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ORIGINAL PAPER

Microbial hitchhikers on intercontinental dust: high-throughput sequencing to catalogue microbes in small sand samples

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Abstract Microbiological studies on the intercontinental transport of dust are confounded by the difficulty of obtaining sufficient material for analysis. Axenic samples of dust collected at high altitudes or historic specimens in museums are often so small and precious that the material can only be sacrificed when positive results are assured. With this in mind, we evaluated current methods and developed new ones in an attempt to catalogue all microbes present in small dust or sand samples. The methods used included classical microbiological approaches in which sand extracts were plated out on a variety of different media, polymerase chain reaction (PCR)-based amplification of 16S/18S rRNA sequences followed by construction of clone libraries, PCR amplification of 16S rRNA sequences followed by high-throughput

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sequencing (HtS) of the products and direct HtS of DNA extracted from the sand. A representative sand sample collected at Bahaï Wadi in the desert of the Republic of Chad was used. HtS with or without amplification showed the most promise and can be performed on ≤100 ng DNA. Since living microbes are often required, current best practices would involve geochemical and microscopic characterisation of the sample, followed by DNA isolation and direct HtS. Once the microbial content of the sample has been deciphered, growth conditions (including media) can be tailored to isolate the micro-organisms of interest.

Keywords Chad · Deserts · Eukaryota · 16S amplicons · Metagenomics

1 Introduction

Historically, true deserts in which evaporation exceeds rainfall by wide margins have been the main sources of airborne dust. Under conditions that favour transport, this dust moves above the planetary boundary layer and can travel long distances including over continents and oceans (see Shao 2008; Shao et al. 2011). Since respiratory problems are sometimes associated with this dust when it falls back to land, it has been speculated that human pathogens can hitchhike on the dust (e.g. Griffin 2007).

Our interest is in this dust, which, for example, falls sporadically on Central Europe and the East coast of the Americas, is in the microbial load that it carries (see Engelstaedter et al. 2006; Kellogg and Griffin 2006). Ultimately, we would like to know what micro-organisms are present at the desert sources of the dust and which of them survive intercontinental travel. This information is necessary to better understand how airborne diseases spread globally. We began this work by studying the historic dust that is housed in the Ehrenberg collection (see Gorbushina et al. 2007) and quickly ran up against two technical difficulties—methods for analysing the microbial content of soils are based on virtually unlimited supplies and, until very recently (Griffin et al. 2011), were limited to classical microbiological culture-based techniques (e.g. Griffin et al. 2007; Gorbushina et al. 2007).

As in vitro-based methods provide living cultures for further experimentation and proof that the microbes are indeed alive, they are indispensable. Yet these same techniques fail to deliver a complete catalogue of all micro-organisms present in a sample. Recent developments in high-throughput DNA sequencing (HtS) have revolutionised biology and are potentially powerful tools for microbial ecology. Several major impediments must be overcome before HtS can be applied to dust however including the (often vanishingly) small quantities of material available for analysis and the long list of substances that render isolation of DNA difficult from soils (Daniel 2005; Kennedy 2009). As intercontinental dust originates from desert soils, the problems in extracting high-quality DNA from dust and soil are virtually the same.

Thus, to fully answer the questions "What micro-organisms are present at the desert sources of the dust and which of them survive intercontinental travel?" detailed catalogues of all microbial inhabitants in the soils that are at the origin of intercontinental dust must be established. To do this, we used a relatively abundant desert sand sample (≈50 g) that was collected in the Republic of Chad near a region that is reputed to be at the origin of much of the world's dust (Giles 2005). In order to validate new HtS techniques based on the Illumina™ platform, we compared our methods to well-established ways of cataloguing microbes in soils. Direct sequencing of DNA isolated from soil revealed about one hundred times more micro-organisms (including aerobic, anaerobic, heterotrophic, lithotrophic, phototrophic) than current techniques.

2 Materials and methods

2.1 Sand

Desert sand sample C3 was collected aseptically by the International Committee of the Red Cross at Bahaï-Wadi, which is near Iriba [781 m above sea level (asl)], a town in the Wadi Fira Region of the Republic of Chad (Favet et al. 2012, in preparation). It was stored dry in a closed 50-ml centrifuge tube at room temperature before use. The subsample used for scanning electron microscopy (SEM) (Hitachi S-2300N, Tokyo, Japan) was air-dried and coated with platinum (Gorbushina et al. 2004).

2.2 Geochemistry

Acid digestions of 25 mg sand/dust were analysed for Al, Ba, Ca, Cr, Fe, K, Mn, Na, P, Sr, Ti, Y, Zn and Zr by inductively coupled plasma optical emission spectrometry (ICP-OES). Sc was used as an internal standard for all elements except K and Na. Sector field inductively coupled plasma mass spectrometry (SF-ICP-MS) was used to analyse the trace elements Ag, Bi, Cd, Cs, Li, Mo, Ni, Pb, rare earth elements (La-Lu), Rb, Sb, Sn, Tl, U, V, Y in low resolution and Co, Cu, Ni and V in medium resolution with In as the internal standard (Schnetger 1997). Some isotopes needed correction due to oxide interference (interfering isotopes are shown in brackets): ^{107}Ag (^{91}Zr), ^{109}Ag (^{93}Nb), ^{111}Cd (^{95}Mo) and ^{114}Cd (^{98}Mo). Oxide formation was calculated from blanks containing only the interfering isotope. Due to the use of a cooled double-spray chamber, oxide formation for Mo, Nb and Zr was typically low (0.3–0.8 %). Analytical precision was better than 3 % relative standard deviation for all elements and both methods. Accuracy was $\leq$7 % for Cd, Cu, K, Na, Ni, P, Pb, U and V, $\leq$11 % for Ag and Sn and $\leq$5 % for all other elements. Owing to the use of hydrofluoric acid, Si was evaporated during acid digestion and was calculated as SiO_2 based on the difference in all other constituents to 100 %. This leads to an overestimation of SiO_2 if volatile compounds (water, carbonates, organic carbon) are present in significant amounts.

