



Molecular identification of the bacterial burden in Sahara Dust samples

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Abstract

The Canary Islands are exposed to dust originating in the Sahara and Sahel regions. Saharan dust (SD) aerosols also cross the Atlantic and affect the Caribbean, Central America, and the south-eastern United States and the north-eastern Amazon basin. SD includes bioaerosols that act as vectors of a variety of micro-organisms (fungi, viruses and bacteria) and their biologically associated products (e.g. spores, mycotoxins, endotoxins).

These are potentially pathogenic to terrestrial and aquatic organisms, as well as to humans. Dust loads transported into the atmosphere have been estimated to range between 500 million tons and 1 billion tons annually. Classic methodology used for the identification of these organisms in SD samples includes the culturing and isolation of viable specimens followed by biochemical analyses. Most recently, molecular biology techniques such as DNA extraction, PCR, and DNA sequencing have complemented this methodology.

Still, these approaches depend on the culturing viability of the micro-organisms that are in the collected samples. In this study, we developed a new method that overcomes this limitation. By utilising as a model for environmental testing SD samples collected in Gran Canaria at two different dust cloud intensities, we effectively include the cloning of

heterogeneous DNA to the experimental scenario. Our results suggest that the diversity of bacterial species seems to be related to the intensity of the dust cloud event.

We report the bacteria: *Morganella morganii*, *Rahnella aquatilis*, *Corynebacterium mucifaciens*, *Staphylococcus kloosii/carnosus*, *Propionibacterium acnes*, *Serratia fonticola*, and *Shigella flexneri* (not previously identified in SD samples) and confirm previously reported genera: *Pseudomonas*, *Bradyrhizobium*, *Sphingomonas*, and an Arctic Sea-origin bacterium. This is the first report describing this methodology for bacterial identification from air-collected samples, containing more than a single bacterial species. This work addresses the air quality at which countries located within the SD trajectory are exposed to, by using a new methodological tool and from a non-conventional environmental/biological standpoint. We expect that a better understanding of SD bacterial burden will contribute to the development and implementation of more efficient management measures, in order to decrease public health issues presented during SD events.

Key words: Bacterial identification; environmental health; environmental sampling; public health management; Sahara Dust.

Introduction

Because of the proximity of the Canary Islands to the African continent, this archipelago is exposed to the presence of African dust throughout the year. However, the maximum frequency of dust events in this region occurs in the winter months (November-February) and it accounts for over 35% of the total annual dust flux (Dorta et al., 2005). Saharan dust (SD) aerosols rapidly (7-10 days) cross the Atlantic Ocean and impact on the Caribbean, Central America, and the south-eastern United States primarily between the months of May and October, and the northeastern Amazon basin from February to April (Swap, 1992; 1996). Dust loads transported into the atmosphere have been estimated to range between 500 millions tons to 1 billion tons, annually (Moulin et al., 1997; Taylor, 2002). Currently, there exist many efforts directed towards the identification and reduction of ambient concentrations of particulate material as a mechanism for improving human health. However, the biological burden present in SD has the potential of spreading a wide variety of microorganisms (fungi, virus, and bacteria), and their biologically associated natural products (spores, mycotoxins, endotoxins). These can be pathogenic to plants (Nagarajan, 1990), marine environments (Shinn, 2000; Mos, 2001; Garrison et al., 2003; Weir-Brush et al., 2004) and humans (Griffin et al., 2001; Kellogg et al., 2004). The National Institute of Allergy and Infectious Diseases have identified airborne dust as the primary source of allergic stress worldwide. Interestingly, areas such as the Aral Sea and the Caribbean, where SD exposure is common, are associated with the highest incidence rates of asthma in the world (Bener, 1996; Howitt, 2000).

Previous studies have utilized a broad range of technical approaches to identify the micro-organisms, transported by SD. These methods include, the culturing of viable specimens, microscopic analyses, and a combination of DNA extraction, polymerase chain reaction (PCR) of the cultured colonies, and DNA sequencing testing (Culturing-PCRSequencing method: CPS). Fungi, bacteria, and viruses, which can be pathogenic to humans, have been isolated and identified in cultures and by means of the CPS technique (Griffin, 2001 and 2003; Kellogg et al., 2004). However, it has been postulated that the species present in SD that can be identified through currently available methods contributes to less than 1% of the total or real burden comprising the tested samples (Eilers et al., 2000; Griffin, 2001).

Some of the limitations of the CPS methodology include the relatively large amount of sample required for obtaining sufficient initial culture material. The sampling of the fine particulate matter with a diameter of 2.5 (PM_{2.5}) or 10µm (PM_{10.0}) usually results in the recovery of small concentrations of dust. The amount of dust necessary from each sample for starting a culture in the different selective growth media is usually not achieved in a single collected filter. Another limitation encountered when using the CPS method is that in order to grow, the organism needs to be alive and viable.

