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Relic DNA is abundant in soil and obscures estimates of soil microbial diversity

Paul Carini[1]*, Patrick J. Marsden[2], Jonathan W. Leff[1,3], Emily E. Morgan[1], Michael S. Strickland[4] and Noah Fierer[1,3]*

Extracellular DNA from dead microorganisms can persist in soil for weeks to years[1–3]. Although it is implicitly assumed that the microbial DNA recovered from soil predominantly represents intact cells, it is unclear how extracellular DNA affects molecular analyses of microbial diversity. We examined a wide range of soils using viability PCR based on the photoreactive DNA-intercalating dye propidium monoazide[4]. We found that, on average, 40% of both prokaryotic and fungal DNA was extracellular or from cells that were no longer intact. Extracellular DNA inflated the observed prokaryotic and fungal richness by up to 55% and caused significant misestimation of taxon relative abundances, including the relative abundances of taxa integral to key ecosystem processes. Extracellular DNA was not found in measurable amounts in all soils; it was more likely to be present in soils with low exchangeable base cation concentrations, and the effect of its removal on microbial community structure was more profound in high-pH soils. Together, these findings imply that this 'relic DNA' remaining in soil after cell death can obscure treatment effects, spatiotemporal patterns and relationships between microbial taxa and environmental conditions.

Microbes play critical roles in terrestrial biogeochemistry and the maintenance of soil fertility. Microbiologists, biogeochemists and ecologists now routinely use DNA-based approaches such as amplicon (marker gene) sequencing, quantitative polymerase chain reaction (qPCR) and shotgun metagenomics to determine the composition and diversity of soil microbial communities. These methods have advanced our understanding of terrestrial microbiology in a myriad of ways by (1) revealing that thousands of microbial taxa can inhabit a single gram of soil[5–7], (2) uncovering novel soil microbial diversity[8,9] and (3) identifying putative functions of undescribed taxa[10].

Linking the activities of microbes to soil processes necessitates distinguishing viable cells (both metabolically active and dormant cells) from those that are dead. Previous work has shown that after a cell dies, amplifiable extracellular DNA can persist in soils for weeks to years (reviewed in refs 1–3). The longevity and size of this extracellular DNA pool is controlled by a myriad of complex physical and biological factors. For example, soil mineralogy, pH and ionic strength can control the sorption of DNA to the soil matrix, as well as the molecular integrity of the DNA itself[11,12]. Sorbed extracellular DNA has reduced accessibility to microbes, thereby limiting its removal from the environment by anabolic or catabolic processes or as a source of transformable genetic material (reviewed in ref. 13). Although it is well-established that soils can harbour significant amounts of extracellular DNA, we do not understand how this extracellular DNA pool may influence DNA-based assessments of soil microbial communities.

Given the potential for extracellular DNA to persist in soil, we hypothesized that extracellular DNA may obscure DNA-based estimates of the diversity and structure of soil microbial communities[14,15]. Differentiating between DNA originating from living microbes versus extracellular sources is important, because ecological definitions of community-level diversity and structure should only encompass organisms that are alive in a sample. Here, we define 'relic DNA' as DNA that is either extracellular or in cells that are no longer intact and thus removed by the photoreactive viability PCR indicator propidium monoazide (PMA)[4,16]. We report the amount of microbial DNA derived from relic DNA pools and show how relic DNA affects the observed richness and composition of microbial communities. We also identify which soil characteristics are linked to greater relic DNA effects and discuss the implications of relic DNA on molecular analyses of microbial communities in soil and other environments.

Results and discussion

We investigated the effect of relic DNA on microbial diversity estimates in 31 soils collected from a wide variety of ecosystem types across the USA selected to encompass a broad range in edaphic characteristics (Supplementary Table 1). Sub-samples of each soil were either treated with PMA ($n = 5$) or left untreated ($n = 5$). PMA is a photoreactive DNA intercalating dye that is generally excluded by cells with intact membranes, but binds relic DNA (extracellular DNA and DNA of cells with compromised cytoplasmic membranes[4]). When exposed to light, intercalated PMA permanently modifies DNA, rendering it un-amplifiable by PCR[4]. We used qPCR to calculate the amount of relic DNA by subtracting the abundance of amplifiable prokaryotic 16S rRNA genes or fungal internal transcribed spacer 1 (ITS) amplicons in PMA-treated samples (DNA from intact cells) from the abundance of these amplicons in untreated samples (total DNA = DNA from intact cells + relic DNA). Microbial communities were characterized by high-throughput sequencing of amplified rRNA gene regions (16S rRNA for prokaryotes or the ITS region for fungi) from both PMA-treated and untreated soil sub-samples. We compared estimates of microbial richness, overall community composition and the relative abundances of individual taxa after standardizing all libraries to equivalent sequencing depths.

