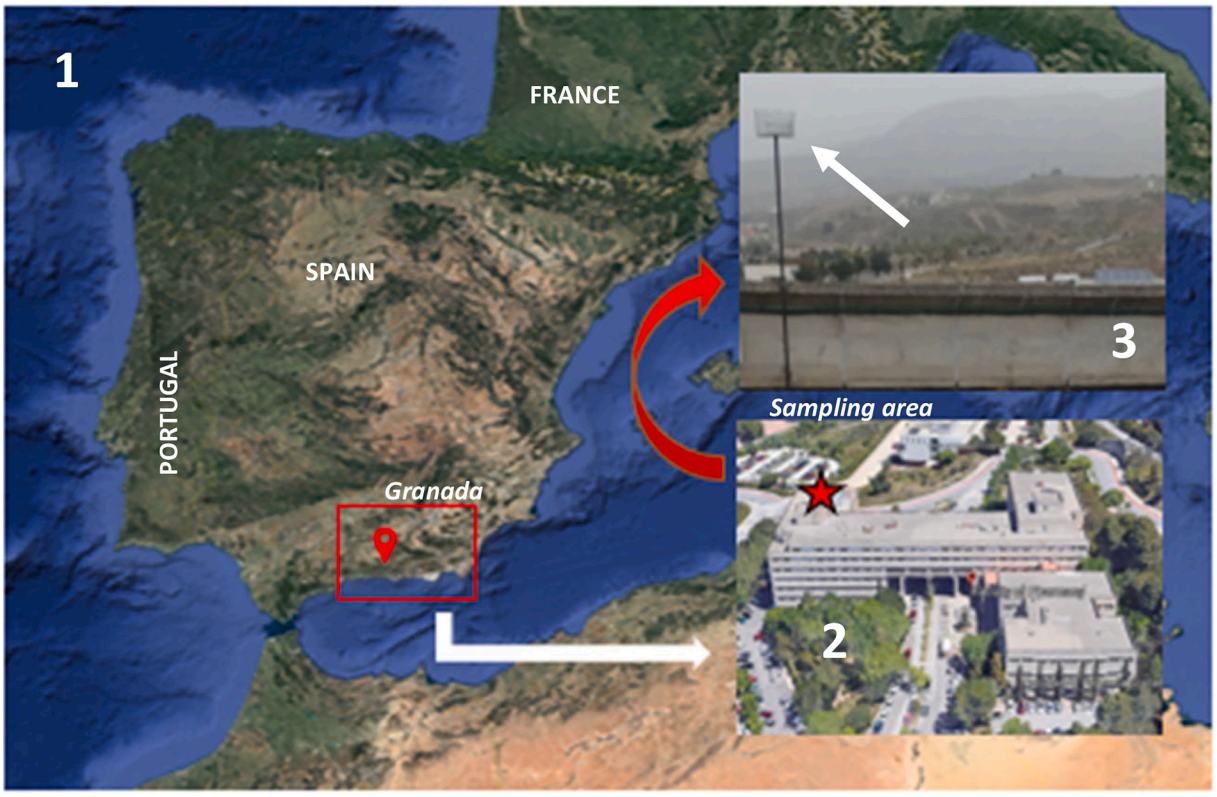

Fig. 1. 1) Location of the sampling area. 2) Faculty of Pharmacy (University of Granada) 3) Sampler (marked with an arrow) installed on the building rooftop. Images 1 and 3 were retrieved from *Google Earth*.

70 % ethanol and cleaned with DNA-exitus (AppliChem GmbH, Darmstadt, Germany) to exclude potential DNA contamination. Afterwards, half of the filter was used for DNA extraction. For this, the DNA extraction ChargeSwitchTM Forensic DNA Purification Kit (Invitrogen, Carlsbad, USA) was used with the following changes. Half of the PVC membrane was bead beaten with InnuSPEED lysis tube W (Analytik Jena, Jena, Germany) together with 1 mL of lysis buffer (included in the Kit). For the enzymatic lysis two steps were done. The first one was performed with Lysozyme (SIGMA-ALDRICH, Darmstadt, Germany) solution (10 mg/mL) and an incubation at 37 °C for 60 min. Afterwards, the Proteinase K lysis was performed as described in the kit's manual at 55 °C for 90 min. Isolation with beads was following the kit's manual. The gDNA was eluted two times, the first time with 100 μl elution buffer at RT for 5 min, the second time with 100 μl at 65 °C for 5 min. Both elutes were unified and reduced to a volume of 30–40 μl on a thermoshaker (VWR, Radnor, USA). DNA concentration was determined with the Denovix Ultra High Sensitivity Assay (DeNovix Inc., Wilmington, USA) and the Qubit 3 photometer (Invitrogen, Waltham, USA). For sequencing, 50 ng of sample were sent to the sequencing facility (StarSEQ GmbH, Mainz, Germany). Samples, including a positive control (mock community, ZYMO RESEARCH EUROPE GmbH, Freiburg, Germany) and a negative control were sequenced on a MiSeq platform using MiSeq reagent V3 for the ITS1 region (Fungi; primer pair ITS1f and ITS2) and the V4-V5 region of the 16S rRNA gene (Prokaryotes; primer pair 515F and 909R). Read length was 300 nt and 25 % PhiX were spiked in as control. Sequences were analysed using the r package DADA2 1.26 (Callahan et al., 2016), creating amplicon sequence variants (ASV) instead of OTU clustering to get better insights in the community diversity and structure. Prokaryotic sequences were rarefied to an even depth of 13,000 sequences after removing chloroplasts, mitochondria and potential contaminants (*Escherichia*, *Klebsiella*, *Shigella*, *Pajariellobacter*, *Staphylococcus*, *Ottowia*, and *Cloacibacterium*). Fungal sequences were rarefied to 85,000 sequences.

Classification (dada2 package) and histogram (ggplot2 package) were performed in the statistical soft-ware R.

2.6. Isolation of culturable microorganisms from filters

The study of culturable microorganisms was carried out on 8 red rain samples (Table 1) and 2 control samples (Supplementary Table 1). Both red rain samples (LLB3-LLB15) and the rain control sample (RC) were filtered through 0.2 μm pore size Millipore filters. Thereafter, each filter was deposited on plates with 10 % Trypticase Soy Agar (TSA) medium and incubated at room temperature. The air control sample (AC), containing microorganisms retained in a filter, was immediately deposited on a plate under the same conditions as explained above. The serial dilution method was used for the isolation of microorganisms, and a preliminary identification was carried out by Gram staining.

2.7. Identification of isolated culturable microorganisms

Isolated microorganisms were identified by partial sequencing of 16S rRNA, using the "DNA Xtrem" kit to extract DNA from each strain. Subsequently, universal bacterial primers F27 and R1492 were used for PCR amplification, and afterwards, PCR products were purified using the X-DNA purification kit (QIAquick PCR Purification Kit 250). Direct sequence determination of PCR-amplified DNA was carried out with the ABI PRISM dye-terminator, cycle-sequencing, ready-reaction kit (Perking-Elmer) and an ABI PRISM 377 sequencer (Perking-Elmer) according to the manufacturer's instructions. The obtained sequences were visualized with Chromas 2.6.6 software (Chromas) and compared to reference 16S rRNA gene sequences available in the GenBank, EMBL and DDBJ databases, using the BLAST search (Altschul et al., 1990) and the EzBioCloud server (Yoon et al., 2017).