The identification of fungal species is also time consuming; it can take several weeks to grow cultures. Finally, it has been suggested that not all the culture media provide the nutritional supplements required by all the species existent in a sample. To overcome some of these limitations, we developed an innovative method termed PCS, to assess the identity of the most common fungal and bacterial species present in SD samples. PCS involves sequentially performing of DNA extraction, PCR, cloning, and DNA sequencing. The PCS method is performed by using DNA extracted directly from a collection filter, which is normally contains a heterogeneous mixture of SD micro-organisms. The PCR

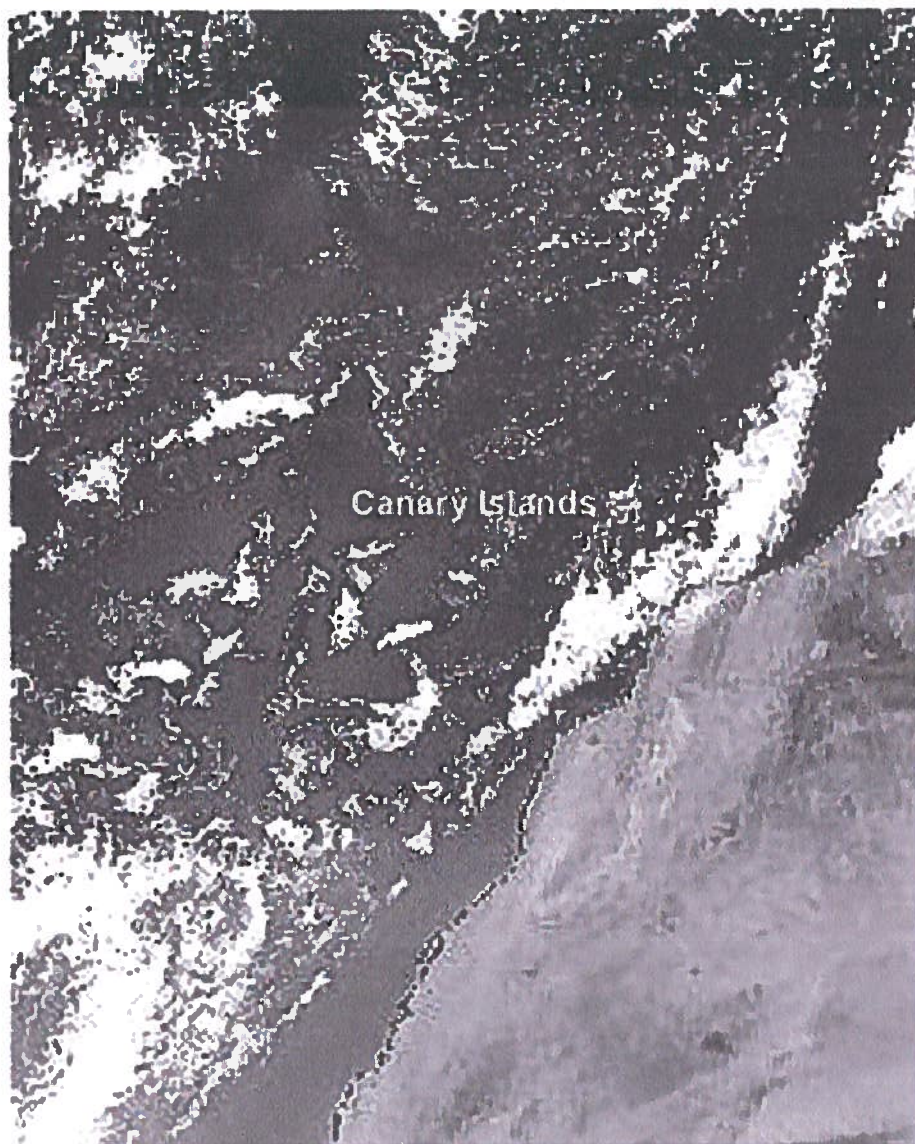
product obtained by using universal primers is cloned into a plasmid vector. Positive clones are purified and sequenced, and the obtained sequences are submitted to the NCBI BLAST database for confirmation of identity.