Relic DNA represented a large fraction of microbial DNA in many soils. Across all 31 soils, 40.7 ± 3.75% (mean ± s.e.; $n = 155$) of amplifiable prokaryotic 16S rRNA genes were derived from the relic DNA pool ('All soils' Fig. 1a). Similar patterns were observed for fungi,

[1]Cooperative Institute for Research in Environmental Sciences, University of Colorado, Boulder, Colorado 80309, USA. [2]Department of Chemistry, Metropolitan State University of Denver, Denver, Colorado 80217, USA. [3]Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, Colorado 80309, USA. [4]Department of Biological Sciences, Virginia Tech University, Blacksburg, Virginia 24061, USA.
*e-mail: paul.carini@gmail.com; noah.fierer@colorado.edu

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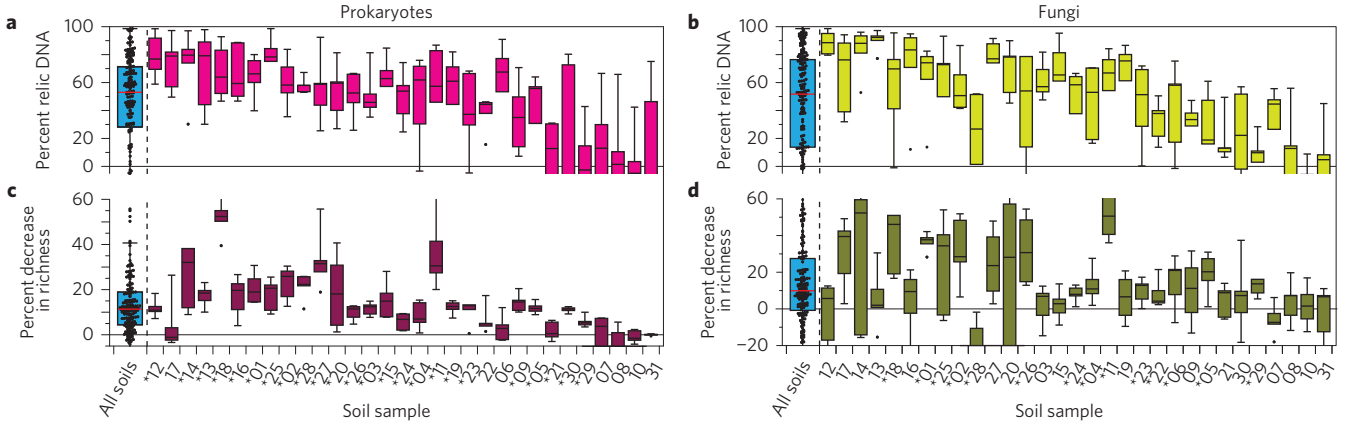

Figure 1 | Relic DNA inflates soil microbial diversity estimates. a,b, Percent of total prokaryotic 16S rRNA gene copies (**a**) or fungal ITS copies (**b**) in the relic DNA pool, as quantified by qPCR. **c,d**, Percent decrease in total prokaryotic (**c**) or fungal (**d**) richness after removal of relic DNA, as estimated from rarefied amplicon sequence data. Soils are ordered from left to right by decreasing mean percent prokaryotic relic DNA, with the 'All soils' category illustrating the distribution of the percent relic DNA and percent richness decrease across all soils. Across all soils, we detected significant differences in richness with relic DNA removed; the mean reductions in richness were 40.7% and 40.5% for prokaryotes and fungi, respectively (two-tailed *t*-test $P \leq 0.05$ in both cases). Soil sample numbers marked with an asterisk had significant differences in richness after relic DNA was removed (two-tailed *t*-test $q \leq 0.05$). Box plots illustrate interquartile range ± 1.5 × interquartile range. The horizontal line in each box plot is the median. Outliers (>1.5 × interquartile range) are shown as points, except in the All soils category, where all points are shown. Some box plots are truncated; see Supplementary Dataset 1 for complete data set and the abundance of 16S rRNA gene copies or ITS amplicon copies per gram of soil for each subsample. Supplementary Table 1 shows the mean percent of relic DNA and mean percent decrease in richness for each soil.

