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Rationale: Bauxite residue restoration success has been largely assessed on visual aboveground indicators and soil physico-chemical properties while microbial biomarkers have been mostly overlooked. The rationale of this study was to identify the status of bacterial communities in two restored bauxite residue deposit sites in comparison to a non-restored un-vegetated site and to identify potential bacterial biomarkers. The target audience for this study are readers dealing with bauxite residue and mine tailings restoration and bioremediation microbiologists.

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Abstract

Bauxite residue is the alkaline by-product generated when alumina is extracted from bauxite ores and is commonly deposited in impoundments. These sites represent hostile environments with increased salinity and alkalinity and little prospect of re-vegetation when left untreated. This study reports the establishment of bacterial communities in bauxite residues with and without restoration amendments (compost and gypsum addition, re-vegetation) in samples taken in 2009 and 2011 from 0-10 cm depth. DNA fingerprint analysis of bacterial communities based on 16S rRNA gene fragments revealed a significant separation of the untreated site and the amended sites in both sampling years. 16S amplicon analysis (454 FLX pyrosequencing) revealed significantly lower alpha diversities in the un-amended in comparison to the amended sites and hierarchical clustering separated the un-amended site from the amended site. The taxonomic analysis revealed that the restoration resulted in the accumulation of bacterial populations typical for soils including *Acidobacteriaceae, Nitrosomonadaceae*, and *Caulobacteraceae*. In contrast, the un-amended site was dominated by taxonomic groups including *Beijerinckiaceae, Xanthomonadacae, Acetobacteraceae* and *Chitinophagaceae,* repeatedly associated with alkaline salt lakes and sediments. While bacterial communities developed in the initially sterile bauxite residue, only the restoration treatments created diverse soil-like bacterial communities alongside diverse vegetation on the surface.

1. **Introduction**

Globally, mineral and ore processing residues (tailings) can occupy significant areas of land. Tailings can exhibit properties such as toxic levels of heavy metals, pH extremes, high electrical conductivity, lack of nutrients and poor structure and are often devoid of vegetation [1, 2]. In the aluminium industry, an alkaline by-product called bauxite residue is generated when alumina is extracted from bauxite ores. Its production is estimated at about 120 mega tonnes (Mt) per annum [3] and these residues are commonly deposited in nearby engineered impoundments. These sites represent hostile environments with increased salinity and alkalinity and little prospect of re-vegetation when left untreated. These bare areas are susceptible to wind and water erosion and can be a potential source of contamination to surrounding environments [1].

Placement of non-polluted materials as a 'soil' cover for tailings may reduce environmental hazards, but can be expensive and impractical due to the large areas that tailings sites can occupy and is generally recommended for tailings with extreme properties e.g. acidic and high metal concentrations [2]. Generally, less extreme tailings, can be ameliorated and support vegetative growth [4, 5]. This re-vegetation can stabilize tailings' surface and is often considered a suitable technique for achieving long term reclamation [2, 6]. Consequently, methodologies for ameliorating tailings and residues to promote vegetation establishment has received considerable attention [1, 2, 4].

Judging re-vegetation success has been largely based on visually distinguishable aboveground indicators and soil physico-chemical properties while microbial biomarkers have been mostly overlooked [7]. More recently, attention increasingly focuses on soil development within these habitats and the role of soil biota.

Microorganisms play crucial roles in soil formation, energy transfer, nutrient mobilization and cycling, vegetative reestablishment and long-term ecosystem stability [8] and a number of mine tailings studies have emphasized a strong association between the establishment of a stable plant community and the abundance and composition of soil microbiota [6]. A robust assessment of the sustainability of vegetative covers in restoration scenarios therefore requires information on the microbial community and its activity [9].

Traditionally, soil microbiota activity has been studied by substrate respiration [10] or enzyme activity [11, 12]. Microbial diversity has been often investigated via specific substrate utilization, usually via cultivation steps [13] which are time-consuming [14] and provide an incomplete assessment as only cultivable organisms (estimated to account for ~1%) are detected [15]. Recently, biochemical and molecular analytical tools have emerged to characterise soil microbial communities. These include phospholipid fatty acid (PLFA) analysis [14], community level physiological profiling (CLPP) [16] and nucleic acid based techniques, such as polymerase chain reaction (PCR) amplification combined with fingerprinting methods [17]. Microbial communities in restored and recovering natural soils have been characterised using PCR based techniques such as the denaturing gradient gel electrophoresis (DGGE), investigating sulphidic tailings [18], cloning and sequencing of 16S rRNA gene fragments in lead-zinc and copper tailings [19, 20], automated ribosomal intergenic spacer analysis [ARISA] on bauxite mining restoration sites [21], and microarray technology in coal spoil heaps [22]. However, second generation sequencing of sites with bauxite residues or mining waste has not been reported until now despite the advantages of these new high-throughput sequencing tools e.g. pyrosequencing [23].

Establishing sustainable vegetation covers on bauxite residues are a significant challenge to alumina producers [4] and vegetation establishment is inhibited by the high pH and exchangeable sodium percentage (ESP) typical of un-amended residues. Although successful re-vegetation of these residues has been reported [1], knowledge of their microbial successional development and community structure is scarce [24]. In a recent review, Gräfe and Klauber [3] highlighted the knowledge gap with respect to microbial populations capable of establishing on alkaline bauxite processing residues.

The objective of this study was to investigate the bacterial communities of two restored bauxite residue sites in comparison to an un-amended site in order to find out a) if residue restoration and re-vegetation resulted into a sustainable below ground bacterial community structure similar to semi-natural soils and b) to identify potential bacterial restoration indicators absent in non-restored sites, using up to date molecular tools including pyrosequencing.