The use of heterogeneous DNA and the cloning step allows the assessment of more than one kind of DNA per PCR reaction. This is expected to correspond to the presence of the species in the collected sample. In this pilot study, we used the PCS approach to identify bacterial species present in SD samples from the island of Gran Canaria. Methods
Sample collection The Canary Islands are an archipelago of seven islands located 115 km off the northwestern coast of Africa. SD samples were collected at the Pico de la Gorra air-sampling station located at 1,930 m of altitude on the island of Gran Canaria (28°06'N, 15°24'W). The Island of Gran Canaria, located in the Atlantic Ocean at approximately 100 km off the African Coast, is frequently exposed to dust plumes originating in the Sahara and Sahel regions. A high volume pump model CAV-AM (MCV, S.A.) set at 60m³/hour was used to collect particles of 10µm or less, during a 12 hours sampling period.

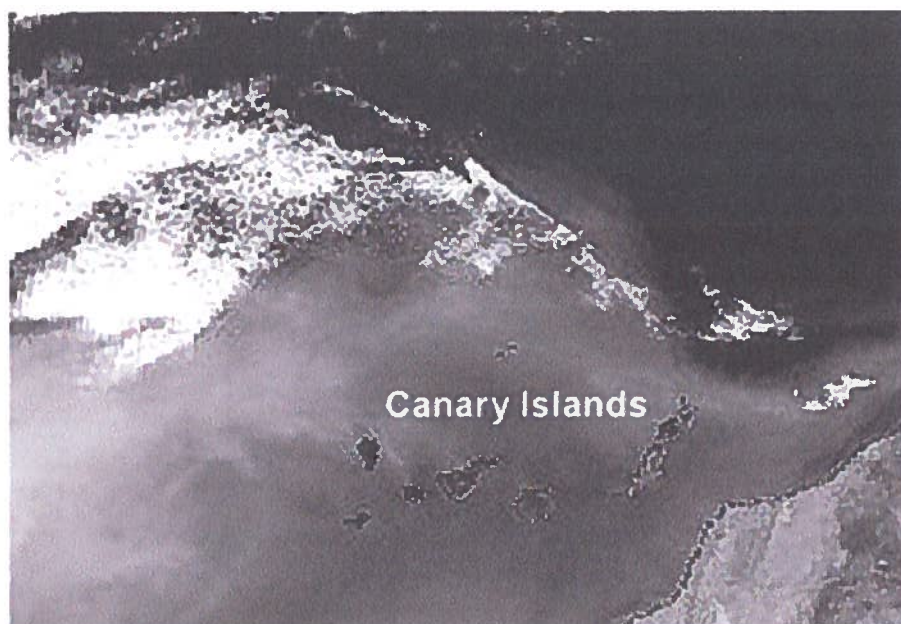
The samples were collected in 8" x 10" Whatman filters (GF/A) that were stored after the completion of the sampling in a sterile bag and frozen at -20°C until DNA extraction. The filters, labelled as SD#15 (3rd June 2002) and SD#7 (1st March 2003), correspond to a low dust concentration (35.72µg/m³) and an intense dust event (4492.9µg/m³), respectively. The occurrence and intensity of the events were confirmed using satellite images (SeaWiFS). The ICoD Dust Regional Atmospheric Model (ICoD/DREAM) and the National Oceanic and Atmospheric Administration (NOAA) Hybrid Single-Particle Lagrangian Integrated Trajectory (HySplit) model were used to determine the surface dust concentration over the Canary Islands and backward trajectories of particles, respectively. DNA extraction of SD samples DNA extraction of SD samples was performed by placing one-half of the filters in a sterile 15ml Falcon tube with 5ml of ultra pure, molecular grade, autoclaved water. The samples were incubated at 37°C for one hour with continuous shaking.