2.8. Statistical analysis of isolated culturable microorganisms

Statistical analysis of isolated microorganism data was performed with IBM SPSS Statistics Software (v21). To that end, Spearman rank correlation (ρ) and Kruskal Wallis test were chosen because of non-normal distribution of the data. The abundance of culturable microorganisms represents the number of isolated culturable microorganisms per sample (Table 2).

3. Results and discussion

3.1. Identification of dust sources linked to red rain episodes

A total of eleven red rain episodes were investigated, with four of them linked to dust intrusions from Northwest and five of them to Northeast Africa; two of these episodes did not have an African origin (Table 1). PM10 concentrations during the dust events were variable and ranged from 23.6 to 58.2 $\mu g/m^3$ (Table 2), with the most intense dust event registered in February 2017. Dust-surface concentration maps and air mass backward trajectories of some dust events are shown in Fig. 2.

3.2. Electron microscopy analysis

Red rain samples contained mineral particles washed out by raindrops of variable size. They were mainly composed of 90 % clay (<2 μm) and 10 % silt (2–50 μm), as suggested by analysis of the SEM images. Fig. 3 (LLB15) shows several fields of dust-airborne particles with an average particle size of 1.2 μm. Dust particles with rounded and subrounded surfaces were observed in both fields, denoting aeolian transport.

EDX analyses of mineral dust contained in red rain samples indicated that the major constituents were O, Si, Al and Mg (Fig. 3). The presence of Ca, Na, K and Fe was also noteworthy. Sample spectra suggest a great abundance of silicates such as clay minerals (i.e. kaolinite), whereas an intermediate proportion of quartz was also found. In addition, there were possible carbonates, iron oxides, sodium feldspars and other silicates in minor proportions. These mineral phases were previously described in other red rain studies (Ávila et al., 1997; Fiol et al., 2005; Rodríguez-Navarro et al., 2018).

We have also observed large particles known as iberulites (Díaz-Hernández and Párraga, 2008), typically occurring during Saharan dust intrusions (Fig. 4). These particles are quasi-spherical in shape and show a core (internal zone), a rind (external zone) and a typical depression called vortex, the latter being shown in Fig. 4a. During our sampling period, we could detect that iberulites sometimes drop during red rain episodes, provided that rainfall is not heavy enough to disintegrate them. Under these weather conditions, soil dust particles with attached microbes relocate within the water droplets, with a major concentration of fine-grained (clays) and coarser materials in the rind and the core, respectively. The subsequent evaporation of the water droplet is crucial for the configuration of its final morphology (Diaz-Hernández and Sánchez-Navas, 2016; Párraga et al., 2021). According to these authors, iberulites have a low density (0.65 g/cm^3) and a porosity about 50 %, so they could act as flying spheres, being able to remain in the air for long time and cover large distances.

The iberulite shown in Fig. 4a comprises spore-like forms in a tiny hollow of its crust (Fig. 4b). In addition, other cavities along the external clayey rind are also observed and could shelter microorganisms. Therefore, iberulites could act as carriers of microorganisms, which may offer protection from harsh environmental conditions and a habitat rich in nutrients and minerals (Párraga et al., 2021).

SEM-microstructure analysis of red rain samples (LLB0) also suggests the presence of different biological material (Fig. 5). Fig. 5a might show a biofilm with EPS structures that glue mineral particles together; a bacterium located close to the biofilm was identified as biological sign.

Table 2
Spearman correlation coefficients (ρ) (n = 8) for PM10 concentration, abundance (number) and type of isolated microorganisms of red rain samples (LLB3-LLB15).

	PM10 (μg/m^3)	Abundance of culturable microorganisms		Gram+ bacilli		Gram- bacilli		Gram+ cocci
PM10(μg/m^3)	1.000	−0.868	**	−0.626		−0.913	**	−0.169
Abundance of culturable microorganisms		1.000		0.788	*	0.918	**	0.228
Gram+ bacilli				1.000		0.565		0.665
Gram− bacilli						1.000		0.000
Gram+ cocci								1.000

* Significant at 0.05.
** Significant at 0.01.