X-ray fluorescence (XRF) for chemical analyses (including Si) and X-ray diffraction (XRD) for the analysis of mineral compounds and elemental analysis (C, H, O, N, S) were used. Elements in very small samples including compacted powders and single grains were mapped using energy dispersive X-ray spectroscopy (ED-XRF) or electron probe microanalyser (EPMA)-based techniques.

2.3 Amplifying, cloning and sequencing algal, bacterial and fungal rRNA genes

Amplification-based techniques for bacteria were performed exactly as described by Lapanje et al. (2010). Algae and fungi were studied morphologically and further identified by partial sequencing of the nuclear rRNA genes using the following primer pairs: (1) for fungi—ITS1F (5′-CTT GGT CAT TTA GAG GAA GTA-3′)/ITS4 (5′-CGC CGT TAC TGG GGC AAT CCC TG-3′) (Larena et al. 1999) and (2) for algae, the forward primer used was A-1512-5′ (Beck 2002). DNA was isolated from algae as described by Beck and Koop (2001). Double-stranded Sanger sequencing reads ($\approx$875 bp in length) comprising the 3′ part of the SSU (128 bp), the entire 658 bp ITS region (including 5.8S) and 89 bp of LSU were obtained.

PCR products were purified on Macherey–Nagel columns (Macherey–Nagel GmbH & Co. KG, Neumann-Neander-Straße, 52355 Düren, Germany) and sequenced using the Big Dye Terminator Reaction Kit 3.1 (Perkin-Elmer Inc., 940 Winter Street, Waltham, Massachusetts 02451, USA). Sequences were obtained on an ABI 3730 48 capillary sequencer (Applied Biosystems Inc., 850 Lincoln Centre Drive, Foster City, CA 94404, USA). Fragments were assembled with the aid of the Staden package (http://staden.sourceforge.net/). Valid sequences were compared with those available at GenBank using BLASTn with standard settings.

2.4 Amplification of 16S rRNA sequences followed by HtS (Illumina™)

2.4.1 Isolation of total DNA

Total DNA was isolated from 0.25 g of sand using the MO BIO PowerSoil™ DNA Isolation Kit as described by the manufacturer with the following modifications: 40 µl of solution C1 was added to the tubes, heated for 10 min at 65 °C and then vortexed at full speed for 10 min. The volume of solutions C2 and C3 was decreased to 100 µl. Solution C4 was replaced by solution CB3 (CB3 is a stronger binding solution that enhances the recovery of small DNA fragments from degraded samples (Kennedy 2009)), and 2 ml was added per gram of soil. In the final step, the sample was eluted in buffer C6 which was incubated for 3 min on the membrane at room temperature before centrifugation. DNA was purified using the MO BIO PowerClean™ DNA Clean-Up Kit according to the manufacturer's instructions with the exception of the final step, where the volume of purified water added to elute the DNA was decreased from 50 to 30 µl.

2.4.2 PCR amplification

Primers: Forward-515F-SBS3-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT**GTGTGCCAGCMGCCGCG**

GTAA (the bases marked in bold constitute the universal 16S rRNA primer).

Reverse-806R:SBS12-1 (number after the dash indicates the barcode number) CAAGCAGAAGACGGCATACGAGAT**AAGGTGG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT**GGGGACTACVSGGGTATCTAAT**.

The first set of bases marked in bold constitutes the region where the barcode was inserted, while the second set marks the universal 16S rRNA primer. PCR was performed at an initial denaturation temperature of 94 °C for 3 min, followed by 20 cycles of 94 °C for 45 s, 50 °C for 30 s and 65 °C for 90 s. A final elongation step at 65 °C was run for 10 min. PCR products were purified using the Qiagen™ PCR Purification Kit following the manufacturer's protocol.

2.4.3 *Illumina™ sequencing*

Sequencing was conducted on an Illumina HiSeq 1000™ machine (Illumina Inc., 9885 Towne Centre Drive, San Diego, CA 92121, USA) with two 101-base-pair, paired read, cycles. The sequences obtained were first trimmed for quality using a modified version of Trim2 (Huang et al. 2003—(available at: https://gist.github.com/1006830), and the first 11 bases of the primer region of each paired read were removed to prevent biases due to the degenerate bases in the primer sequences. Since all samples were sequenced in a multiplexed Illumina™ run, barcode sequences were used to remove each sample from the total sequencing output. The database used for 16S rRNA analysis was downloaded from RDP (Cole et al. 2009) and formatted using TaxCollector and the PANGEA pipeline (Giongo et al. 2010a, b). The following criteria were applied: only sequences longer than 1,200 bases were included; references in the database matches had to contain complete taxonomic information, and the following were filtered out—sequences that were 100 % identical, sequences of cloning vectors, chloroplasts and Eukarya. Sequences were compared to the RDP database using CLC reference assembly version 4.0 with paired-reads parameters and global alignment. Ninety-eight percentage of each sequence had to align to a reference in the RDP, and the following similarity cut-offs were used to classify the data further: 80 % for domain and phylum; 90 % for class, order and family; 95 % for genus; and 99 % for species.

2.5 HtS Illumina™ of unamplified DNA

A number of separate isolations of DNA from sample C3 using the technique described above were bulked to provide 4.8 µg of total DNA ($A^{260nm}/A^{280nm} = 1.95$) that was used to construct a "paired-end DNA library" following the Illumina™ "Paired-End DNA Sample Prep Kit" protocol. Sequences from paired-end Illumina™ runs were trimmed based on quality scores using the CLC Genomics Workbench version 4.0.3. Trimmed reads in the FASTA format were modified to have an A or B in the header corresponding to each pair of reads (using the script src/add_letters.py). BLASTx (2.2.23+) was used to find matching protein sequences in the SEED database. Only the two best hits with an E-value greater than 10^{-6} were reported. All data are available at MG-RAST (Chad S2 4480704.3; Chad S3 4480703.3).