2. Materials and methods

2.1 Sampling site

Sampling took place at the Aughinish Alumina Ltd. bauxite residue disposal area (BRDA) in Co. Limerick, southwest Ireland. Here, a series of re-vegetation trials have been conducted to investigate closure techniques. An area of the BRDA where residues were deposited in 1993 was chosen for the current study. Three treatments were investigated. At site J, re-vegetation took place in 1997 with 120 t ha^{-1} compost and 45 tonnes ha^{-1} gypsum amendment. Site R was re-vegetated in 1999 with 120 t ha^{-1} compost and 90 tonnes ha^{-1} gypsum amendment, while site M with bare residue was not amended with gypsum and compost and not seeded. During the refining process the residues are separated into a fine fraction (mud) and coarse fraction (sand) which are disposed of separately. Amendment procedures involved incorporating the coarse fraction residue sand (25% w/w) back into the top 20 cm of the residue mud. This was followed with the gypsum and organic amendment. Following a weathering period of three months to sufficiently lower pH and ESP, treatments were seeded with a mixture of *Lolium perenne*, *Festuca rubra*, *Agrostis stolonifera*, *Holcus lanatus*, *Trifolium repens* and *Trifolium pratense* at 80 kg ha^{-1}. Sites have been unmanaged since re-vegetation. The pH, electrical conductivity (Ec), available cations sodium, calcium, potassium and magnesium, exchangeable sodium percentage (ESP), available phosphorus (Olsen P), total organic carbon and nitrogen content of the samples taken in 2011 from 0-10 cm were measured in triplicate (sites J, R, M) using methods as described recently by Courtney and colleagues [4].

2.2 DNA extraction and amplification

Samples taken in triplicate in 2009 and 2011 from all three sites from 0-10 cm depth were subjected to DNA extraction and amplification. Each sample was a mixture of

sieved (4 mm) residue collected from five random locations within the radius of 1 m for each sampling site Samples were brought to the lab on the same day and cooled immediately.

DNA extraction was carried out using the Ultra Clean Soil DNA extraction kit from MoBio (Carlsbad, CA) according to the manufacturer. Obtained DNA was quantified using a Nano Drop ND-1000 (Thermo Scientific, Waltham, MA). DNA from the J and R site was subsequently diluted 5 times to obtain DNA concentrations of 1-10 ng/µl while the same DNA concentrations were obtained from the M site without further dilution.

All PCRs were conducted in a G-Storm GS2 thermo-cycler (Somerset, UK) with primers obtained from Metabion (Munich, Germany). DNA was amplified via PCR for Denaturing Gradient Gel Electrophoresis analysis using established protocols with 0.5 U of Dreamtaq polymerase, 1 x buffer with 2 mM Mg, 0.2mM dNTP each (all Fermentas, Germany) and 0.4µM primer each in a total volume of 25 µl. Primers for DGGE analysis were GC-341F and 518R (Muyzer *et al*., 1993), and a touchdown PCR protocol was employed with the following cycle conditions: 20 cycles 94°C denaturation (45s), 60-50°C (45s) annealing, 72°C extension (45s) and subsequent 18 cycles as above with an annealing temperature of 50°C.

For 454 FLX pyrosequencing, a nested PCR approach was employed using the universal primers V4F (5'AYTGGGYDTAAAGNG3') and V5R (CCGTCAATTYYTTTRAGTTT3') in the first PCR reaction with 0.5U of Robust Taq, 1x PCR buffer with 1.5 mM Mg, 0.2 mM dNTPs each (all Kappa Enzymes, Woburn, MA), 0.4 µM primer each in a volume of 25 µl. The PCR conditions were as follows: 25 cycles of 94°C (45s) denaturing, 55°C annealing (45s) and 72°C extension (60s). The resulting PCR product was diluted 10 times in ultrapure sterile

water and used as template DNA for the nested PCR with tagged LibL primers using the same PCR conditions for 18 cycles. The primers incorporated a proprietary 19-mer sequence (GCCTGCCAGCCCGCTCAG) at the 5′ end to allow emulsion-based clonal amplification for the 454 pyrosequencing system. Unique molecular identifier (MID) tags were incorporated between the adaptamer and the target-specific primer sequence (i.e. as for V4F and V5R), to allow identification of individual sequences from pooled amplicons. After purification with the Agencourt AMPure PCR purification system (Beckman Coulter, Indianapolis IN), the quantity of DNA extracted was assessed using the Quant-It Picogreen dsDNA reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions and a Nanodrop 3300 fluorospectrometer (Thermo Scientific). Amplicons were subsequently sequenced on a 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd, Burgess Hill, UK) in line with 454 protocols at the Teagasc sequencing centre.

2.3 Quantitative amplification

Quantitative PCR was conducted to quantify the number of 16S rRNA gene copies per sample as described previously [25] with the primer pair 341F and 518R. Specific quantification of the *Acidobacteriaceae* was conducted with the primers Acid31 ([26] 5'GATCCTGGCTCAGAATC) and 357R (reverse complement of 341F). PCRs were conducted with a 2x DyNAmo SYBR green master mix (Fermentas), 0.3 pmol primer each and 1 µl of DNA template in 10 µl reactions in a qPCR microtiter plate (Sarstedt, Nuembrecht, Germany) using a Lightcycler 2 480 (Roche). PCR conditions and the application of standards were as described previously [25] with 40 cycles of 95°C denaturing, 55°C annealing and 72°C extension temperatures and copy numbers ranging from 10^2 to 10^8 per reaction.

2.4 Gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was carried out on 200 x 200 x 1 mm gels with a denaturant gradient of 35–65% using urea and formamide as denaturing agents with 10% 37.5:1 acrylamide, bis-acrylamide (Biorad, Hercules, CA) in 1 times TAE buffer in a Scie-Plas TV200 DGGE apparatus (Cambridge, UK). Electrophoresis was carried out for 16 h at 63 V and 60°C. Gels were stained with SybrGold (Invitrogen, Carlsbad, CA) for 30 minutes.

2.5 Data analysis

DGGE gels were digitalised and band patterns analysed with the software package Phoretix 1D (Nonlinear Dynamics, Newcastle, UK). Obtained band pattern matrixes were exported for detrended correspondence analysis (DCA) and permutation tests (Monte-Carlo with 9999 repeats) as described previously [27]. Correlations with the physico-chemical (environmental) data were tested using a canonical correspondence analysis (CCA) and verified using a permutation test approach. A one-way ANOVA (using the Tukey post hoc test) was carried out to test variances in the physico-chemical data.

Sequence reads from the 454 FLX pyrosequencer were first analysed using the Qiime pipeline [28]. Briefly, operational taxonomical units were clustered with a similarity cut off at 97% and diversity analysis was calculated, resulting in alpha and beta diversity analysis based on sequences that exceeded 54,000 reads in total. A phylogenetic tree, for calculation of Unifrac distances, was generated using the FastTree program [29]. Taxonomic analysis of sequences was implemented with a

combination of BLAST [30] against the 16S-specific SILVA database (version 100) and MEGAN 4 [31] with a bit-score cut-off of 86. Sequences of selected families were exported into Mega 5 [32] for alignment and import of related sequences using the BLAST tool. Re-aligned sequences were used for maximum likelihood tree generation (Jukes-Cantor).