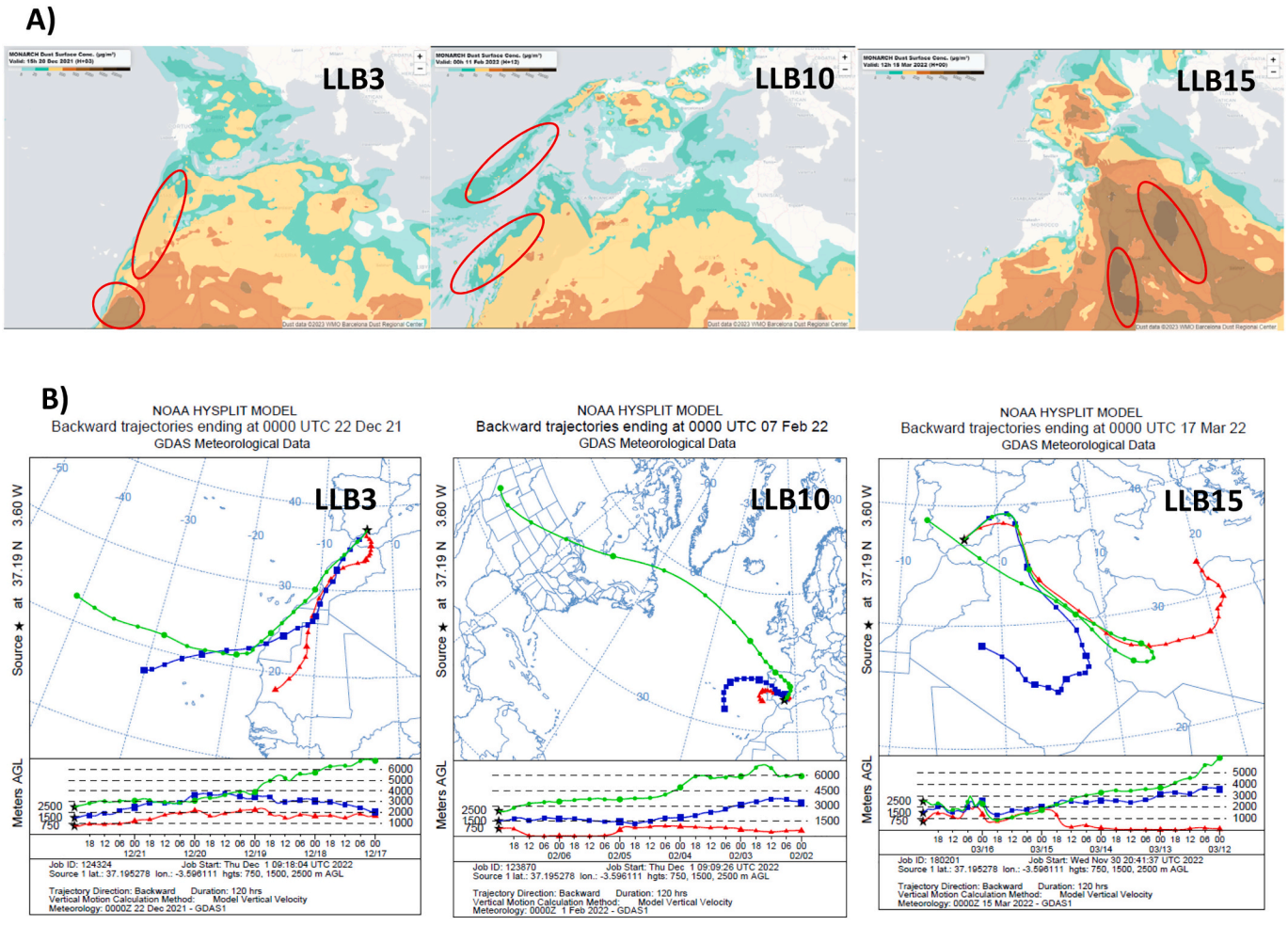

Fig. 2. Identification of dust-source origin of three dust intrusions associated to samples LLB3, LLB10 and LLB15. A) Dust concentration maps (MONARCH model, dust surface concentration μg/m^3): dust plumes are marked with red circles. B) Backward trajectories: 750 m (red), 1500 m (blue) and 2500 m (green).

Fig. 5b likely shows a pollen grain, corroborating again the presence of biological material in our samples. The biological signs identified are in agreement with other works (Kadar et al., 2014; Nastasi et al., 2020).

Interactions between microorganisms and clay minerals are ubiquitous in nature, and some microorganisms are known for their capacity to degrade, alter or decompose clay minerals to obtain nutrients (Li et al., 2019). Additionally, the metabolism and secretion of metabolites, such as extracellular polymeric substances (EPS) or slime (Wedlich, 2023) may enhance this process of clay mineral dissolution, as they facilitate bacterial adhesion to clay and other mineral particles (Biswas et al., 2017; Li et al., 2019; Rogers et al., 1998). In fact, these EPS have also been observed in biological soil crusts present in semiarid and hyperarid soils (Azua-Bustos et al., 2018; Weber et al., 2022) and it is thought that these substances may play an important role in the transport of microorganisms ensconced in iberulites (Párraga et al., 2021). The description of biological microsites in our samples (Fig. 5a) would support this reference.

Fig. 6 shows new evidence of biological material, illustrating in this case features of microbial resistance. Fig. 6a likely depicts a fungal spore, being this feature a key mechanism for survival during their long-range transport linked to Saharan dust intrusions. On the other hand, isolated rod-shaped nanobacteria were also observed (Fig. 6b); their length ranged between 200 and 250 nm, whereas their diameter varied from 30 to 50 nm. So far, nanobacteria had not been described in red rain samples. Their occurrence in atmospheric dust, however, fits to previous observations, which described nanobacteria to live in inhospitable environments, such as places with excessive heat, groundwater, and hot springs. Furthermore, they can live in multi-extreme environments under adverse conditions for life: at extreme pH (0,25), temperature (90 °C), redox potential, salinity and heavy metal content (Gómez

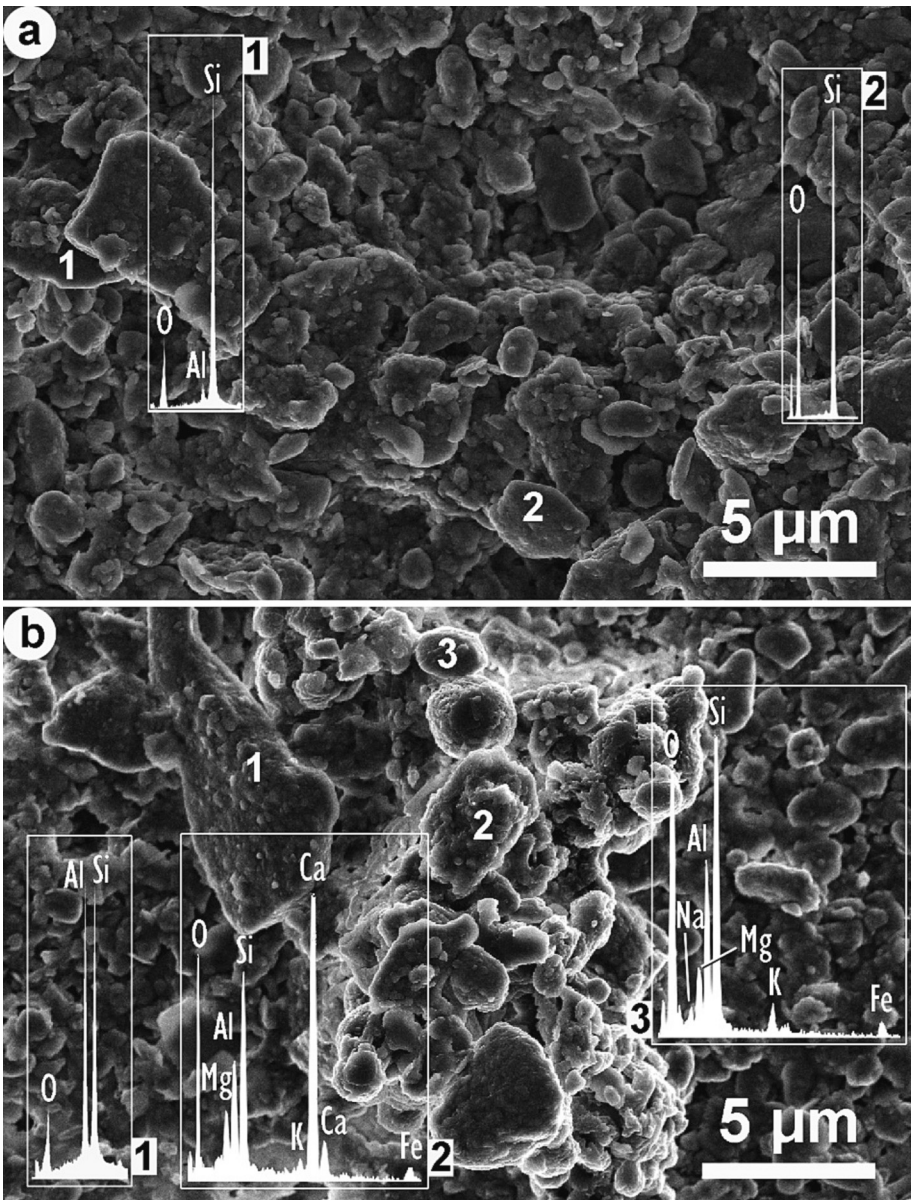

Fig. 3. Sample LLB15 (March 2022). An example selected from the red rain samples collection. Dust-borne particles of different size and chemical (mineralogical) composition. General roundness of particles edges can be seen (aeolian modelling). The agglomerated and stratified aspect is due to the preparation technique.
a) Field of particles with an average size of 1.17 μm (0.18–4.91 μm). Chemical composition: 1. EDX, Si, O (Quartz) 2. EDX, Si, O (Quartz).
b) Field of particles with an average size of 1.22 μm (0.18–9.82 μm). Chemical composition: 1. EDX, Si, O Al, Mg, K, Fe (Aluminosilicate, possible kaolinite, with iron oxides) 2. EDX, Ca, Si, O Al, Mg, K, Fe (Carbonate coated with aluminosilicates and iron oxides) 3. EDX, Si, O, Al, Mg, Na, K, Fe (possible sodium feldspar).

et al., 2019).