2.6 Culture-based techniques

The media used are listed in Table 2 (details are given in Appendix 1). Approximately 50 mg portions of sand were suspended in physiological saline containing 0.001 % (v/v) Tween 80, shaken for 1 h (at 160 rev min^{-1}, 26 °C), kept overnight at 4 °C and the next day shaken again for 2 h. Portions (100 µl) were streaked out on the different media listed in Table 2 and incubated at 25 °C 3–10 days. Vegetative cells were eliminated and germination of spores forced by subsequently incubating the suspensions at 70 °C for 30 min and then plating them out on R2A and TSA. All purified isolates were stored on Microbank™—Blue Colour Beads (Pro-Lab Diagnostics, 20 Mural Street, Units #3 & 4, Richmond Hill, ON, L4B 1K3 Canada).

A sterile loop full of a culture grown on agar was placed in sterile H_20, boiled briefly and then subjected to PCR using universal prokaryote primers (27F-1385R) to amplify almost the entire 16S rDNA gene fragment (ca 1,400 bp). Sequences obtained by Fasteris SA, 1228 Plan-les-Ouates, Switzerland (info@fasteris.com), were blasted against the EMBL and GenBank accessions (NCBI Blast: http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). If this procedure failed to make a positive identification, a kit-based procedure (MO BIO) was used to isolate DNA from the culture, which was amplified and sequenced using the same procedures.

2.6.1 Rhizobia: "trapping" with legumes

Acacia albida (syn *Faidherbia albida* (Delile) A. Chev.—native to Africa), *A. espinosa* (North America), *A. saligna* (Australia), *A. schweinfurthii* (East Africa) and *A. tortilis* (East Africa) were used as "traps" along with *Vigna unguiculata* (West Africa), the most promiscuous legume known (see Pueppke and Broughton 1999). To do this, 1 g of sand was weighed into and distributed evenly across the surface of sterile vermiculite contained in 2-l "Leonard" jars that were planted with seedlings of all six plants (see Fig. 1S). After covering the vermiculite with sterile, plastic beads, the plants were allowed to grow under standard conditions until nitrogen deficiencies were pronounced in the control (uninoculated) plants (see Pueppke and Broughton 1999). When present, nodules were harvested and sterilised in 70 % (v/v) ethanol for 10 min, washed in sterile H_20, placed in 5 % (v/v) H_20_2 for 10 min, washed and sliced open with a sterile scalpel and some of the contents removed with a fine, sterile Pasteur pipette. The tip of the needle was drawn across the surface of a Petri dish containing YM3 agar (Broughton and John 1979), then broken off and left on the agar. Petri dishes were incubated at 26 °C. Individual colonies were purified and used to inoculate *V. unguiculata* plants growing in Magenta™ jars filled with sterile, nitrogen-free nutrient solution (Broughton and Dilworth 1971). DNA was extracted from rhizobia that were reisolated from nodules and characterised using the PCR techniques described above.

2.6.2 Algae/fungi: isolation

Two different dilutions (1:1, 1:100) of the suspensions in physiological saline described above were streaked out on different media (Table 2) and incubated at 27 °C for extended periods (min 6 weeks). BG11 medium was used for the isolation of algae/fungi (incubation at 18 °C, light intensity 60 μmol m^{-2} s^{-1}). Different fungal types were studied morphologically and further identified by partial sequencing of the nuclear rRNA genes (vide infra). Putative algal colonies were restreaked on BG11 and MBB agar and incubated for four weeks at 18 °C (12 h days–12 h nights—light intensity 60 μmol m^{-2} s^{-1}). Bright-field micrographs of specimens mounted in water were taken with a Nikon D2Xs camera (Nikon Corp., Fuji Bldg., 2-3, Marunouchi 3-chome, Chiyoda-ku, Tokyo 100-8331, Japan) connected to a Zeiss Axioplan microscope (Carl Zeiss MicroImaging GmbH, Carl-Zeiss-Promenade 10, 07745 Jena, Germany).

3 Results

As our long-term goal is to catalogue all microbes that are present in selected desert sources of intercontinental dust, we compared those methods that are currently used for identification purposes. Red brown, clumped desert sand collected near Bahaï Wadi (see Table 1; Fig. 1) was chosen for in-depth analysis since it contained the highest number of cultivable bacteria among all the samples that were collected in the Republic of Chad (see Favet et al. 2012) and was sufficiently abundant to permit multiple analyses. In contrast to all other Chad samples, which comprised individual sand grains, sample C3 had a crust-like texture made up of smaller mineral particles held together by biogenic material (Fig. 1a–c). Only diatoms could be identified morphologically (Fig. 1d). Table 1 also shows that the sand contains, with the possible exceptions of iodine and molybdenum,

Table 1 Geochemical properties of the Bahaï Wadi dust/sand sample C3

Major components (%)	Micro-nutrients (ppm)	Others (ppm)
SiO_2—64.2	Co—17	As—2
Al_2O_3—14.3	Cr—97	Ba—376
Fe_2O_3—5.2	Cu—24	Ni—45
K_2O—1.8	Fe (5.2 %)	Pb—22
MgO—1.3	I—n.d.	Rb—121
TiO_2 – 0.9	Mn—(0.1 %)	Sr—95
CaO—0.7	Mo—3	U—6
Na_2O—0.6	Se—n.d.	V—78
P_2O_5—0.1	Zn—80	Y—34
MnO—0.1		Zr—363
Total—89.2		