Quantitative PCR data were subjected to a univariate analysis of variance using SPSS (IBM, Armonk, NY) in order to test differences in sequence abundance as described previously [25].

Alpha diversity in the form of Shannon diversities and Chao1 index were subjected to comparative analysis using a non-paired T test (equal variances not assumed). Differences in abundances of sequences were tested (SPSS) for selected phylogenetic groups (family and phylum level) via *i*) a one-way ANOVA (using the Tukey post hoc test) to differentiate between sampling sites and *ii*) univariate analysis of variance (using the Tukey post hoc test) to differentiate between sampling sites and sampling years. Next generation sequences were deposited in the ENA sequence read archive (ERP002349).

3. Results

3.1 DNA fingerprinting

The visual inspection of the DNA fingerprints allowed separation of the profiles according to the sampling site with the naked eye. The number of detectable bands from M site profiles was in the range of 40 while approximately 50 bands were detectable in profiles from the R and J site (Supporting Fig. S1). Detrended Correspondence Analysis (DCA) of the DNA fingerprints from samples taken in 2009 and 2011 identified clear separations of the microbial communities in the un-amended and the amended sites and identified differences between the two amended types (Fig. 1a, b). Monte-Carlo permutation tests revealed that in 2009 and 2011, the bacterial communities in the M site were significantly different from the R and J site ($P<0.04$). While in 2009 the J and R site communities were significantly different ($P<0.04$) this was not the case in 2011 ($P=0.09$).

3.2 Alpha and beta diversity

The 454 pyrosequencing of 16S amplicons allowed the estimation of alpha and beta diversities of the M, J and R sites from 2009 and 2011 using Qiime [28]. The determined alpha diversity of the un-amended site was estimated to be in the region of 447 to 492 (Chao1) and 6.42 to 6.43 (Shannon) while the alpha diversity of the R and J series varied from 1116 to 1836 (Chao1) and 7.94 to 8.46 (Shannon) (Supporting Table 1). The Shannon diversity and Chao1 index of the un-amended site was significantly lower when compared to the restored sites ($P<0.01$). Furthermore, the Shannon diversity and Chao 1 index in the restored site dropped significantly in 2011 when compared to 2009 ($P<0.01$). Principal coordinate analysis (PCoA, unweighted

Unifrac distance matrix) (Fig. 2) separated the M site from the amended treatments (J, R) very clearly. However, the separation between the 2 amendment types was less pronounced than the separation by sampling year (2009 and 2011). Nevertheless, a clear separation of the M site by sampling year (2009 and 2011) was observed.

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3.3 Surface properties of bauxite residues

Physico-chemical analysis of samples taken in 2011 confirmed significantly lower pH, Ec, ESP ($P<0.05$) in the restored bauxite residue sites, as published previously [4]. Furthermore, significant increases in organic carbon, magnesium and nitrogen
285 ($P<0.05$) were revealed in the restored sites (Table 1). Canonical correspondence analysis (CCA) and permutation tests uncovered that DGGE fingerprints were significantly affected ($P<0.05$) by all environmental factors measured other than potassium and phosphorus (Supporting Fig. S2).

290 3.4 Taxonomic analysis

Taxonomic analysis revealed that remediation resulted in the accumulation of bacterial populations typical for soils in the J and R sites that include high numbers of Verrucomicrobia, Acidobacteria and Proteobacteria (Fig. 3). While Proteobacteria and Verrucomicrobia were also abundant in the un-amended site, several other taxonomic
295 groups dominated the M site too, such as the Planctomycetes (2009 only), Bacteriodetes and Actinobacteria. The lowest abundances of Acidobacteria were recorded in the M site in both sampling years (below 5%) (Fig. 3).

At the family level, significantly increased proportions of *Chitinophagaceae*, *Beijerinckiaceae*, *Xanthomonadaceae* and *Acetobacteraceae* were identified across
300 the M site ($P<0.05$, Table 2). Significantly increased proportions of the candidate

group BRC1 ($P<0.05$, Table 2) were also observed. After the creation of phylogenetic trees of these individual family groups (data not shown), representative sequences from major clades were chosen for BLAST analysis. Closer inspection of the closest related sequences were found to be A) *Chitinophagaceae* sequences from alkaline and saline lakes such as Mono lake, California and Kulunda Steppe lakes, Siberia (e.g. AF449772, EF622438, [33]; B) *Beijerinckiaceae* sequences from Lonar soda lake, India (e.g. JQ480103) and alkaline, hypersaline lakes of the Wadi An Natrun, Egypt (e.g. DQ432346, [34]); C) *Xanthomonadaceae* sequences from alkaline, saline soil (e.g. JQ427801), alkaline ikaite columns, Greenland (DQ028387, [35]; D) *Acetobacteraceae* sequences from polluted Manzala Lakes, Egypt (AB355047, [36]); E) BRC1 sequences from the alkaline lake Alchichica, Mexico (JN825632, [37]) and Guerrero Negro hypersaline microbial mat, Mexico (JN512713, [38]). In contrast, sequences identified in higher percentages in the J and R sites only were associated with the families of the *Nocardioidaceae*, *Acidobacteriaceae*, *Nitrosomonadaceae*, *Caulobacteraceae*, *Anaeroplasmataceae* and on the phylum level the candidate group of WS3 (Table 2). These increases were significant for the *Acidobacteriaceae*, the proteobacteria *Nitrosomonadaceae* and *Caulobacteraceae* and the candidate phylum WS3 ($P<0.05$). Furthermore, significant increases in the *Nocardioidaceae* (J and R, $P<0.05$) and the *Anaeroplasmataceae* (R only, $P<0.05$) were identified in the restored sites in 2011 (Table 2). Many of the representative sequences from major clades (taken from calculated trees, data not shown) were associated with sequences isolated from A) cropland soils (e.g. EF651169, cotton, Australia), B) grassland (e.g. EU134658, tallgrass prairie, USA), C) crop (e.g. AM157250, maize, France) and D) tree rhizospheres (e.g. EF018650, aspen, USA). Interestingly, many of the sequences obtained from the *Anaeroplasmataceae* family in this study from the J and R site were

closely associated with sequences found in fungal endophytic bacteria (e.g. JN791233, Italy, AMF-colonized thalli of liverworts). High abundances of *Acidobacteriaceae* sequences were found in the J and R site, exceeding 20% of the total amount of assigned sequences.