3.3. Metabarcoding analyses

The prokaryotic community of dust samples was characterized by a similarly high relative abundance of *Bacteroidota*, *Bacillota* and *Pseudomonadota* and low abundance of *Gemmatimonadales*, *Chloroflexi*, and *Myxococcota* (Fig. 7A). The high abundance of *Bacillota* and *Bacteroidota* during dust intrusions of North African origin is a well-known phenomenon (Petroselli et al., 2021; Polymenakou et al., 2008; Triadó-Margarit et al., 2019). The genus *Bacillus* (phylum: *Bacillota*) made up ~6.2 % of the total prokaryotic community (Fig. 7B). This is in line with studies showing that snow covered by dust originating from Africa shows a high relative abundance of *Bacillus* (Courville et al., 2020; Weil et al., 2017). Both samples from NW Africa also showed higher proportions of *Clostridiaceae* within the *Bacillota* compared to the sample from NE Africa. The presence of *Clostridiaceae* and the genus *Bacillus* also supports the discovery of potential bacterial spores in the red rain samples by FESEM, since both are known for their capability to form endospores to endure unfavourable environmental conditions like scarcity of nutrients or water. Additionally, members of *Bacillaceae* (*Bacillus*) were quite abundant in the bacteria recovered by isolation techniques, supporting the hypothesis that some members of the red rain associated microbiome travelled as spores. In total, the bacterial genera isolated from red rain samples made up ~11.1 % of the sequences recovered by metabarcoding (LLB0 = 6.89 %, LLB1 = 14.0 %, LLB2 = 12.5 %) (Supplementary Table 2). Furthermore, ~13.7 % of the sequences belonged to genera known to have EPS producing species (LLB0 = 10.7 %, LLB1 = 12.4 %, LLB2 = 18.1 %) (Supplementary Table 3). The calculated observed ASVs per sample correlated positively with increasing PM10 concentrations during sampling. Sample LLB0 showed the highest number (1559) of observed ASVs, followed by LLB2 (781)

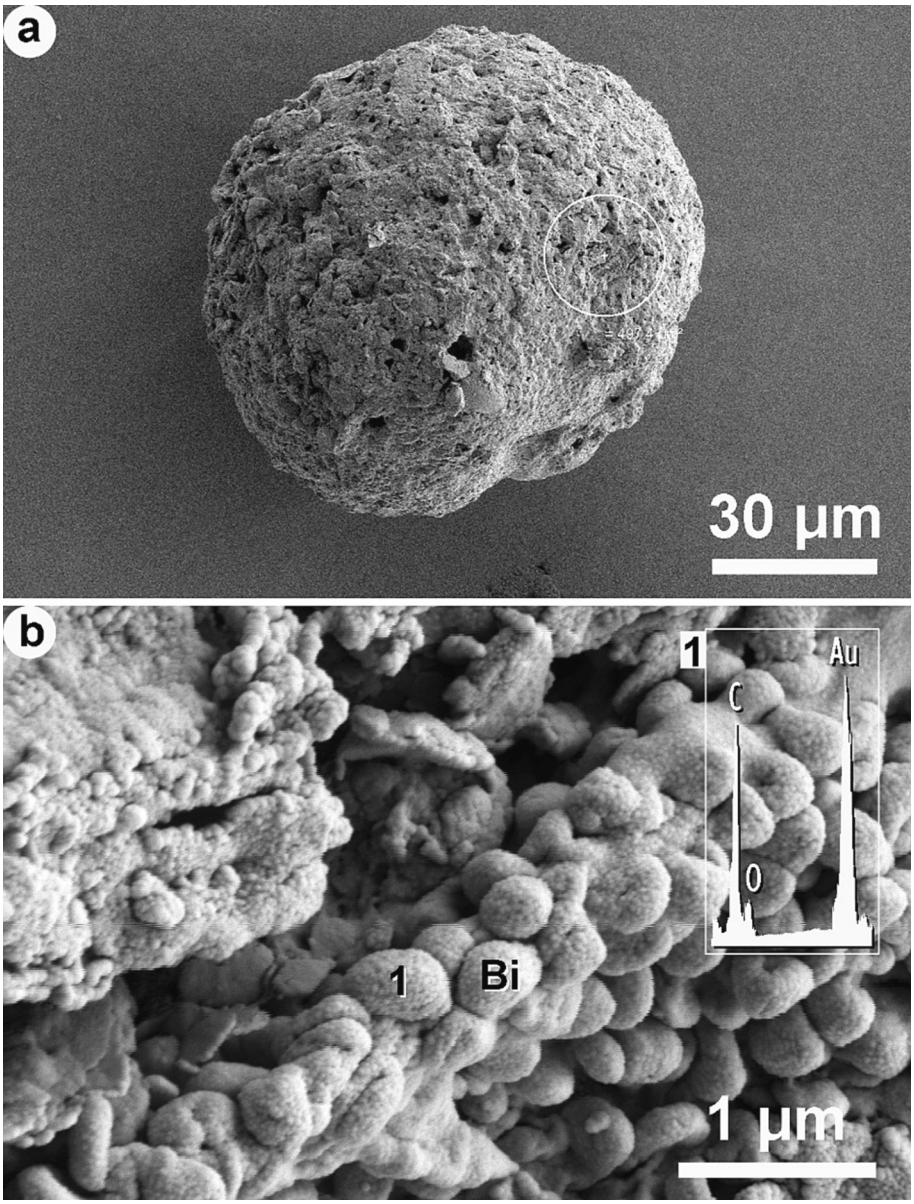

Fig. 4. Sample LLB0 (Feb 2017). Iberulite with potential biological features on its surface.
a) Iberulite of 110 μm diameter and pseudo-spherical morphology. The surface appearance is compacted by an external rind where fine clay particles are predominant. The vortex (marked with V) can be observed.
b) Detail of the upper image in the field marked with a circle. Biological features (Bi) shown into a depression in the external rind of the iberulite, closely resemble a bunch of bacterial spore-like forms (EDX clearly shows two peaks of Au and C).

and LLB1 (83 observed ASVs). This correlation was previously described for air masses in the Mediterranean (Mazar et al., 2016) and is considered to be due to an addition of alien ASVs/species to the local prokaryotic bioaerosol community.

The fungal community of red rain samples (LLB0, LLB1, and LLB2) was dominated by *Ascomycota* at the phylum level (Fig. 8A) and here the biggest proportion was attributed to the *Pleosporaceae* (Fig. 8B). Other authors showed a trend of increasing proportions of *Ascomycota* in continental air (Fröhlich-Nowoisky et al., 2012), being consistent with our results.

The composition on class level was also similar to snow samples taken during dust influence in the Alps, with the biggest proportion belonging to the class of *Dothideomycetes* (Weil et al., 2017). With regard to *Ascomycota*, samples with NE Africa dust influence showed lower abundance of *Cladosporiaceae* and a higher abundance of *Sporormiaceae*. This could indicate that the different land covers in the source locations influenced the transported fungal community. The *Basidiomycota* in all three red rain samples were dominated by *Filobasidiaceae*. The sample collected during NE African dust influence showed a higher abundance of *Chytridiomycota* at phylum level, compared to the other two samples collected during NW Africa dust influence. *Spizellomycetales*, which made up the biggest proportion of the *Chytridiomycota*, are associated with vegetation and soils.