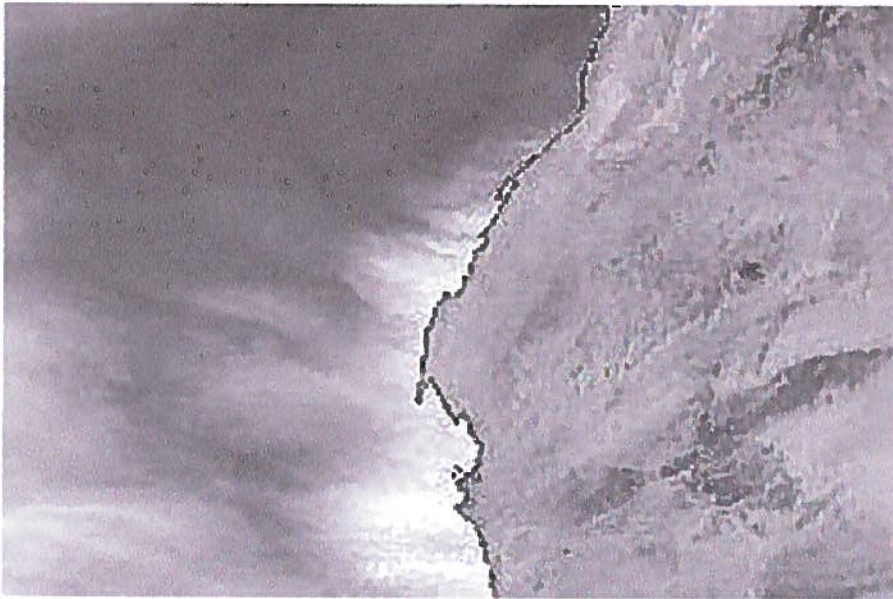
The total DNA extraction was carried out from 500µl of the eluted sample using Epicentre's SoilMaster™ DNA Extraction Kit, following the manufacturer's instructions. PCR amplification and electrophoresis The microbial PCR amplification from the SD DNA samples was performed using the bacterial universal 16S primers: P4 (5'-AACGCGAAGAACCCTTAC-3') and P5 (5'-CGGTGTGTACAAGGCCCGGGAA-CG-3'), with a Triple-Master® PCR Kit (Eppendorf AG, Hamburg, Germany) in a PCR reaction volume of 20µl. Each reaction contained 3.2µl of 10X High Fidelity PCR Buffer, 0.4µl of each 10mM DNTP; 0.5µl of each primer (10.0µM), 0.3µl of TM enzyme (5U/µl), 10.1µl of water and 5µl of extracted DNA. PCR was performed in an Eppendorf Gradient Mastercycler® (Eppendorf AG, Hamburg, Germany) following these steps: denaturation for 5 min. at 94°C and 35 cycles of amplification using a step programme of 20 sec. at 94°C, 20 sec. at 54.0°C; 5 min. at 72°C; a final extension of 10 min. at 72°C; and a hold step at 4°C.

Positive and negative controls were included in each reaction using *Escherichia coli* bacterial DNA and water as templates, respectively. Electrophoresis of the PCR products (9µl) was performed in a 3% agarose gel with TAE buffer at 100 volts for 90 minutes, stained with ethidium bromide, and visualized under UV light. PCR product cloning, plasmid purification, and DNA sequencing The obtained amplicons were cloned using the TOPO TA Cloning® kit (Invitrogen Corp., Carlsbad, CA), following the specified protocols. The *E. coli* 5F' competent cells were chemically transformed according to the manufacturer's specifications (Invitrogen Corp., Carlsbad, CA), and plated on Luria-Bertani (LB) plates containing 100µg/ml of ampicillin. Ampicillin-resistance and -galactosidase bluewhite screening was used to identify white, recombinant colonies. Twenty-one (21) recombinant colonies were individually picked from SD#7 and SD#15 transformations. Colonies were grown overnight with constant shaking at 37°C in 5ml of LB media containing ampicillin (100µg/ml). Plasmid purification was performed using the (QIAGEN Inc., Valencia, CA), following manufacturer's instructions. The DNA sequencing was performed at Florida State University using the Big Dye Terminator protocol with the M 13 Forward and Reverse primers. *E. coli* DNA (control reaction) was included for sequencing, in order to verify the accuracy of the generated sequences. Sequence alignment, editing, and species identification The obtained DNA sequences were aligned with the P4 and P5 16S rRNA universal primer sequences and edited by using BioEdit software. Edited sequences were submitted to NCBI-BLAST (www.ncbi.nlm.gov/BLAST/) for bacterial identification. The sequences were analysed by terms of individual matches and the percentage of homology with any existing sequence in the database and were submitted to the GenBank database.