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3.5 Quantitative PCR

A quantitative PCR approach was used to verify the high numbers of *Acidobacteriaceae* in the J and R site when compared to the M site and a grassland reference soil. For the J and R site and the representative grassland site (unmanaged
335 grassland, Woburn experimental farm, UK) 15-24, 17-24 and 18-28% of the total 16S rRNA gene copy numbers could be attributed to the *Acidobacteriaceae*, respectively (Table 3) which were all significantly higher ($P<0.05$) than the abundance of sequences associated to the *Acidobacteriaceae* from the M site (0.6-2.8%).

4. Discussion

The analysis of the microbiota in restored or untreated bauxite residue has been sparsely investigated [1] even though sustained plant growth is largely dependent on microbes recycling and mobilizing soil macro-nutrients [39-42]. The use of microbiota as an indicator for successful restoration efforts has recently become of interest as the presence or absence of certain microbes could provide insight into the advancements of restoration efforts [43, 44]. This study investigated the restoration (bioremediation) progress of bauxite residues through the analysis of bacterial communities and compared it to a bare bauxite residue treatment.

The analysis of the bacterial communities employing the PCR-DGGE technique revealed significant differences between the restored and bare sites in both sampling years and significant correlations between most environmental factors, including ESP, pH, total nitrogen and organic carbon content, and the fingerprints were detected. Earlier investigations of the bauxite residue site in 2005 and 2008 found similar diversities and maturity indices of plants and nematodes in the restored sites. However, the J site appeared to have a higher overall nematode diversity [4, 45]. Significant differences between the two restored sites were also detected in this study in 2009 but not in 2011. DCA biplots from bacterial communities in the M, J and R site in this study suggest that J may have moved towards the state of R in the 2009 to 2011 period. Although site R was restored two years later than J, higher gypsum application rates were used at the R site. This may have resulted in improved physico-chemical conditions [5, 46] thus accelerating the microbial activity further than in the J site. Indeed, calcium, magnesium and nitrogen content was significantly higher in the R site when compared to J, although pH and organic carbon were not (Table 1). The use of gypsum to reduce the alkalinity of bauxite residues to promote plant

365 growth and initiate restoration has been successfully used before [1, 5] but only rarely has microbiota been employed as an indicator or driver for restoration in remediated residues [24, 43]. Recently, microbes received higher attention in mine tailings, and soils and sediments polluted by mining operation [18, 19, 22, 47]. There, alkalinity can reduce or prevent microbial activity at pH levels of 10-12.

370 The recent development in next generation sequencing including pyrosequencing is now often used to conduct in depth characterisation of microbial communities [48, 49]. Alpha diversity analysis in this study showed that the Shannon diversity and Chao 1 index from sites R and J had values similar to a large selection of German soils [50], thus suggesting the presence of an alpha diversity in the restored sites 375 similar to soils. A significant decrease of the Shannon diversity and Chao1 index in the restored sites in 2011 was detected when compared to 2009 (Supporting Table 1). This could be explained by an increased dominance of bacterial groups typically found in soils [51] at the expense of earlier residue colonisers.

The beta diversity analyses clearly separated samples from the M site and the restored 380 sites and between the two sampling dates in the case of the M site. However, separation of the R and J site was less pronounced than the effect of the sampling year (Fig. 2). Fingerprinting methods such as DGGE have been used successfully for nearly two decades to study microbial diversities [52, 53] but these methods have their limitations by displaying a finite number of different bands (different types of 385 bacteria), usually less than 100 [17, 54]. Despite these limitations, analysis of the obtained DGGE profiles in this study revealed results strikingly similar to the beta diversity calculated on the basis of the pyrosequencing results. This congruence of beta level diversity from pyrosequencing and fingerprinting results was also observed recently in mangrove micro-sites [55]. These findings demonstrate that PCR-DGGE is

still a justifiable, economical preferred method of choice for basic comparative community analysis when sequence information is not initially required.

The identification of the major bacterial phyla present in the three sites in 2009 and 2011 clearly showcased the dramatic change achieved through the restoration efforts (Fig. 1-3). A detailed analysis of bacterial families representing at least 1% of the overall bacterial sequences in one of the 6 sample types revealed that some families were significantly higher in abundance in the M sites or in the J and R site or were most abundant in J and R in 2011 (Table 2). *Chitinophagaceae*, *Beijerinckiaceae*, *Xanthomonadaceae*, *Acetobacteraceae* and members of the candidate division BRC1 sequences were only found in abundance in the M site and in the past have been closely associated with alkaline lakes, hypersaline mats and other environments of high salinity, high pH and often low carbon content [33-35, 37, 38]. These findings imply that over the years the initial sterile bauxite residue with a pH of around 12.5 [4] was colonised by bacteria normally dominating aquatic environments with similar chemical characteristics. Indeed, the non-restored bauxite residue had a high clay and silt content with a low porosity and was prone to water logging, thus resembling sediments more than soils. Low levels of organic carbon and nitrogen in this environment may be responsible for the significantly higher abundance of the *Chitinophagaceae*, that include chitinolytic bacteria, and the *Beijerinckiaceae*, with its nitrogen fixing members, The lack of input of organic carbon and pH neutralization prevented the succession of the M site towards a soil like habitat. Nevertheless, organic acid producing *Acetobacteraceae* were significantly more abundant in the non-restored site, suggesting that modest pH reductions over the years in this site to pH 10 may have been accomplished in part through bacterial activity. Restoration efforts in the J and R site transformed the sites [1, 4] and their bacterial communities

that resembled semi-natural soil environments. Detailed analysis of bacterial families most abundant in the J and R site were found to be closest related to sequences found largely in soils and rhizospheres of grassland plants and crops.

As noted above, the decrease of the alpha diversity in 2011 may be in part traced back to the emergence and higher abundance of key bacterial groups important in soils. More specifically, for instance, the amount of *Acidobacteriaceae* that make up a large part of the Acidobacteria in the J and R site exceeded 20% and was highest in 2011 in the R site. These findings were in accord with the quantitative PCR results obtained for the *Acidobacteriaceae* in the M, J and R site from 2011 that showed highest abundances in the R site and significantly higher abundances when compared to the M site (Table 3). The abundance of Acidobacteria in soils is correlated with soil pH [56]. Lauber and colleagues found that while Acidobacteria in soils with a pH of five and lower could make up more than half of all soil bacteria, Acidobacteria in soils with a pH of seven to eight may represent 20% of the total bacteria [57]. The results from this study were in accordance with the findings of Lauber and colleagues as pH levels of the R and J site were in the range of pH eight and the abundance of the Acidobacteria in R and J was in the range of 20%.