3.4. Identification of microorganisms isolated from red rain samples

A total of 44 microorganisms were isolated from red rain samples after a preliminary characterisation (Fig. 9). Most of them were bacilli (with 45 % being Gram-positive and 43 % Gram-negative); 11 % of Gram-positive cocci were isolated, whereas Gram-negative cocci were not observed. Additionally, 23 % of bacilli were spore-forming, and around 64 % of the isolated microorganisms were pigmented bacteria.

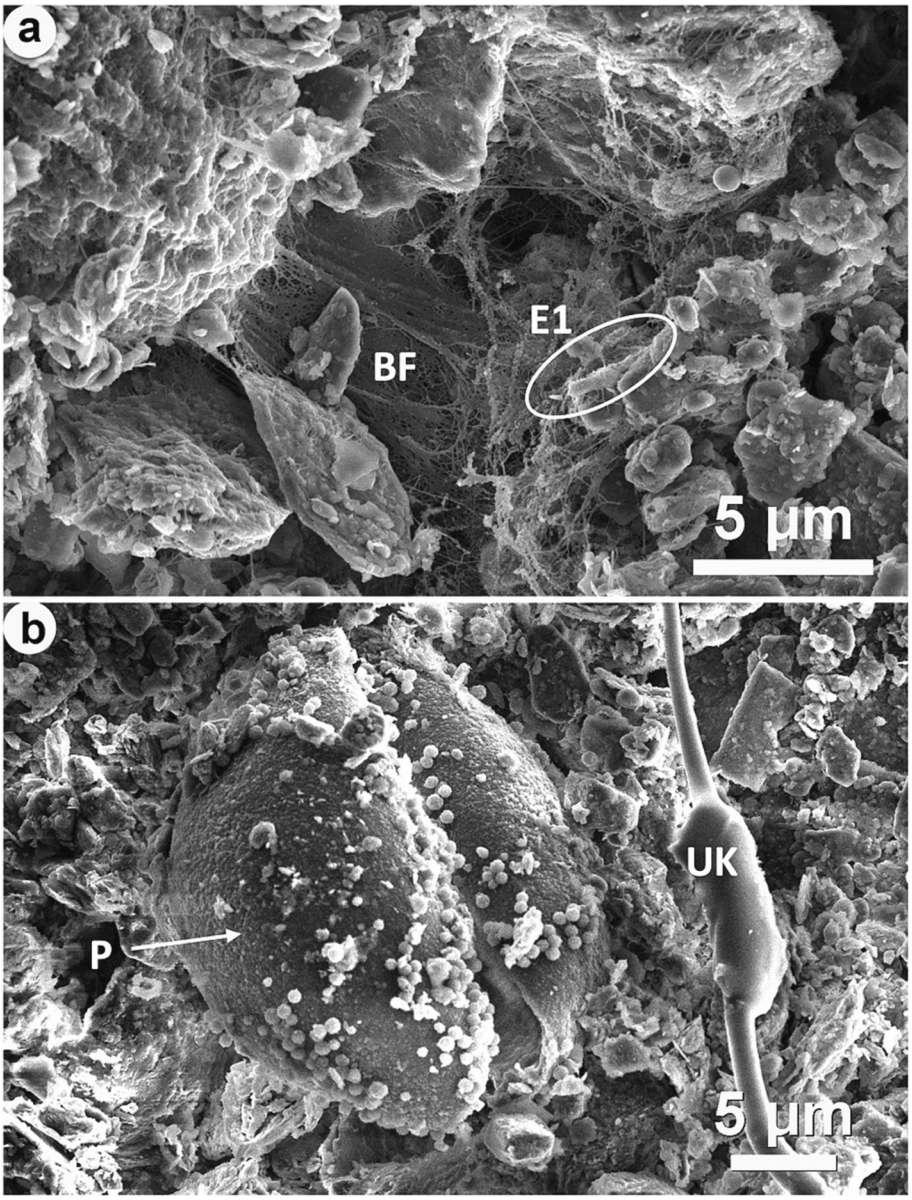

Fig. 5. Sample LLB0 (February 2017). Evidence of biological material in red rains.
a) Filamentous network (possible biofilm made up of EPS) (BF), joining mineral particles. A possible bacterial body (E1).
b) Possible pollen grain (P) of 21.6 μm size. Unknown biological material (UK) of 26.8 μm length in the image.

The analysis of data per month showed that around 75 % of the microorganisms were isolated during the months of February and March (Fig. 9B). 75 % of the isolated microorganisms were collected during dust events from northeast Africa (Fig. 9C).

The relationships between PM10 concentration, abundance (number) and type of isolated culturable microorganisms per sample were investigated by means of correlation analysis. In Table 2, correlation ranks show a strong positive correlation of the number of isolated microorganisms with gram-negative bacilli ($\rho = 0.918$). Besides, there was a strong negative correlation between PM10 concentration and the number of the isolated gram-negative bacilli ($\rho = -0.913$).

Saharan dust intrusions are usually characterized by a high level of PM10 particles in the air (Querol et al., 2009). As previously described, red rain samples were mainly composed of clay-size particles (<2 μm; mean = 90 %) according to the granulometric analysis (Fig. 3). Our results suggest that PM10 concentration is negatively correlated with the number of gram-negative bacilli. Therefore, PM10 concentration and size-particle might have an influence on the bacterial diversity transported in Saharan dust plumes, previously observed by other authors (Petroselli et al., 2021; Polymenakou et al., 2008).

A Kruskal Wallis test was used to assess whether the dust source provenance and/or the sampling date had an influence on the abundance and type of isolated microorganisms per sample. Test results revealed that the month of the red rain episode did not affect the abundance or type of isolated microorganisms per sample. In contrast, dust source provenance had a significant impact on the isolated microorganism diversity. In fact, the number of isolated Gram-negative bacilli was significantly higher in samples originating from NE Africa ($H = 6.054$; $p = 0.048$) (Fig. 10). In addition, most of the isolated microorganisms are considered to be soil microbes, and therefore the mineralogical composition and the organic matter of soils from different dust sources might alter the microbial biodiversity observed after red rain episodes.