a



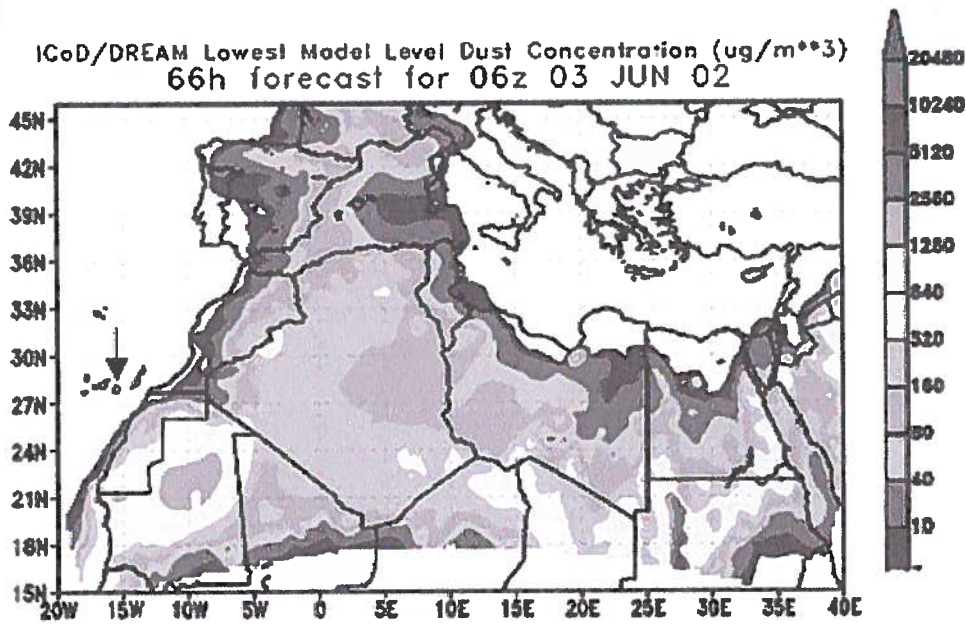


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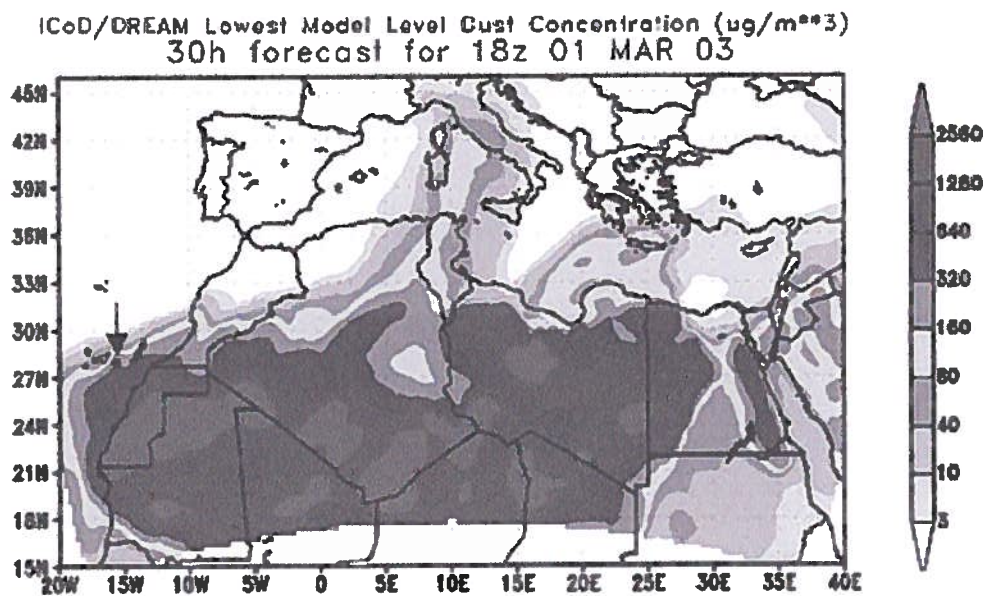
Results The Sea-WiFS images (Figures 1.0a and 1.0b), as well as the ICoD DREAM models (Figure 2.0a and 2.0b) of dust concentrations, confirmed the differences in the intensity of the dust loads affecting Gran Canaria during the sampling dates of this study. The origin of the sampled particles is different, as shown by a backward trajectory analysis of dust particles travelling at 500, 1000, and 2000 (Figures 3.0a and 3.0b). The PCR fragments obtained from the amplification of the heterogeneous DNA collected in the filter, showed products of variable length when visualised in the agarose gel analysis (data not shown).

The cloning step allowed us to obtain more than 21 colonies per sample, but we selected randomly only 21 colonies of each sample for our purposes. In theory, each one of the selected clones has the potential to represent a different 16S rRNA identity. The PCR products, which ranged from 433-445 bp, were confirmed as revealed by the DNA sequencing of each clone (42). The obtained sequences were assigned with the accession numbers DQ453569-DQ453603 (Table 1.0) at the GenBank database. Thirteen genera were identified from the 21 clones selected from the SD #7 sample transformation (Table 1.0). As well, five genera were identified from the 21 clones of SD #15 (Table 1.0). The percentages of homology for SD #7 vary from 93% the lowest to 100% the highest. All the positive clones of the SD#15 showed 99 % homology with the corresponding matches in the database with the exception of one clone that showed a 98% homology (DQ453597). However, eight clones from the SD#15 resulted as false positives for the DNA insert, a phenomenon not observed in the SD#7. Discussion Because of their proximity to the African Continent, the Canary Islands are exposed to intense Saharan dust storms throughout the year.