where 40.5 ± 4.12% (mean ± s.e.; $n = 155$) of fungal ITS amplicons originated from the relic DNA pool ('All soils' Fig. 1b). Several lines of evidence suggest that these estimates of the percent of relic DNA are conservative. First, because some dead cells retain intact membranes, PMA probably underestimates dead cell concentrations[17,18]. Second, when we experimentally added extracellular DNA to soil, our approach completely removed the added extracellular DNA from most soil samples, but only reduced extracellular DNA spiked into a soil with large amounts of relic DNA (Supplementary Fig. 1). This demonstrates that our approach is effective across a broad range of soil types and is conservative in soils where relic DNA is abundant. Conversely, it is unlikely that our approach removed DNA from living cells because (1) studies have shown the effects of PMA on intact microbial cells are minimal and PMA primarily targets extracellular DNA or DNA from cells that are no longer intact[4,16,18,19]; (2) our own tests confirmed PMA treatment did not significantly reduce the amount of DNA from live *Escherichia coli* or *Saccharomyces cerevisiae* cells spiked into buffer (Supplementary Fig. 2); and (3) we did not observe relic DNA in all soils (Fig. 1a,b), suggesting that PMA treatment did not inflate estimates of the percent relic DNA by entering live cells.

Removal of relic DNA significantly reduced estimates of soil microbial diversity. Across all samples, the total prokaryotic richness was reduced by 13.9 ± 1.20% (mean ± s.e.; $n = 155$) after relic DNA was removed (Fig. 1c). In 24 soils, the prokaryotic richness was significantly lower, by up to 55%, after relic DNA was removed (two-tailed *t*-test *q* value of ≤0.05) (Fig. 1c). The percent decrease in the number of prokaryotic taxa after relic DNA was removed was positively correlated with the proportional abundance of 16S rRNA genes present in the relic DNA pool (Supplementary Fig. 3a). That is, soils with more relic DNA tended to have lower richness once relic DNA was removed. Similar results were observed when we analysed fungal DNA; fungal richness decreased by an average of 12.4 ± 1.97% (mean ± s.e.; $n = 152$) after relic DNA was removed (Fig. 1d). The percent decrease in fungal richness after relic DNA removal was significant in 14 soils (two-tailed *t*-test *q* value of ≤0.05) and reduced estimates of fungal diversity by up to 52% (Fig. 1d).

Removal of relic DNA can substantially reduce estimates of soil microbial diversity, indicating that commonly used molecular methods for assessing soil microbial diversity may inflate richness estimates due to the detection of DNA from cells that are unlikely to be viable. However, similar to previous studies[20], the removal of relic DNA did not result in a significant reduction of prokaryotic diversity in several soils (Fig. 1c), suggesting that, in some soils, the diversity detected in the relic DNA pool is reflective of the diversity obtained in the total DNA pool. Similarly, although an average of ~40% of the total fungal DNA was derived from relic DNA pools (Fig. 1b), the effect of relic DNA removal on fungal richness was variable across the soils examined, as highlighted by the lack of a significant correlation between the percent fungal relic DNA and the percent decrease in richness after relic DNA removal (Supplementary Fig. 3b). These between-sample differences in the magnitude of the effects of relic DNA on estimates of fungal diversity may be a product of differences in the temporal variability of fungal communities at individual sites.