By means of 16S rRNA sequencing, 18 out of 44 isolated microorganisms could be identified (Fig. 11) (Supplementary Table 5); 3 isolated microorganisms were also identified from control samples (air and

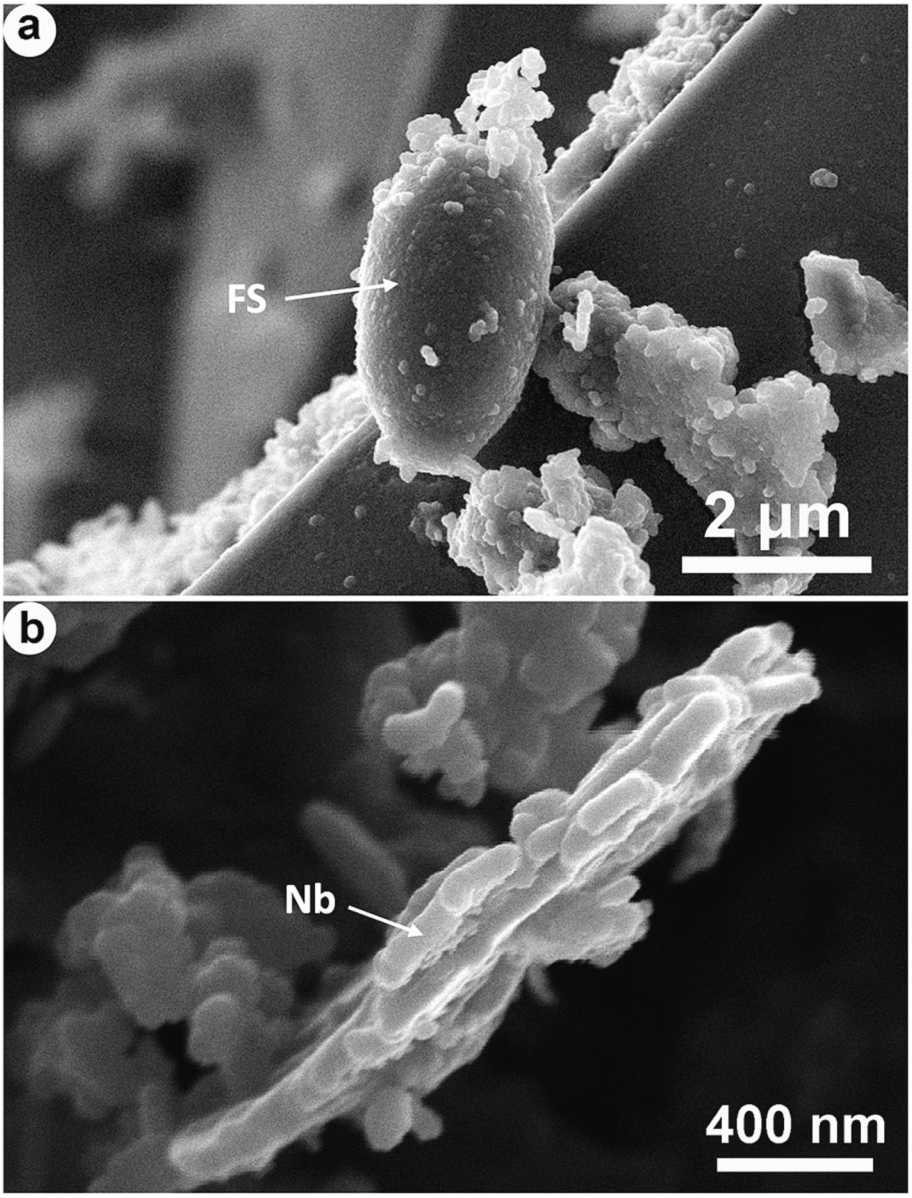

Fig. 6. Sample LLB15 (March 2022). New evidence of biological material in red rains.
a) Ellipsoidal-shaped fungal spore (FS) of 3.7 μm size.
b) Bacillar-shaped nanobacteria (Nb) with an average length of 250 nm, over mineral particles (some ultrafine particles <100 nm). Self-replication is observed.

rain controls) (Supplementary Table 4). According to our results, 41 % of the isolated microorganisms belonged to the phylum *Bacillota*, whereas 59 % of them corresponded to the phylum *Pseudomonadota*. In the results of metabarcoding, 18 % belonged to *Bacillota* and 23 % to *Pseudomonadota*. In general, these results show a greater diversity at the phylum level than those previously obtained in Southern Spain (Sánchez De La Campa et al., 2013).

The most abundant genus within the phylum *Pseudomonadota* was *Pseudomonas* (17 %). Culturable microorganisms belonging to other genera such as *Acidovorax*, *Massilia* and *Sphingomonas* were also found (Fig. 11A), being consistent with other works (Federici et al., 2018; Petroselli et al., 2021). Microorganisms corresponding to the genera *Sphingomonas* and *Acidovorax* have previously been associated with Saharan dust events (Petroselli et al., 2021). Different species of *Massilia* were isolated from red rain (LLB14) and air control samples (AC), with previous studies describing this genus to occur in extreme environments (Chuvochina et al., 2011).

Bacillus (17 %) was the most abundant genus within the phylum *Bacillota*, and other culturable microorganisms belonging to the genera *Peribacillus*, *Planococcus* and *Priestia* were also identified (Fig. 11B). In the metabarcoding approach, *Bacillus* made up ~6.3 % of the whole procaryotic community. In this case, our results show a greater variety of culturable microorganisms when compared with previous works (Itani and Smith, 2016; Sánchez De La Campa et al., 2013). Among the above-mentioned genera, it should be highlighted microorganisms of the genus *Priestia* are resistant to UV radiation (Gupta et al., 2020), while members of *Planococcus* occur in extreme environments (An et al., 2015; González-Toril et al., 2020). Different species of *Priestia* were isolated from red rain (LLB 5) and control samples (RC).

The presence of spore-forming microorganisms resistant to heat, desiccation and UV radiation could justify the long-distance transport of microorganisms associated with Saharan dust events (Griffin, 2007; Sánchez De La Campa et al., 2013). Additionally, pigmentation could act as a protection mechanism of bacteria against UV radiation (Favet et al., 2013; Kellogg and Griffin, 2006) during the atmospheric transport (Griffin et al., 2011), being a noticeable feature frequently observed in

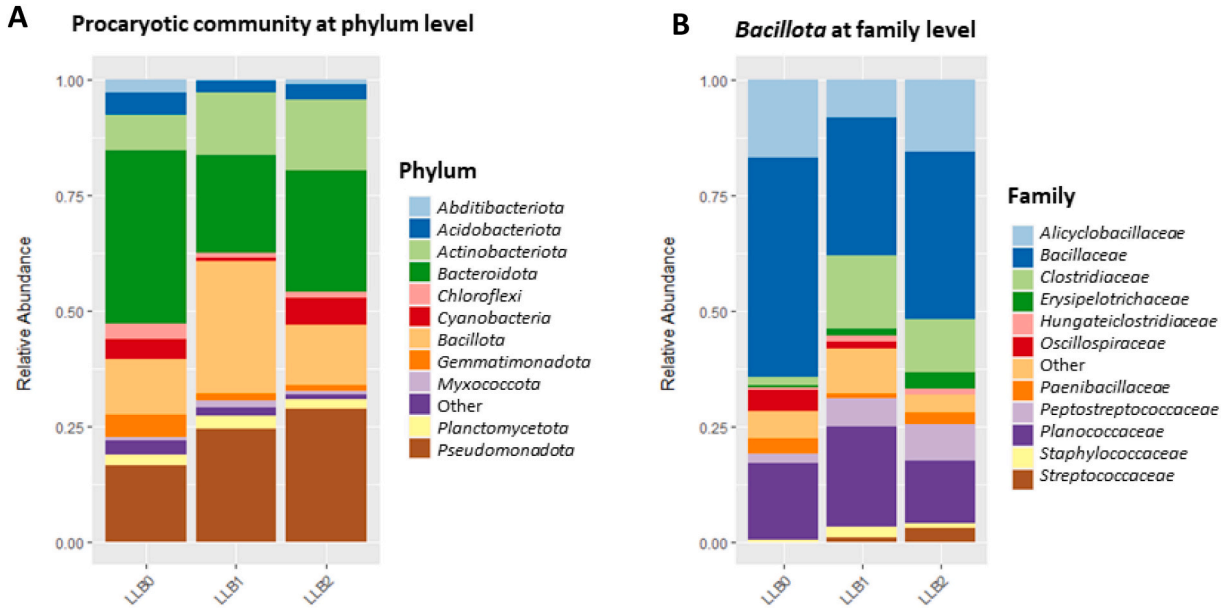

Fig. 7. Relative abundance of prokaryotic ASVs at phylum level (A) and ASVs classified as *Bacillota* at family level (B) for three samples. Only the 11 most abundant groups are shown, all others are lumped in "Other".