Nitrosomonadaceae were significantly more abundant in the restored sites suggesting that nitrification may be an important process in these sites. The significant higher abundance of *Caulobacteraceae* in the restored sites is in accord with the chemical analysis of the sites as members of this family have a reportedly low tolerance to salts [58]. *Anaeroplasmataceae* of the phylum Tenericutes showed higher abundances in 2011 in the J and R sites with highest numbers found in the R site in 2011 (significant, $P<0.05$, Table 2). A closer inspection of the sequences attributed to this family identified sequences closely related to endobacteria from arbuscular mycorrhizal fungi

(AMF; FJ984707 and others, [59]; FJ984707, [60]). The likelihood of increased AMF colonization in 2011 suggest that, in accordance with the other findings, both restored sites have developed into soil-like sites similar to a semi-natural soil with AMF activity, promoting plant growth [61] and thus completing the restoration development.

While previous investigations into the sites from this study have found evidence of successful restoration including plant cover and nematode diversity in 2005 and 2008, respectively, this study on the bacterial diversity revealed that between 2009 and 2011 both restored sites were still developing, becoming more like semi-natural soils exemplified by the most recent increased abundance of *Acidobacteriaceae* and *Anaeroplasmataceae*. While this study cannot precisely predict further developments in the restored bauxite residue sites, the provided evidence indicates that in 2011 site J and in particular site R were in a state that could be regarded equivalent to a semi-natural soil. The omission of restoration treatment as exemplified in site M demonstrated that although bacterial colonization took place, there was no detectable trend of the M site towards becoming a semi-natural, soil-like environment anytime soon.

Since no DNA samples prior to 2009 exist for this site, this study cannot reveal earlier microbiota states. In order to determine if restoration efforts could result in outcomes similar to the R site in 2011 but within a shorter period of time, new long-term (<10 years) studies would be necessary.

This project provided insight into the development of the bacterial community in restored and un-amended bauxite residue. While the application of soil microbes has been used in the past to improve bio-remediation of bauxite residue [1, 24], very little is known about bacterial communities in non-amended and restored bauxite residue

and this study is the first of its kind to provide in depth bacterial diversity analysis employing pyrosequencing. These and complementing data obtained from community fingerprinting and quantitative PCR detailed a picture of a successful restoration after a 12 year period with the use of gypsum and compost leading to a bacterial community rich in Acidobacteria and other typical soil bacteria including AMF endosymbionts. These bacterial groups may serve as indicator organisms for future restorations of bauxite residues and other mine processing wastes or tailings.

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Supporting Information Available

Supporting information is provided in form of a supporting table with information about the alpha diversity indices, calculated. Two supporting figures are provided showing the DGGE fingerprints and the CCA of the environmental data with the DGGE fingerprints from the 2011 sampling. This information is available free of charge via the Internet at http://pubs.acs.org/.

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Figures and tables

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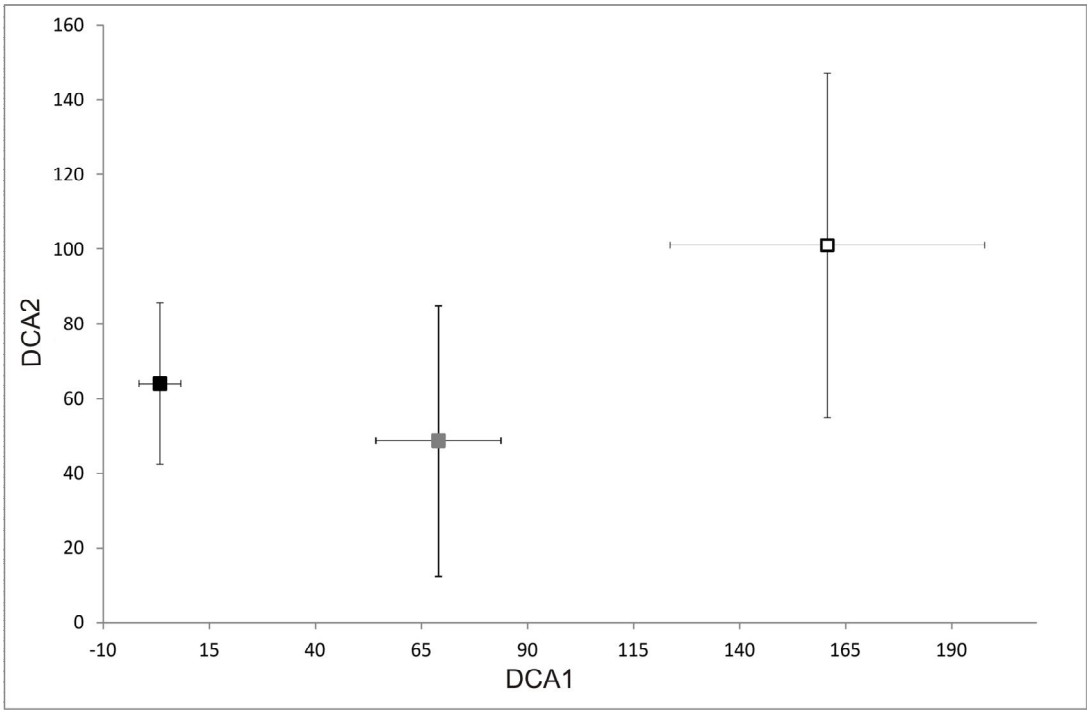
Figure 1
Detrended Correspondence Analysis (DCA) of Denaturing Gradient Gel Electrophoresis (DGGE) matrices of bacterial 16S rRNA gene fragments from bauxite residue sites M (white box), J (grey box) and R (black box) sampled at Aughinish Aluminia, County Limerick, Ireland in a) 2009 and b) 2011. Error bars indicate standard deviation.

Figure 2
Principal Coordinate Analysis (PCoA) of bacterial community sequences based on 16S rRNA gene amplicons from bauxite residue sites sampled in 2009 (black) and 2011 (grey) from site M (circle), R (diamonds) and J (squares). PCoA was calculated using an unweighted Unifrac distance matrix and visualised with King.