Sample C3 was collected by "Sandman B" on April 30, 2007, at Bahaï Wadi [N15.37.473; E23.00.626 781 m above sea level], which is near Iriba, a town in the Wadi Fira Region of the Republic of Chad. The sample was a clumped, red brown sand devoid of all traces of vegetation (see Favet et al. 2012 for further details). *n.d.* not detected

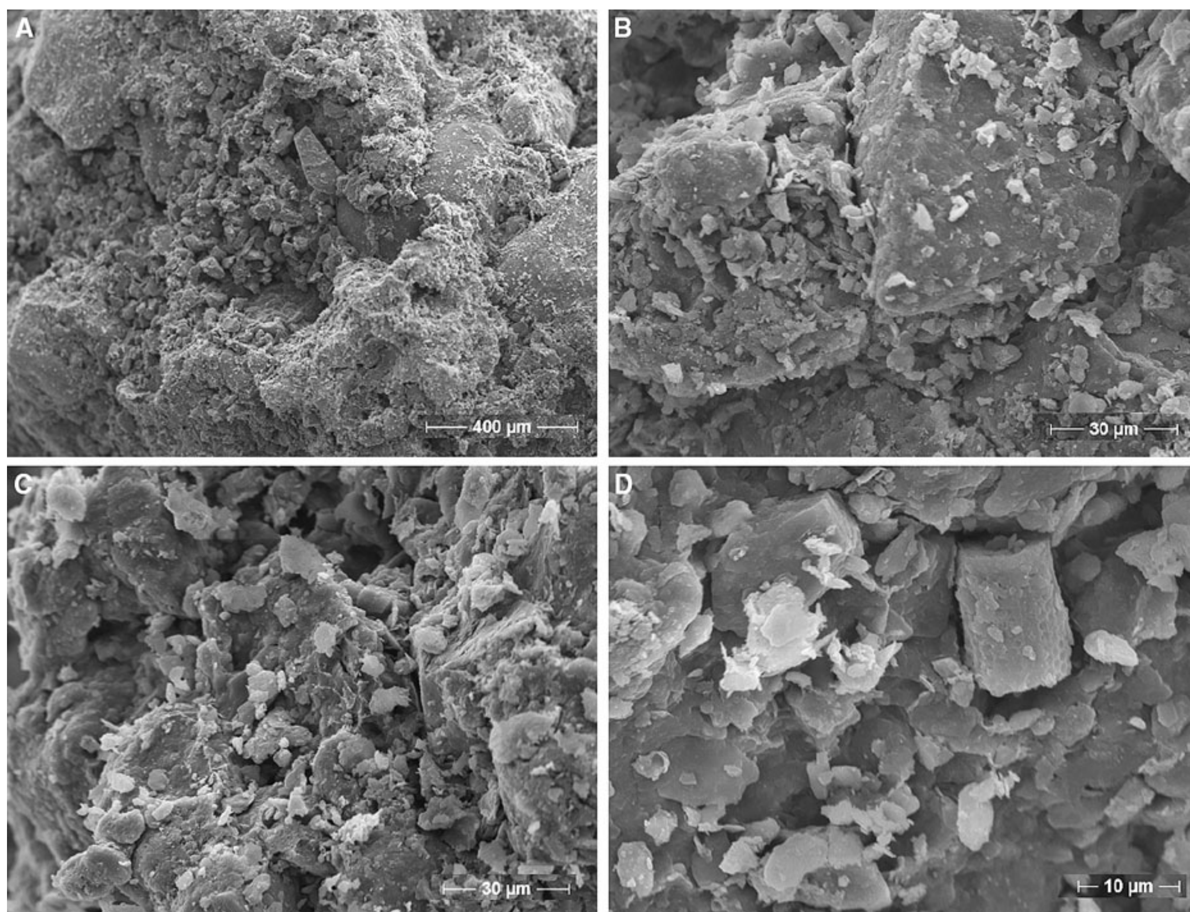

Fig. 1 Scanning electron microscopic (SEM) images showing a desert crust from Bahaï-Wadi sample C3 held together by microbial growth and clay minerals. **a** Individual sand grains were clumped together by microbial biomass and clay particles. As the sample was air-dried (not fixed), the structure of the extracellular substances was not preserved and appears in the images as "small-grained particles" that cover the sand, binding it into a mechanically stable crust. **b–d** Smaller crust particles consisting of clay as well as cellular and extracellular biological material. Note that surfaces devoid of biological material are not present. **d** Higher magnification showing a diatom that in contrast to all other cells has preserved its shape in this air-dried preparation

sufficient nutrients to support the growth of most auxotrophic organisms.

3.1 Cultivation on agar

Classic techniques of plating out suspensions on various solid media that support heterotrophic growth were tried first. Of the media tested (Table 2), R2A revealed the highest total numbers of microbes [expressed in colony-forming units (CFU) of bacteria present per g sand] despite the fact that Maxted is reputed to support larger diversities. Sample C3 contained 2.2×10^6 CFU g^{-1}, and more than a quarter of the isolates were able to form spores [*Bacillus drentensis*, *Ba. megaterium*, *Ba. pumilus*, *Ba. subtilis* and *Ba. thuringiensis*]. Representatives of the *Microbacteriaceae* (*Curtobacterium citreum)*, *Sphingomonadaceae* (*Sphingomonas yabuuchiae*) and *Streptomycetaceae* [*St. diastaticus*, *St. rochei* and *St. violorubens*] were also present. As befits inhabitants of hot, dry deserts, some of the isolates were coloured—colonies of *Cu. citreum* were dark yellow, while those of *Sphingomonas* spp. were greenish yellow. It should be noted however that only heterotrophic and aerobic bacteria can grow on the media tested.