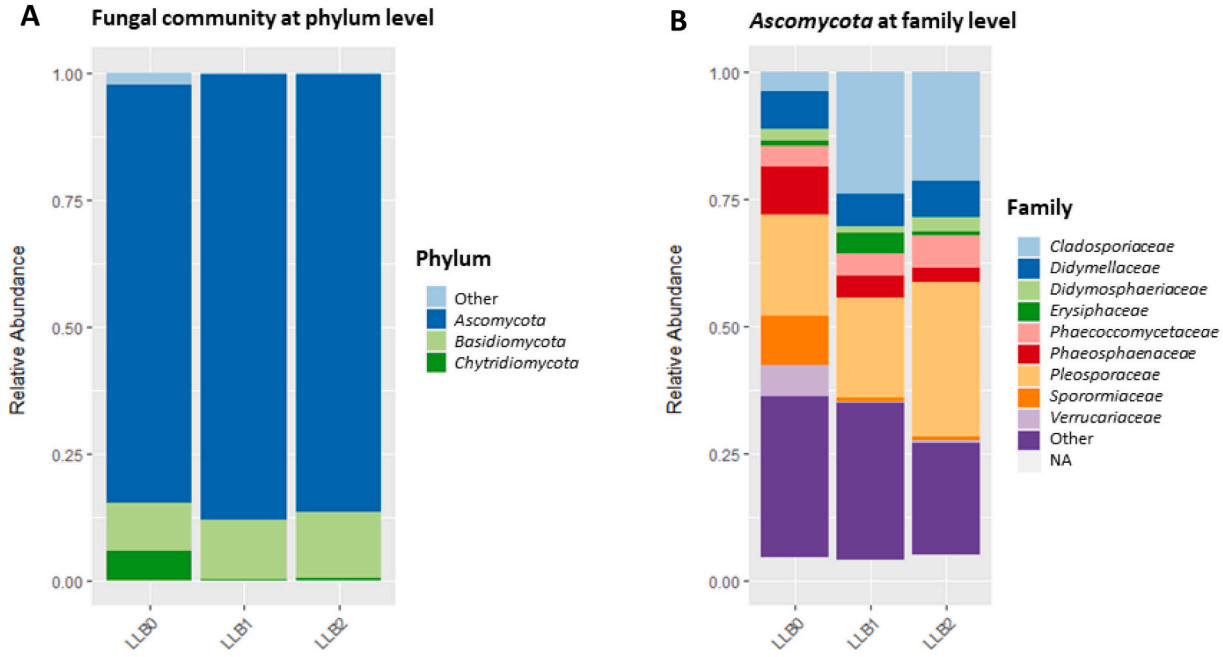

Fig. 8. Relative abundance of fungal community at phylum (A) and for Ascomycota at family (B) level for three red rain samples. Only the 11 most abundant groups are shown, all others are lumped as "Other".

bacteria isolated from our samples.

Red rain episodes might enhance bacterial survival, given that the high relative humidity in the atmosphere favours their growth. The aggregation of loose mineral particles through water uptake and EPS production could be involved in the iberulite and other polymineral aggregate formation, as it was previously commented (Stanier et al., 2004; Párraga et al., 2021). In contrast, bacteria could endure harsh conditions when they are involved in long-distance transport. Thus, the growth rate reduction bound to nutrient limitation could trigger some survival mechanisms such as pigmentation or spore production(Bren et al., 2013).

Among the species identified, it is important to highlight that some species such as *Peribacillus frigoritolerans* and *Bacillus halotolerans*, which are characterized by surviving in extreme conditions, were found in samples from three different episodes (LLB4, LLB5 and LLB14). In fact, *Peribacillus frigoritolerans* was isolated and described for the first time in arid soils from Morocco (Delaporte and Sasson, 1967). On the other hand, the presence of halotolerant microorganisms was detected in Asian desert dust (Maki et al., 2008, 2010). Likewise, *Stenotrophomonas bentonitica* was also found in one sample (LLB14), being previously isolated from a bentonite formation in Almería (Sánchez-Castro et al., 2017).

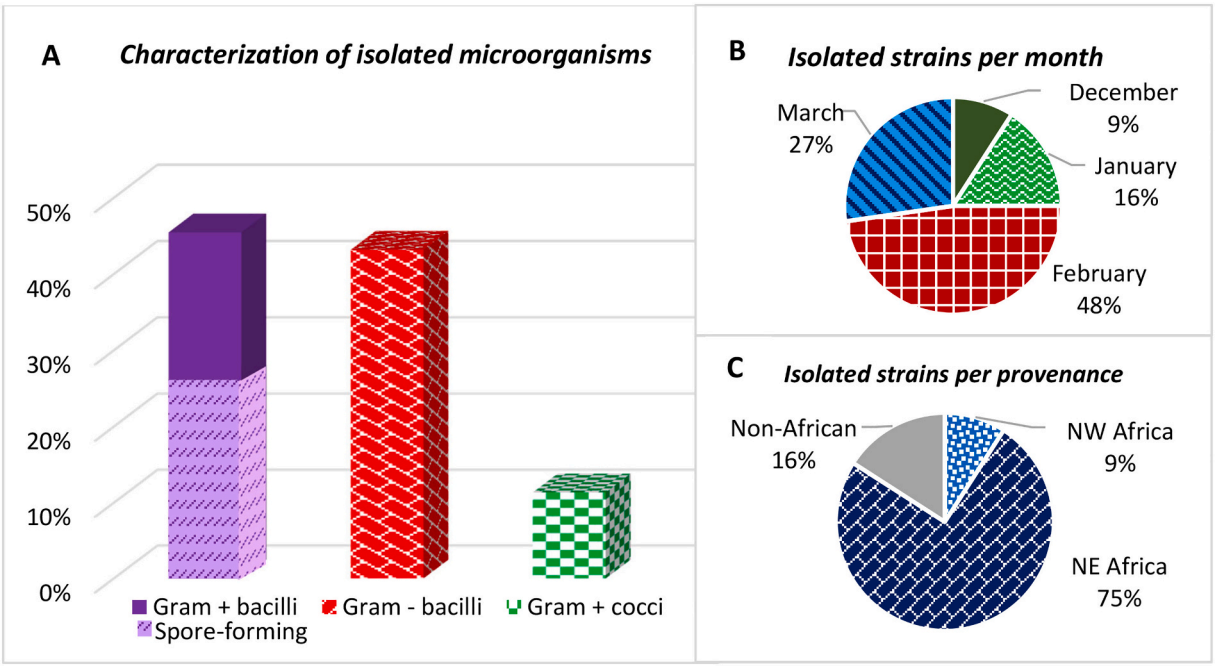

Fig. 9. Isolation of culturable microorganisms from red rain samples. A) Classification according to Gram staining (A). Isolated microorganisms' abundance (number) per month of sampling (B) and dust provenance (C).