Figure 3
Abundance of sequences allocated to major bacterial phyla after taxonomic analysis of 16S rRNA gene amplicons from bauxite residue sites M (black bars), J (dotted bars) and R (checked bars) from samples taken in a) 2009 and b) 2011.

Fig1A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90
115
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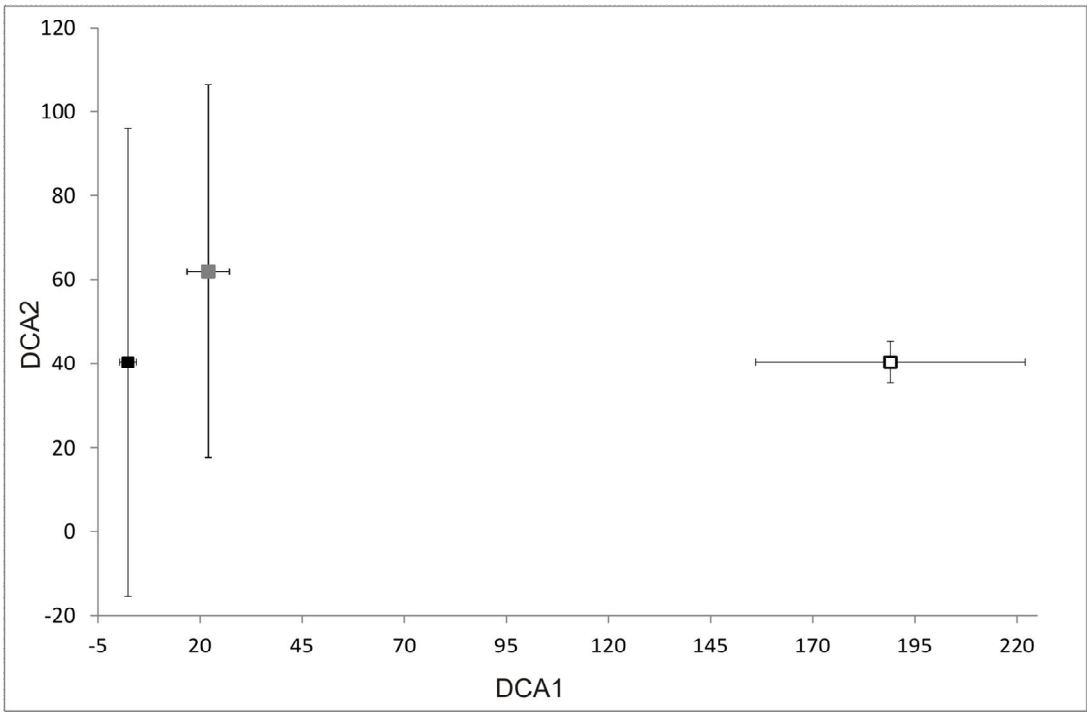
165
190
DCA1

Fig1B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80
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40
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0
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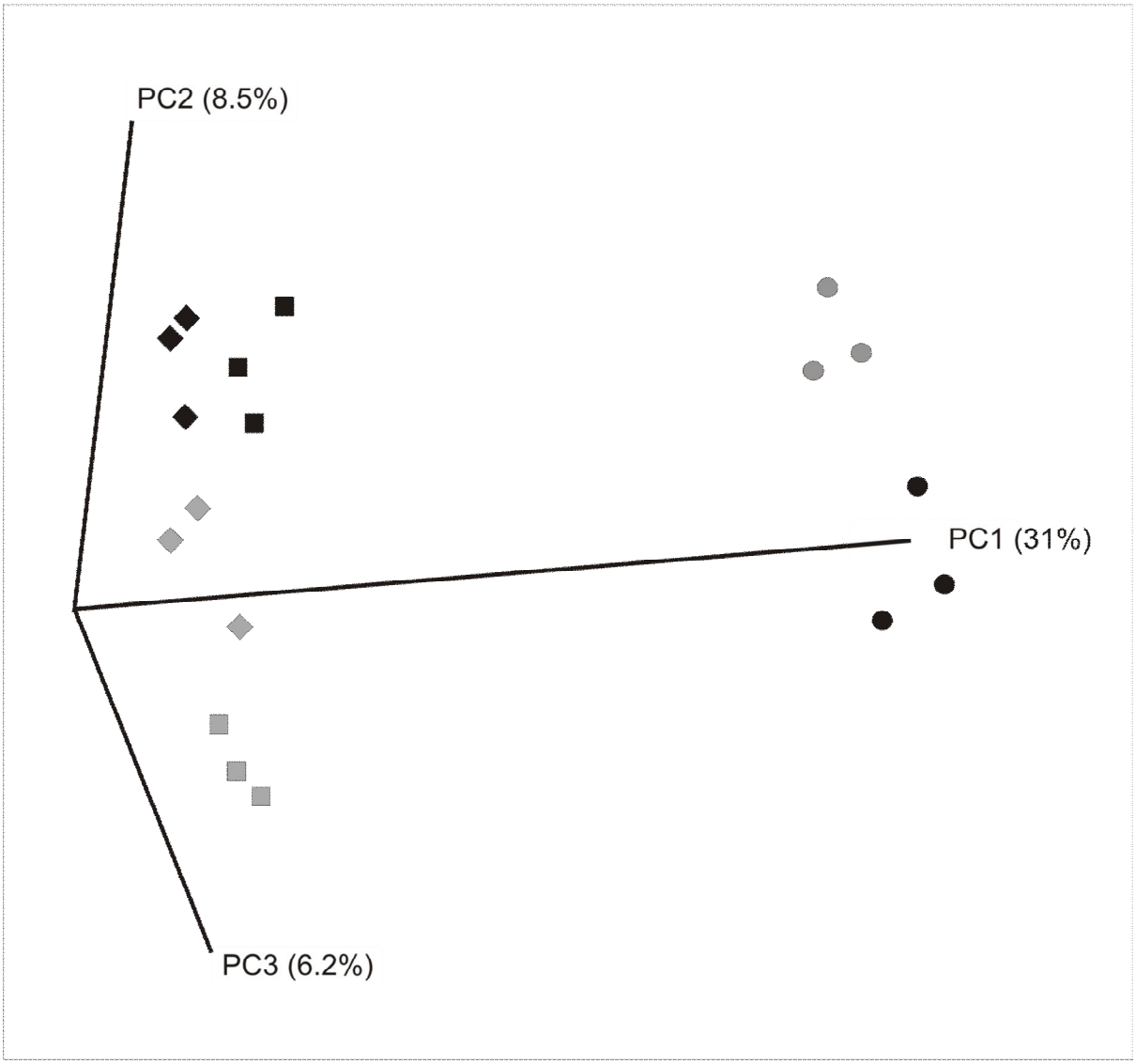
20
45
70
95
120
145
170
195
220
DCA1