Table 2 List of the culture media and conditions used (their exact compositions are listed in Supplementary Table 1)

Name	Abbrev.	Used for	Incubation temperature (°C)	Other conditions
Blue-Green11	BG11	Algae	18	Light intensity—60 μmol m^{-2} s^{-1}, 12 h days, agitated, aerobic
Czapek-Dox	CzD	Fungi	27	Aerobic
Dichloran-glycerol-18	DG-18	Xerophilic fungi	27	Aerobic
Dichloran rose bengal chloramphenicol	DRBC	Fungi/yeasts	27	Aerobic
Erythrol-chloramphenicol agar	ECA	Fungi	27	Aerobic
Maxted	Mxt	Bacteria	25	Aerobic
Malt-extract agar	MEA	Fungi	27	Aerobic
Modified Bold’s basal medium	MBB	Algae	18	Light intensity—60 μmol m^{-2} s^{-1}, 12 h days, agitated, aerobic
Plate count agar	PCA	Bacteria	25	Aerobic
Peptone yeast glucose vitamins	PYGV	Fungi	27	Aerobic
Reasoner and Geldreich (1985) 2A	R2A	Bacteria	25	Aerobic
Tryptone soya agar	TSA	Bacteria	25	Aerobic
Yeast mannitol 3	YM3	Rhizobia	26	Aerobic

As legume trees, particularly *Acacia* spp., are common in the desert regions of Africa, we used them as “baits” and “traps” to see whether symbiotic bacteria (rhizobia) were present in the sand. *Bradyrhizobium* spp. and *Sinorhizobium* spp. (Rhizobiales) were isolated from nodules of the “trap” plants *Acacia albida*, *A. espinosa* and *Vigna unguiculata.* (see Supplementary Fig. 1).

Cultivable fungi (*Ascomycota*) including *Aspergillus flavus*, *Cochliobolus lunatus*, *Fusarium* spp. (probably including *Fu. equiseti*), *Trichoderma inhamatum* and *Ulocladium* spp. were isolated using the six media chosen for fungi (see Table 2). Two algal isolates that were morphologically assigned to the “*Chlorella*” morphotype (Bourrelly 1966; Ettl and Gärtner 1995) were isolated. Sanger sequencing of the 3′-18S nrDNA and the ITS region resulted in a sequence almost identical (97 % similarity—all differences were located in ITS1 or ITS2 and not the 18S or 5.8S ribosomal genes themselves) to that of *Micractinium reisseri* (*Chlorellaceae*, *Trebouxiophyceae*) and to the ‘European’ algal symbiont of *Paramecium bursaria* (Hoshina et al. 2010).

3.2 DNA analysis

PCR amplification of ribosomal RNA genes is a widely used, culture-independent method for identifying micro-organisms. Two methods were used: (1) amplification followed by cloning of 16S/18S rRNA sequences and (2) amplification of 16S rRNA gene followed by high-throughput (Illumina™) sequencing. We also developed a third method—high-throughput sequencing (HtS) of unamplified DNA.

Table 3 gives an overview of the bacteria found using these methods. Amplification followed by cloning is not very efficient (only 32 reads and 13 genera identified) including [in operational taxonomic units (OTUs)] *Blastococcus*, *Clostridium*, *Enhydrobacter*, *Escherichia/Shigella*, *Gemmatimonas*, *Hydrogenophaga*, *Kribbella*, *Marmoricola*, *Micrococcus*, *Rubellimicrobium*, *Rubrobacter*, *Pseudonocardia and Salmonella*. Unidentifiable species in the order Actinomycetales and the families Anaerolineaeceae, Enterobacteriaceae, Geodermatophilaceae and Micromonosporacea were also present.

We were surprised that the Bacillaceae, which were dominant in culture, were not found using cloning techniques. On the other hand, the *Escherichia/Shigella* group, which easily grow on R2A, was not found in culture and is well represented here. Unfortunately, the success of DNA-based methods is also dependent on matching sequences being present in public databases. More than 60 % of the 16S rRNA

Table 3 Comparison of three sequencing-based methods

	16S and cloning	16S HtS	Direct HtS
Total "reads"	32	5,511	11,022,664
Classified	32	4,384	5,136
Unclassified	0	1,127	11,017,528
Bacteria	32	4,384	5,116
Archaea	0	0	20
Phylum	5	16	16
Class	7	25	25
Order	/	44	45
Family	/	98	98
Genus	13	270	260

Amplification of 16S rRNA sequences followed by cloning the amplicons (16S & cloning); high-throughput sequencing of 16S rRNA amplicons (16S HtS); and direct high-throughput sequencing (Direct HtS) of unamplified DNA] for identifying bacteria in Chad sample C3

sequences obtained above matched entries in databases, but only three of thirty-three fungal sequences could be attributed to a particular OTU. These were *Dothidotthia aspera*, *Phoma exigua* var. *exigua* and *P. macrostoma*.

Since the cultivation- and cloning-based procedures failed individually to identify major groups of bacteria in the sand, we explored newer techniques reputed to uncover most microbes present in a sample. Whole-genome amplification (WGA) was tried but in our hands introduced large biases in the types of micro-organisms found (unpublished). As a consequence, we were obliged to develop new methods to catalogue all microbes present in a sand sample. Many different methods were tried, including various ways of isolating DNA from sand, but the most successful were based around HtS (see "Materials and methods") and revealed the presence of at least one hundred times more microbes than were found using techniques that included cloning (Table 3). Two variants were the most successful—one in which 16S sequences were amplified while the other used unamplified DNA. The first yielded a total of 5,511 reads, 4,384 of which could be classified. Although at first sight the technique using unamplified DNA gave almost two thousand times as many reads ($\approx$11 million), only 5,136 of these could be classified. Both techniques identified about 260 genera in 16 phyla (see Table 3) and the percentages of reads per family were similar (see Table 4), but only direct HtS revealed the presence of Archaea.