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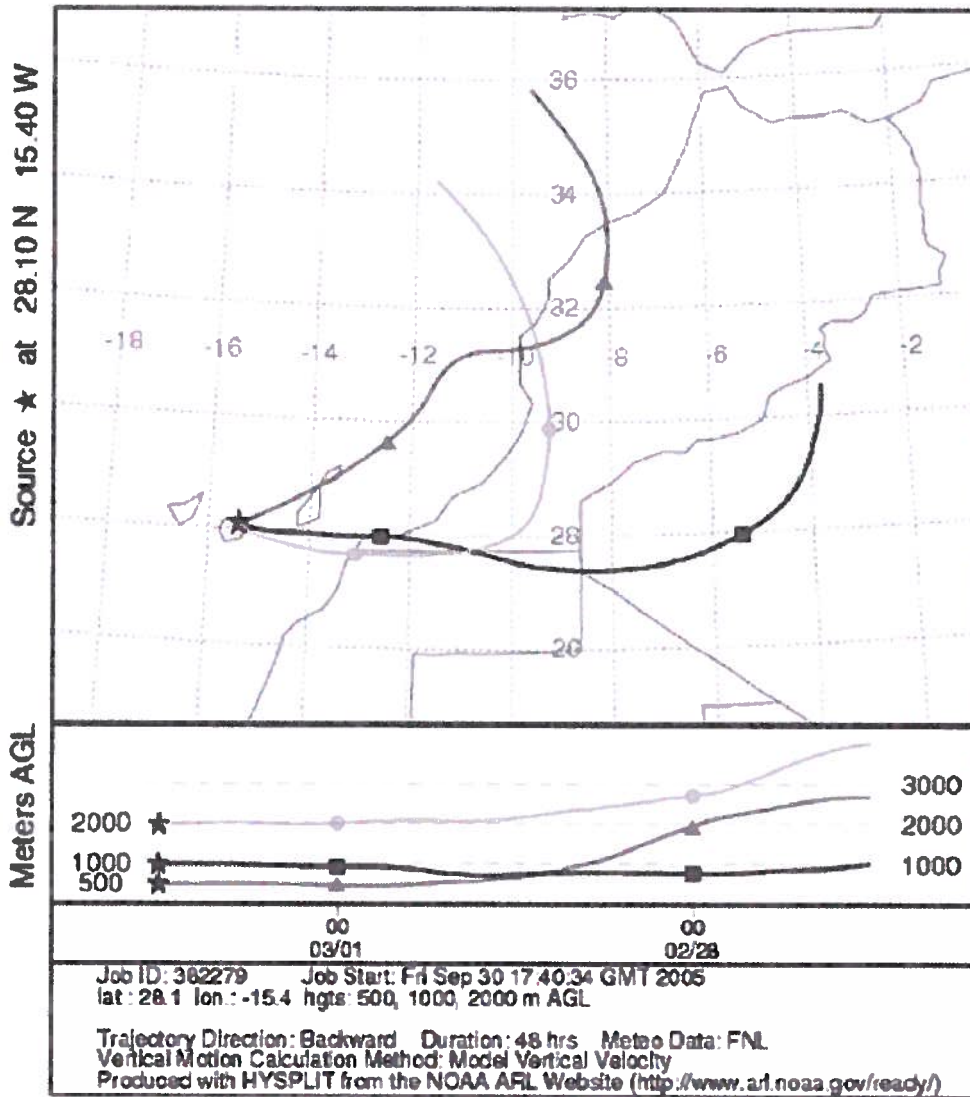


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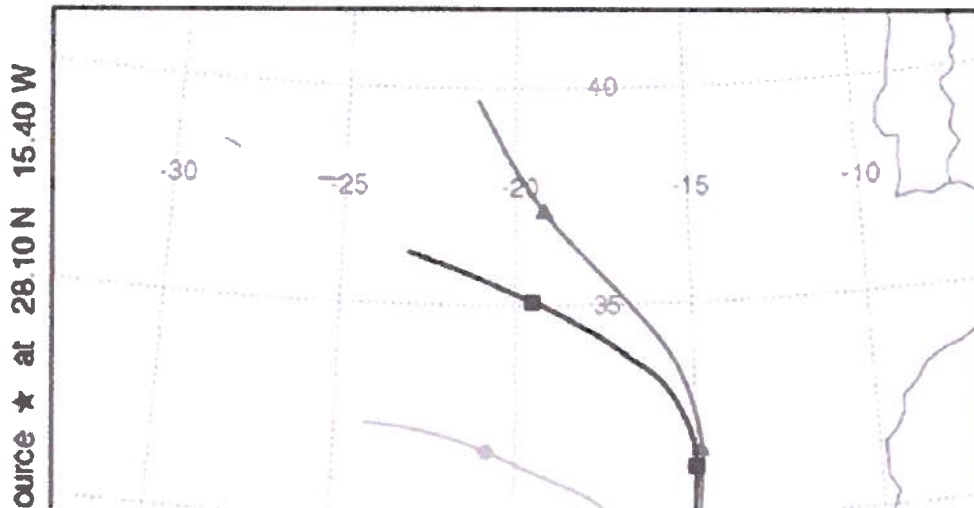