Fig. 2



710

Fig 3A

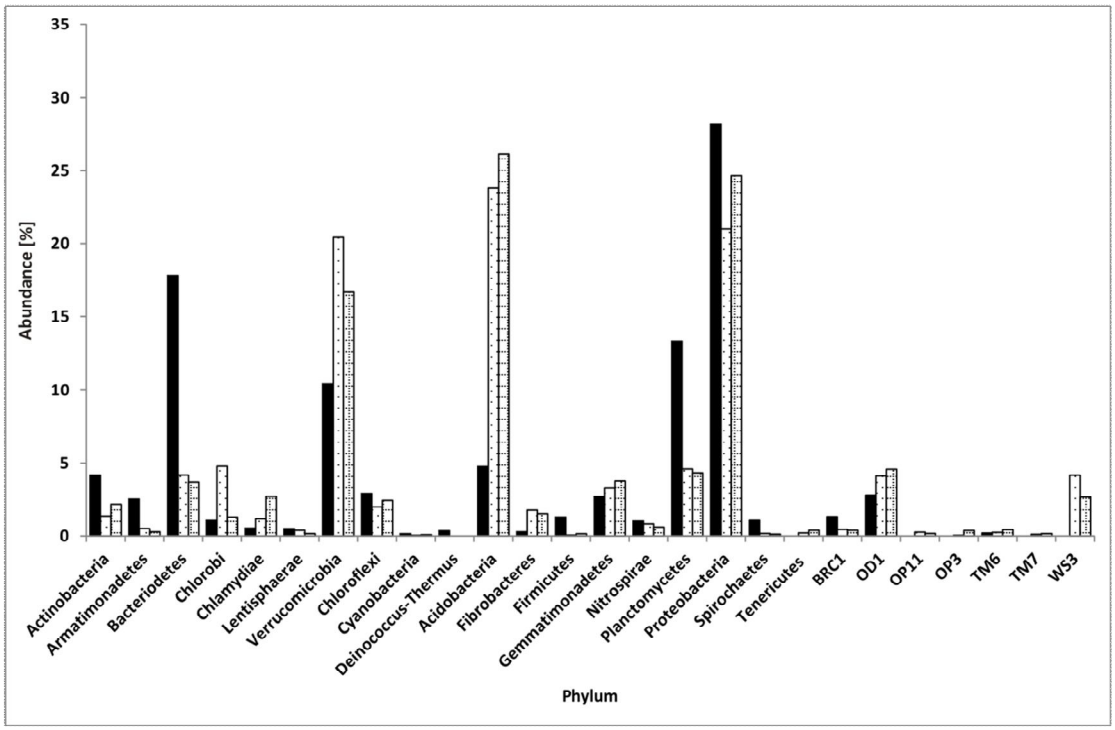
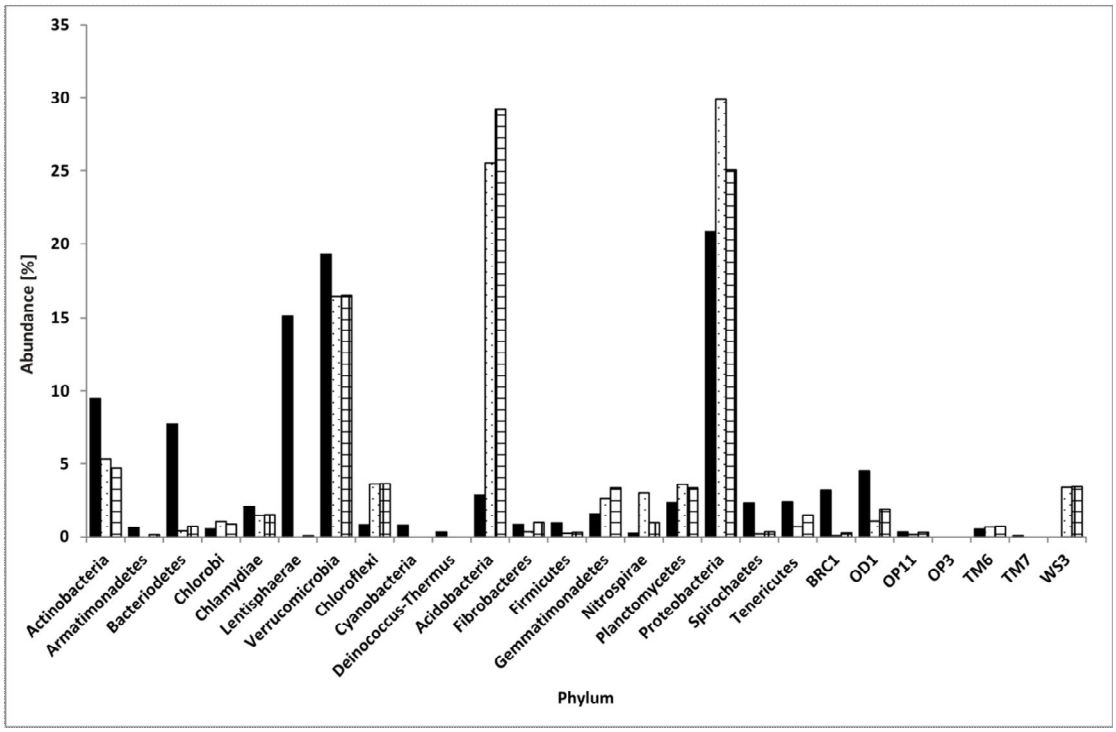

Abundance [%]
35
30
25
20
15
10
5
0
Actinobacteria
Armatimonadetes
Bacteriodetes
Chlorobi
Chlamydiae
Lentisphaerae
Verrucomicrobia
Chloroflexi
Cyanobacteria
Deinococcus-Thermus
Acidobacteria
Fibrobacteres
Firmicutes
Gemmatimonadetes
Nitrospirae
Planctomycetes
Proteobacteria
Spirochaetes
Tenericutes
BRC1
OD1
OP11
OP3
TM6
TM7
WS3
Phylum