Table 4 Comparison of the number of bacterial families (grouped by phylum) revealed in Chad Sample C3 by high-throughput (HtS) sequencing of 16S amplicons or by direct sequencing the DNA

Phylum	16S HtS	Direct HtS
Acidobacteria	2	2
Actinobacteria (incl. Geodermatophilaceae)	35	38
Bacteroidetes	3	3
Chloroflexi	7	7
Deinococcus-Thermus	1	1
Firmicutes	5	5
Fusobacteria	1	1
Gemmatimonadetes	1	1
Nitrospirae	0	1
Planctomycetes	1	1
Proteobacteria	38	34
Tenericutes	1	1
Thermotogae	1	1

Two additional phyla—Chlorobi and Crenarchaeota—were identified by Direct HtS

Eukaryota were identified on the basis of their 18S rRNA sequences and compared to different databases—Silva and the Ribosomal Database Project (RDP) (see Table 4). The RDP database delivered 50 % more "hits" than comparisons with the Silva databanks (2,892 for RDP and 1,925 for Silva). Furthermore, the Silva "hits" were not an exact subset of those made using RDP (the divisions into kingdoms and phyla are different), suggesting that both data-bases are required for thorough analyses (see Tables 4, 5, 6). The Silva database revealed seven kingdoms against 16 for the RDP database. Although the nomenclature varies according to the database used, fungi were well represented in both cases. It should be noted however that the data did not permit the identification of microbes at the species level.

3.3 Microbes found in sample C3

HtS of unamplified DNA revealed 16 phyla of bacteria, but only two phyla dominated the reads. Actinobacteria were twice as numerous as Proteobacteria (50 vs. 20 % of the reads), but both possessed the same number of families (see Table 4). No other phylum made up more than 5 % of the reads. Chlorobi and Thermotogae were poorly represented (at only

Table 5 Eukaryota revealed in Chad Sample C3 by matching direct sequencing of DNA to the Silva and Ribosomal Data Base Project (RDP) databases

	Silva	RDP
Kingdoms	7	16
Phyla	12	
Total no. of Eukaryota	1,925	2,498
Unclassified	0	394
Total no. of reads	1,925	2,892

0.01 % of the total reads, they are not shown in Table 4). Twenty (out of 5,136) reads were of Crenarchaeota (Archaea), but it was not possible to classify the reads at the family level.

All typical actinomycetes including Micromonosporaceae, Nocardiaceae, Nocardioidaceae, Streptomycetaceae, Streptosporangiaceae and Thermomonosporaceae were present. Nevertheless, the dominant families were Geodermatophilaceae (516 of 5,136 reads), Nocardioidaceae (258 reads), Pseudonocardiaceae (116 reads), Rubrobacteraceae (148 reads) and Streptomycetaceae (49 reads). Many of these bacteria possess a rich secondary metabolism that includes the production of antibiotics (Micromonosporaceae, Streptomycetaceae, Streptosporangiaceae and Pseudonocardiaceae). All also play diverse ecological roles and are active in degradation of insecticides (Intrasporangiaceae), polycyclic aromatic hydrocarbons (PAHs) (some Mycobacteriaceae), toluene (Nocardiaceae—*Rhodococcus* spp.), vinylchloride (Nocardioidaceae—*Nocardioides* spp.) and wood (cellulose, lignin, etc.) (Intrasporangiaceae, Promicromonosporaceae).

As expected of micro-organisms living in harsh environments, resistance to physical constraints were common including to: (a) cold (psychrophyly) (Microbacteriaceae—*Cryobacterium* spp.; Nocardioidaceae—*Friedmanniella* spp.; Patulibacteraceae—*Patulibacter* spp.); (b) desiccation (Kineosporiaceae—*Kineococcus* spp., Nocardiopsaceae—*Nocardiopsis* spp.); (c) heat (Acidimicrobiaceae, Rubrobacteraceae, Streptosporangiaceae and Thermomonosporaceae); (d) gamma-radiation (Kineosporiaceae—*Kineococcus* spp.; Rubrobacteraceae—*Rubrobacter* spp.). Also, the nitrogen-fixing symbiotic bacteria *Frankia* was present.

Many of the alpha-, beta-, gamma- and delta/epsilon-Proteobacteria are phototrophs or photo-heterotrophs (i.e. photosynthesise in light under anoxic conditions) [e.g. *Blastochloris* spp., *Rhodomicrobium* spp., *Rhodoplanes* spp. (all Hyphomicrobiaceae), Rhodobacteraceae, Rhodobiaceae and Rhodospirillaceae]. Facultative photo-heterotrophs that require oxygen including *Paracraurococcus* spp. (Acetobacteraceae)

Table 6 Matches of eukaryotic HtS data to the Silva and RDP databases (percentage of reads)

Kingdom	Subkingdom/division	Phylum (Class)	Silva	RDP
Fungi	Dikarya		28.3	24.3
		Ascomycota	n.d.	14.8
		Basidiomycota	n.d.	8.6
	Chytridiomycota		0.16	0.44
		Microsporidia	0.31	0.32
		Glomeromycota	n.d.	0.08
		Others	0.62	n.d.
Viridiplantae	Streptophyta		44.4	38.9
	Chlorophyta		n.d.	1.32
Metazoa		Mollusca	n.d.	25.9
		Bryozoa	n.d.	2.56
		Nematoda	0.16	0.48
		Arthropoda	0.1	0.12
		Chordata	0.47	0.08
		Annelida	n.d.	0.04
		Plathelminthes	n.d.	0.04
Chromalveolata		Ctenophora	n.d.	0.04
	Heterokontophyta	(Bacillariophycea)	0.05	0.04
	Heterokontophyta	(Chrysophyceae)	0.05	n.d.

n.d. no data

and some *Sphingomonas* spp. (Sphingomonadaceae) were also present.