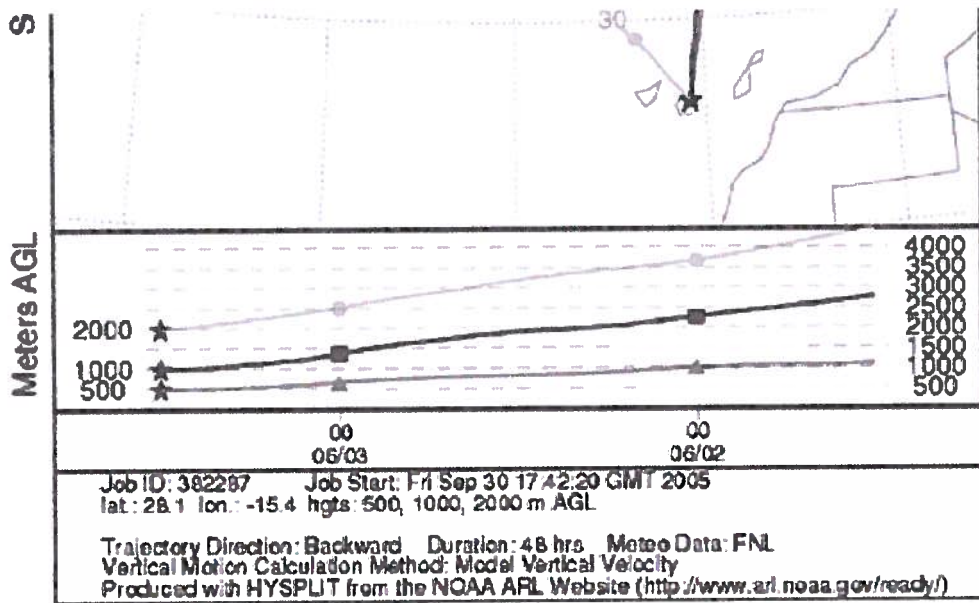
The mass concentration of dust particles in the filter from 1st March 2003 was 128X higher than the mass of dust particles concentration measured in the filter on 3rd June 2002. In addition to the differences in the intensity of the two dust storm events, an important observation is that it is also suggested that the origin of the two sampled dust particles is different (Figures 3.0a and 3.0b). A backward trajectory analysis of dust particles travelling at 500, 1000, and 2000 m confirmed the African origin of the dust sampled on 1st March 2003. However, the origin of the particles sampled on 3rd June 2002 appeared to be oceanic. We selected two different events in order to identify temporal variations in the microbial diversity spread by SD. A 68% higher diversity of bacterial species was observed in the SD#7 sample (Table 1.0), which corresponds to a large dust storm that passed over the island of Gran Canaria (Figure 1.0b).

a NOAA HYSPLIT MODEL
 Backward trajectories ending at 12 UTC 01 Mar 03
 FNL Meteorological Data



b NOAA HYSPLIT MODEL
 Backward trajectories ending at 12 UTC 03 Jun 02
 FNL Meteorological Data





The number of species found in SD #15 was lower than that of SD #7 (5 and 13, respectively) and showed two species with a higher number of clones per species: *Staphylococcus kloosii/carnosus* (6) and *Bradyrhizobium* sp. (4) (Table 1.0). We suggest that the high number of clones per species, identified in SD#15, represent lower diversity. In contrast, in SD#7, we identified a lower number of clones per species, but the representative genera was richer. In conclusion, the number of clones per species was inversely proportional to the identified bacterial diversity. Fifty percent (50%) of the bacteria identified in SD#7 are associated with human health issues. These include: *Pseudomonas*, *Rahnella aquatilis*, *Serratia fonticola*, *Sphingomonas*, *Shigella flexneri*, and *Corynebacterium mucifaciens*. In contrast, only one of the species, *Corynebacterium mucifaciens*, identified in SD #15 is pathogenic to humans. The other four species identified from SD#15 are part of normal or resident microbiota of animals (*Staphylococcus kloosii*; *Propionibacterium acnes*), soil (uncultured soil bacterium) or plants (*Bradyrhizobium*). Interestingly, by comparing the identified species, it is clear that all the species identified in the light dust event sample (SD#15) were also present in the heavy dust event sample (SD#7).