Fig 3B


Abundance [%]
35
30
25
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15
10
5
0
Actinobacteria
Armatimonadetes
Bacteriodetes
Chlorobi
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Verrucomicrobia
Chloroflexi
Cyanobacteria
Deinococcus-Thermus
Acidobacteria
Fibrobacteres
Firmicutes
Gemmatimonadetes
Nitrospirae
Planctomycetes
Proteobacteria
Spirochaetes
Tenericutes
BRC1
OD1
OP11
OP3
TM6
TM7
WS3
Phylum

Table 1 Physico-chemical properties (0-10 cm) from restored (J, R) and bare (M) bauxite residue sites

		M	+/-	J	+/-	R	+/-
	pH	10.32^{A}	0.02	7.86^{B}	0.22	7.73^{B}	0.02
mS cm^{-1}	Ec	2.88^{A}	0.88	0.26^{B}	0.04	0.48^{B}	0.07
	Na	20.69^{A}	5.41	1.06^{B}	0.17	1.24^{B}	0.10
	Mg	0.03^{A}	0.00	0.52^{B}	0.23	1.17^{C}	0.22
	Ca	2.97^{A}	0.18	9.34^{B}	1.09	17.41^{C}	3.23
cmol kg^{-1}	K	0.32^{A}	0.10	0.4^{A}	0.11	0.39^{A}	0.10
	ESP	78.85^{A}	10.26	9.79^{B}	1.89	6.52^{B}	1.55
	org C	0.18^{A}	0.00	2.52^{B}	1.62	3.49^{B}	1.44
%	N	0.03^{A}	0.00	0.24^{B}	0.09	0.53^{C}	0.05
mg kg^{-1}	Available P	2.83^{A}	0.30	8.57^{B}	0.89	9.93^{B}	0.84

Ec = electroconductivity; ESP = exchangeable sodium percentage; mS = milli Siemens; cmol = centimole

ABC= Significantly different ($P<0.05$); +/- = standard deviation

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Table 2: Relative abundance [%] of major bacterial phyla and families in 16S rRNA gene fragment amplicon library

Phylum	Family	M2009	M2011	J2009	J2011	R2009	R2011
Actinobacteria	*Propionibacteriaceae*	0.08	1.46	n.d.	0.06	n.d.	0.06
	Nocardioidaceae	n.d.	0.16	0.12	**1.25**	0.10	**1.44**
Armatimonadetes		2.32	0.55	n.d.	n.d.	0.28	0.16
Bacteriodetes	*Chitinophagaceae*	**7.74**	**4.94**	0.95	n.d.	1.04	0.13
	Rhodothermaceae	1.17	0.20	n.d.	n.d.	n.d.	n.d.
	Cyclobacteriaceae	2.26	0.76	n.d.	n.d.	n.d.	n.d.
	Cytophagaceae	1.41	0.13	1.19	0.19	1.27	0.29
Chlorobia	*Chlorobiales*	0.98	0.47	4.70	1.01	1.25	0.85
Lentisphaerae		0.45	13.33	0.37	n.d.	n.d.	0.09
Chlamydiae	Unclass. *Chlamydiales*	n.d.	0.16	0.51	0.16	1.28	0.27
Verrucomicrobia	*Opitutaceae*	4.60	2.20	2.54	1.29	1.86	1.05
	Methylacidophiliaceae	n.d.	1.23	n.d.	n.d.	n.d.	0.09
	Verrucomicrobia sub div 3	0.73	2.20	1.36	0.48	1.22	0.89
	Verrucomicrobiaceae	0.93	1.00	1.42	0.49	0.80	0.68
	Spartobacteria (class)	1.19	3.75	5.29	6.65	3.82	4.22
Chloroflexi	Anaerolinae (class)	0.10	n.d.	1.09	1.47	1.59	2.38
Fibrobacteres	*Fibrobacteraceae*	0.26	0.74	1.72	0.34	1.48	0.95
Acidobacteria	*Acidobacteriaceae*	4.38	2.54	**21.70**	**23.20**	**20.76**	**26.38**
	Holophagae (class)	n.d.	n.d.	0.24	0.64	3.60	0.95
Gemmatimonadetes	*Gemmatimonadaceae*	2.04	1.24	2.96	2.27	3.35	3.14
Planctomycetes	*Phycisphaeraceae*	11.20	1.22	1.94	0.53	1.19	0.66
	Planctomycetaceae	1.07	0.70	1.09	2.40	1.53	2.05
Proteobacteria	*Beijerinckiaceae*	**1.99**	**3.86**	n.d.	n.d.	n.d.	n.d.
	Rhodospirillaceae	0.53	0.30	0.99	0.49	0.40	0.60
	Caulobacteraceae	n.d.	n.d.	**1.28**	**0.86**	**1.25**	**1.26**
	Acetobacteraceae	**1.24**	**1.09**	0.22	0.21	0.23	0.25
	Nitrosomonadaceae	0.61	0.45	**3.07**	**2.63**	**2.95**	**3.19**
	Xanthomonadaceae	**4.77**	**2.17**	0.24	0.07	n.d.	0.07
	Enterobacteriaceae	n.d.	0.06	n.d.	n.d.	2.28	0.17
	Halomonadaceae	1.35	n.d.	n.d.	0.00	n.d.	n.d.
	Coxiellaceae	0.61	2.40	0.86	0.80	1.68	0.72
	Nitrospinaceae	n.d.	n.d.	0.36	0.76	1.01	0.68
Tenericutes	*Acholeplasmataceae*	n.d.	2.15	n.d.	0.00	0.00	n.d.
	Anaeroplasmataceae	n.d.	n.d.	0.20	0.68	0.37	**1.45**
BRC1		**1.22**	**2.84**	0.43	0.09	0.37	0.27
OD1		2.58	4.00	4.05	1.03	4.50	1.85
WS3		n.d.	n.d.	**4.10**	**3.32**	**2.59**	**3.36**

n.d. = not detected

Highlighted numbers indicate significant differences ($P<0.05$)

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Table 3: Quantitative PCR of bauxite residue sites in 2011
(M, J, R) and a reference soil (S)

Bauxite residue site	*Acidobacteriaceae* / 16S [% gene copy number]	Standard deviation [+/-]	Univariate analysis [P=0.05]
M	0.29	0.31	A
J	13.55	3.98	B
R	15.85	5.01	B
S	15.11	7.98	B