The best represented proteobacterial families were the Acetobacteraceae (115 reads), the Rhodospirillaceae (82 reads) and the Sphingomonadaceae (96 reads). Among the alpha-Proteobacteria were a number of families of the order Rhizobiales that can fix nitrogen under various conditions [Beijerinckiaceae, Bradyrhizobiaceae, Rhizobiaceae (*Rhizobium* spp., *Sinorhizobium* spp.), Phyllobacteriaceae (*Mesorhizobium* spp.), Rhodospirillaceae (*Azospirillum* spp., *Herbaspirillum* spp., *Rhodospirillum* spp.) and Xanthobacteraceae (*Xanthobacter* spp.).

In other words, most of the bacteria identified were truly environmental and well adapted to their desert niches. Yet, to be sure that there were no serious pathogens, we compared the genera identified with the classification of bacteria edited by the Swiss Federal Office of the Environment (see Frey 2011). Some genera present were potential human pathogens: *Bacillus*, *Clostridium*, *Comamonas*, *Mycobacterium*, *Salmonella* and *Sphingomonas*, but *Escherichia* were not identified. *Pseudomonas* spp. include phytopathogens, suggesting that environmental bacteria which are also occasional (opportunistic) pathogens can inhabit this biotope but true pathogens (parasitic or commensals) were very rare and probably only accidentally present. Nevertheless, as noted above, the data do not permit the identification of the microbes to the species level. For this reason, we cannot exclude that pathogenic species are present in the desert sands. If pathogenic fungi are present, they almost certainly belong to risk group 2 since their spores can easily move with wind and survive long-range transport (Toepfer et al. 2012). Yet even then we have no way of knowing whether they present a danger since they were identified via DNA and there is no proof that they are still alive.

The domain Eukaryota contains the kingdoms **Amoebozoa** (single-celled organisms resembling amoebae), **Animalia** (animals), **Chromalveolata** [comprising Cryptophyta, Haptophyta and Stramenopiles which share few morphological characteristics) and the Alveolata [comprising Apicomplexa (parasitic protozoa), Ciliates (common protozoa), and Dinoflagellates (mostly marine, many of which have chloroplasts)], **Excavata** (unicellular eukarya), **Fungi**, **Plantae** (plants) and **Rhizaria** (comprising the groups Cercozoa, Foraminifera and Radiolaria) (Whittaker and Margulis 1978).

This complexity of the Eukaryota group was reflected in the number of matches ("hits") of the Chad C3 sequence data to the two databases used (Table 5, 6). Information obtained from the RDP database comparisons showed that about 40 % of the sequences matched those of plants and 23 % fungi. Almost all the plants were Streptophyta (Bryophyta and vascular plants), while the large majority (88 % of the total "fungal hits" [including Chytridiomycota (0.4 %), Glomeromycota (0.1 %) and Microsporidia (0.3 %)]) were Dikarya (Ascomycota and Basidiomycota). The same trends were visible when the sequence data were compared to the Silva databases—47 % plant sequences with a majority of Streptophyta and 32 % fungal.

Chlorophyta (green algae) made up 1.3 % of the RDP "hits", but they are absent from the Silva comparisons. Bacillariophyta (diatoms) were poorly represented (0.05 % with RDP and Silva) as were Chrysophyceae (golden algae), Amoebozoa and Ciliophora (Ciliata). Worm-like animals including Nematoda, Plathelmintes and Annelida were present. Arthropods [Arthropoda and Chordata] sequences were also found. Surprisingly, some sequences matched those of marine animals—Bryozoa (26 % with RDP) and Ctenophora (0.04 %). Currently, we are unable to explain this observation.

4 Discussion

In reviewing available techniques for isolating soil microbes, Winogradsky said (in French) "The current (culture-based) methods give a false idea about the microbial population of a soil" (Winogradsky 1925). Eighty-five years later, Penny Hirsch and co-workers' conclusions were much the same, i.e. that the soil meta-genome (the genomes of all individual organisms present in a sample) is inaccessible (Hirsch et al. 2010; Delmont et al. 2011). Then, as now, the problem is that the soil habitat is so diverse: oxygen tensions range from atmospheric to zero; water potentials are positive in well-irrigated soils but negative in the presence of solutes; rhizoplanes are rich in nutrients but bare soils cannot support microbial growth; surface soils exposed to the atmosphere react rapidly to changes in precipitation, solar radiation, winds, etc. but a few centimetres below the surface conditions are cool, dark and relatively stable. Soils are thus a mosaic

of vastly different niches jumbled together in close proximity, and each niche has its own microbial flora. Microbes that survive say in the strongly reductive conditions below rice roots cannot be cultivated under the same conditions as those found on the banks of the paddy fields. On the other hand, nucleic acids can be isolated from many soils, and HtS-based techniques are capable of matching much of the sequenced DNA to taxonomic entities. Yet, the presence of taxonomically meaningful DNA sequences in a niche says nothing about the viability of the micro-organisms. And this is a very important criterion in assessing dust loads and health risks, for dead pathogens have very restricted abilities to provoke disease.

Nevertheless, the methods developed here are a large advance over those based on cloning PCR products. At least one hundred times more prokaryotes were identified using HtS (Illumina^{TM})-based techniques. Given the length of the sequence data ("reads") obtained, it made little difference whether the 16S RNA genes were amplified before sequencing or whether the DNA was simply isolated and then sequenced. Nevertheless, direct sequencing has other advantages including (1) only one (instead of two—the second being for eukaryotes) sequencing run needs to be made; (2) microbes that do not possess ribosomes—for example, viruses can be identified this way; and (3) direct sequencing provides snapshots of the niche's global metabolic functions (Fig. 2). Confidence in HtS-based techniques was provided when we refound microbes first identified through cultivation. This was not the case with cloning-based procedures and is the primary reason why we set out to develop new cataloguing techniques.