Sahara Dust Sample #7			
No. of Clones	Matching organism(s)	GenBank Accession number	% homology
1	Arctic sea ice bacterium ARK9973/Marine sediment bacterium OC-12 <i>Bradyrhizobium</i> sp.	DQ453581	99 a
2	<i>Corynebacterium mucifaciens</i>	DQ453573, DQ453583	99
1	<i>Modestobacter</i> sp. Ellin 165 a	DQ453589	99
1	<i>Morganella morganii</i>	DQ453582	97 a
2	<i>Propionibacterium acnes</i>	DQ453569, DQ453572	100
2	<i>Pseudomonas</i> sp.a	DQ453576, DQ453579	98
1	<i>Rahnella aquatilis</i> isolate k	DQ453577	99 a
1	<i>Serratia fonticola</i>	DQ453570	100
1	<i>Shigella</i> sp./ <i>Escherichia</i> sp	DQ453578	99
1	<i>Sphingomonas</i> sp.	DQ453585	100
4	<i>Staphylococcus kloosii/carnosus</i>	DQ453571, DQ453584, DQ453586, DQ453588	97-99
3	Uncultured bacterium clone MP104-01	DQ453574, DQ453575, DQ453580	99
1	<i>Sphingomonas</i> sp'	DQ453587	93 a
Sahara Dust Sample #15			
No. of Clones	Matching organism(s)	GenBank Accession number	% homology
4	<i>Bradyrhizobium</i> sp.	DQ453590, DQ453598, DQ453601, DQ453593	99
1	<i>Corynebacterium mucifaciens</i>	DQ453596	99
1	<i>Propionibacterium acnes</i>	DQ453597	98
6	<i>Staphylococcus kloosii/carnosus</i>	DQ453594, DQ453599, DQ453600, DQ453592, DQ453602, DQ453603	99
1	Uncultured bacterium clone rrw5	DQ453595	99
8	No Insert	False Positives	-

These results may represent a shared or common bacteria burden between these two regions or normal microbial inhabitants of these sites. These data can also be useful to identify the bacterial contribution from different North African geographic areas to a dust cloud crossing the Atlantic Ocean and reaching the western hemisphere. This type of strategy would provide insights about the final bacterial complexity of the dust clouds that arrive at the western hemisphere. The importance of the identification of the bacterial build-up as the final dust cloud is formed has been a constant concern. However, the viability status of the bacteria had always been presented as a methodological limitation for this purpose. With the PCS method, this limitation is overcome because the bacterial DNA can be extracted and analysed independently of the bacterial viability status. The possible existence of a bacterium with an Antarctic origin (marine sea ice brine or ponds) was not surprising.

Similar to the arctic sea bacterium identified in the SD#7 sample (DQ453581), Kellogg et al. (2004) previously reported *Kocuria* and *Planococcus* genera in a sample collected at an African dust event in Mali. It is hypothesised that as a result of changes in the Hadley circulation (past glacial periods), tropical dust (including Saharan) has been transported to the Polar Regions (Chylek et al. 2001).

Conclusions

By using the PCS method, we overcame some of the limitations of the current methods used for the identification of fungal and bacterial species. The identification can be performed independently of the viability status, using small amounts of initial material, and in a relatively brief period of time. In this pilot project, we effectively utilised the cloning of heterogeneous DNA collected from environmental samples at two different intensities of Sahara Dust clouds events. Our results indicate that the diversity of species seems to be related to the intensity of the event. This new method provides an innovative tool for the assessment of species that might not be viable or cultured. In addition, by using this method we can estimate the prevalence of the species in a given sample by identifying the number of clones per species. The identification of additional species can be achieved by increasing the number of selected clones.

* One limitation that we encountered was the false positive results (no insert) in sample SD#15. However, this might represent a technical issue (cells viability or lack of technical experience in the transformation process), rather than a questioning of the applicability of the technique per se.

* It would be beneficial to conduct a sampling in different regions and of dust events with different geographic origins because this would contribute to the elucidation of the bacterial contribution of each region to the final dust cloud. The PCS method can be employed in the characterisation of fungal and viral organisms as well.

* This work addresses the air quality to which countries located within the SD trajectory are exposed. By using this new methodological tool and from a nonconventional environmental/biological standpoint, we expect to provide a better understanding of the SD bacterial burden.

* Our findings could be used to study the presence of these organisms and their correlation with the public health issues at specific geographical regions or sampling sites during a SD event. It might also contribute to the development and implementation of more efficient management measures (e.g. medical prophylaxis for specific biological agents, scheduled vaccinations, use of protective cloth) that will decrease public health issues related to the occurrence of a SD event.

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