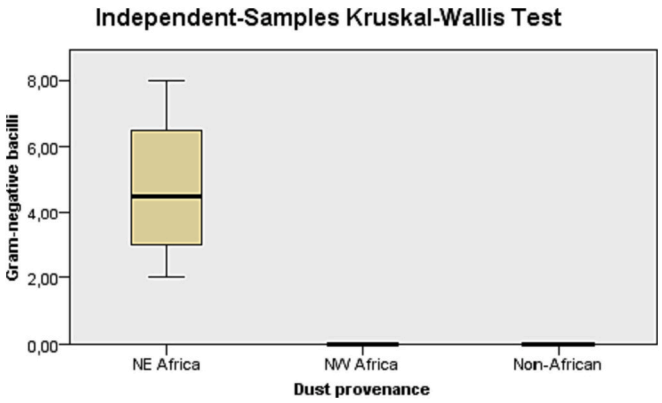

Fig. 10. Kruskal-Wallis Test. Distribution of gram-negative bacilli versus dust provenance. Significant differences observed in red rain episodes from North-east Africa (NE Africa).

4. Conclusions

This work could be considered as the first study focused on the characterisation of the microbial diversity in red rain samples collected in the southeast of the Iberian Peninsula. Therefore, it could initiate progress in this new and unknown atmospheric microbial ecosystem.

The city of Granada is frequently affected by Saharan dust intrusions, owed to its proximity to North Africa, being an ideal location for both atmospheric dust and red rain sampling. Airborne microbes attached to mineral particles along with size-particle distribution and mineralogical composition have been determined by FESEM. Additionally, our images suggest that EPS and biofilm production are evident processes involved in the formation of atmospheric dust aggregates such as iberulites and microsites suitable for microorganisms. Nanobacteria were identified in red rain samples for the first time. The presence of fungal spores and nanobacteria indicate the microbial resistance in atmospheric dust, an inhospitable environment for microbial growth.

The prokaryotic community composition determined by 16S amplicon sequencing was similar to the one found in dust intrusions, demonstrating the connection between red rain episodes and African dust intrusions as related to the microbial composition. Especially the high abundance of *Bacillota* is a feature often found in African dust samples. Around 11.13 % of prokaryotic sequences were attributed to genera which were also recovered by cultivation approaches within this study. Genera of spore forming and EPS producing bacteria were also found in the prokaryotic community. For the fungal community, the results showed a high abundance of *Ascomycota*, and dependent on the origin, the presence of *Chytridiomycota*. To our knowledge, this study presents the first 16S and ITS1 amplicon metagenomes (metabarcoding) of red rain events in the western Mediterranean.

Culturable microorganisms belonging to the genera *Acidovorax* and *Sphingomonas*, being resistant to extreme conditions, were identified by means of 16S rRNA sequencing. The isolation of spore-forming bacilli and pigmented bacteria could justify the long-range transport through atmospheric dust. Some species, such as *Bacillus halotolerans* and *Peribacillus frigoritolerans* were found in different episodes, the latter being remarkable because of its African provenance.

The prevalence of atmospheric desert dust and iberulites in the Mediterranean region along with their associated biological constituents (bacteria, fungi) may play a significant role in the ecosystem and human health and warrants the need for further research in this interesting field.

Credit authorship contribution statement

Azahara Navarro: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Ana del Moral:** Investigation, Supervision, Writing – original draft, Writing – review & editing. **Bettina Weber:** Data curation, Formal analysis, Software, Writing – original draft, Writing – review & editing. **Jens Weber:** Data curation, Formal analysis, Software, Writing – original draft, Writing – review & editing. **Alberto Molinero:** Investigation. **Rafael Delgado:** Conceptualization, Data curation, Supervision, Writing – original draft. **Jesús Párraga:** Conceptualization, Investigation, Supervision, Writing – original draft, Writing – review & editing. **Fernando Martínez-Checa:** Data curation, Formal analysis, Investigation, Methodology, Writing – original draft,

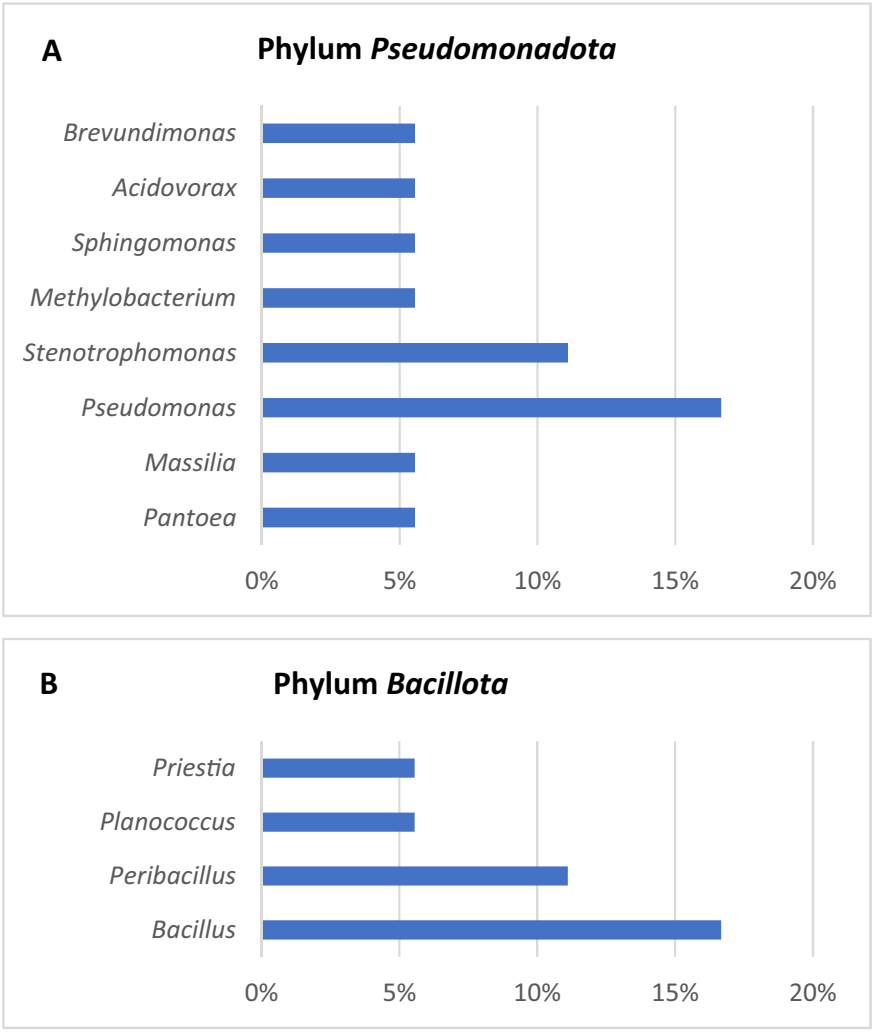

Fig. 11. Isolated microorganisms (genera) belonging to the phylum of A) *Pseudomonadota* B) *Bacillota*.

Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.169745.

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