Two of the methods tested have unique advantages (Fig. 2)—direct HtS can provide metabolic snapshots of the microbial communities, while only culture-based techniques demonstrate viability and thus potential pathogen loads of the samples. For these reasons, current "best practices" would begin by applying direct HtS sequencing techniques to any new sample. Based on the catalogues of microbes found, media and conditions could be tailored to isolate specific groups of microbes (see Table 7). Combined with microscopic and geochemical analyses, a holistic view of microbial life in a sample would be obtained this way. A simplified HtS procedure would use only the RDP database for these comparisons; it worked

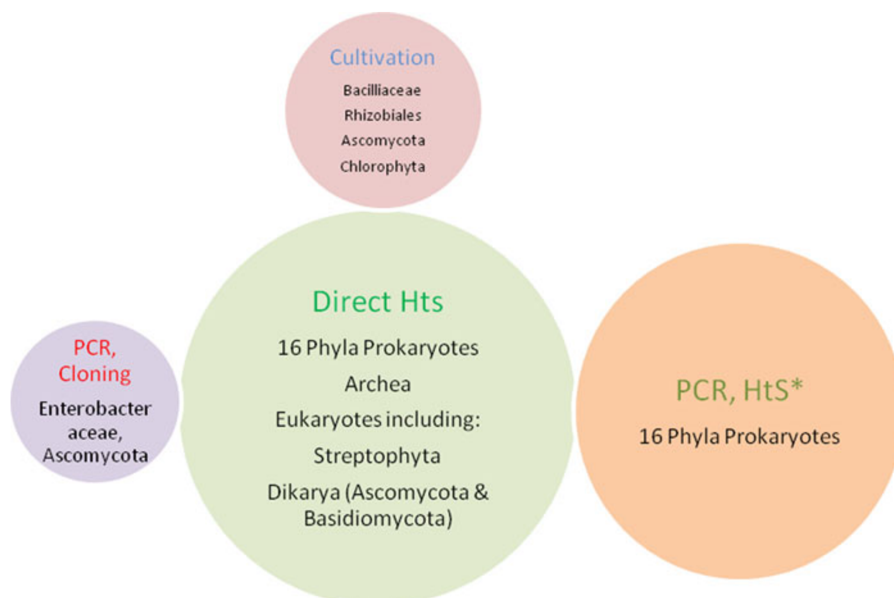

Fig. 2 Comparison of the methods used to catalogue the microbial content of a desert sand. The relative size of the circles is roughly proportional to the size and usefulness of the catalogue obtained. The major groups of micro-organisms that each method is capable of revealing are shown. Although we did not use the technique, it is possible to amplify parts or all of the 18S rRNA genes and subject the products to high-throughput (HtS) sequencing. Such a method would also reveal the presence of eukaryotes in the sample (denoted by an *asterisk* in the *right-hand circle*)

Table 7 Advantages/drawbacks of the methods used

Methods	Examples/types	Information gained	Advantages	General drawbacks	Specific disadvantages
Microscopy	Light/fluorescence (combined staining and/or sectioning)	Matrix—texture and organisation	Spatial organisation of matrix/biomass		Time-consuming, labour-intensive (highly trained specialists needed)
	Electron (SEM and TEM)	Localisation of microbes	All characteristic microbes including eukaryotes revealed		
(Geo)-chemistry	EDAX ICP-OES ICP-MS XRF XRD	Chemical and mineralogical composition of the matrix	Support for designing media/growth conditions		High-end equipment and experts needed
Molecular biology	Amplification of 16S/18S rRNA genes followed by cloning	Limited numbers of dominant microbes	Superseded	1. Completely dependent on databases 2. Inhibitors concomitantly extracted with DNA reduce the number of sequences obtained	Labour-intensive, inefficient Overshadowed by newer methods
	Amplification of 16S/18S rDNA genes followed by HtS	Separate data sets for prokaryotes and eukaryotes	High numbers of sequences obtained Specific groups targeted via primer design		PCR amplification introduces biases Biased towards certain groups Prokaryotes and eukaryotes must be treated separately Requires relatively intact DNA Generates much spurious data
	Direct sequencing	One data set containing all microbes present	No additional steps required = no biases Natural abundances closely reflected Can be performed on fragmented DNA No amplification Reveals prokaryotes and eukaryotes Method of choice		Small samples = limited DNA available for sequencing. Less selective Large genomes more “visible”
Cultivation	Different growth conditions, various media Baiting with various “trap” plants	Living clones of all cultivable strains	Isolates available for further experiments Cultivable microbes can be quantified	Many microbes uncultivable	No estimate of total population Differences in growth rate make slow-growing “environmental” strains difficult to isolate Some microbes exist only as members of consortia that cannot be cultured separately

SEM scanning electron microscopy, *TEM* transmission electron microscopy, *ICP-OES* inductively coupled plasma optical emission spectrometry, *ICP-MS* inductively coupled plasma mass spectrometry, *XRF* X-ray fluorescence, *XRD* X-ray diffraction, *EDAX* energy-dispersive X-ray spectroscopy

well with prokaryotes and gave many more matches with eukaryotes than were found using the Silva databases (see Table 6). Methods for recovering DNA from small amounts of dust/sand/soils need improvement however as does the taxonomic information in the DNA sequence databases. Most of the micro-organisms found were normal inhabitants of the sand of a hot desert. Traces of pathogens were also present, but whether they were opportunistic, environmental bacteria or true pathogens shed by animals (including humans) cannot be ascertained based on the current data.

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