Estimates of microbial community composition were also significantly influenced by the presence of relic DNA. Soil type was the strongest predictor of community differences (permutational multivariate analysis of variance (PERMANOVA) $R^2 = 0.727$, $P \leq 0.001$ for prokaryotes; $R^2 = 0.646$, $P \leq 0.001$ for fungi). That is, we could still discriminate between the distinct microbial communities found in the different soils whether relic DNA was removed or not (Supplementary Figs 4–6). However, the effects of relic DNA removal on community composition across soils was also significant for both prokaryotic and fungal communities (PERMANOVA $P \leq 0.001$ for prokaryotes; $P \leq 0.001$ for fungi). On an individual soil basis, the composition of prokaryotic communities was significantly affected by the removal of relic DNA in all 31 of the soils tested (PERMANOVA $R^2 = 0.10$–0.23, *q* value ≤ 0.05) (Fig. 2a). In 21 of the 31 soils, removal of relic DNA also had a significant effect on the composition of fungal communities (PERMANOVA $R^2 = 0.10$–0.22, *q* value ≤ 0.05) (Fig. 2b). The effects of relic DNA on the composition of both prokaryotic and fungal communities were positively correlated (Supplementary Fig. 7), highlighting that the magnitude of relic DNA effects on community composition were similar for both prokaryotic and fungal communities. Moreover, the mean difference in microbial community composition after relic DNA removal was negatively correlated with the mean abundance of

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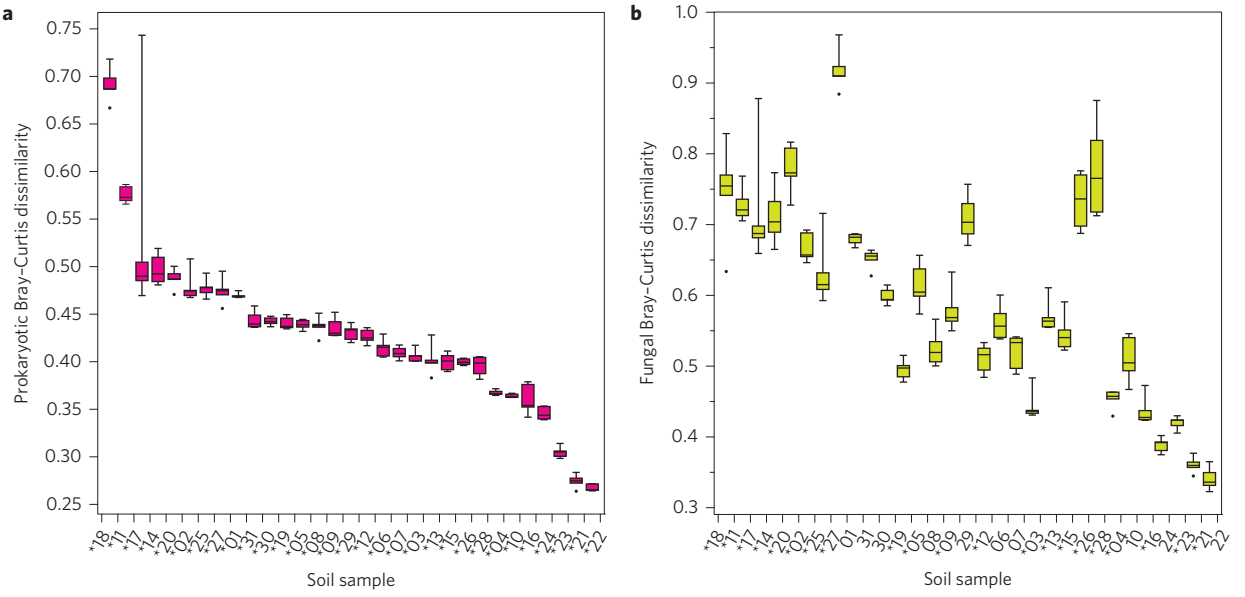

Figure 2 | Relic DNA removal can have a significant effect on community structure within a given soil sample. a,**b**, Mean dissimilarity in soil prokaryotic (**a**) and fungal (**b**) communities after relic DNA removal, relative to untreated soils. Soils in **a** and **b** are ordered from left to right by decreasing order of the mean dissimilarity for prokaryotic communities. Asterisks next to soil sample numbers denote significant community differences between relic and total DNA pools (PERMANOVA $q \leq 0.05$). Box plots illustrate interquartile range ± 1.5 × interquartile range. The horizontal line in each box plot is the median. Outliers (>1.5 × interquartile range) are shown as points. Supplementary Table 1 presents mean Bray–Curtis dissimilarities for each soil.

16S rRNA gene or ITS amplicons per gram in untreated soil (Spearman's $\rho = -0.48$, $P = 0.006$ for prokaryotes and $\rho = -0.93$, $P < 0.001$ for fungi). This correlation is probably the result of the communities in replicate sub-samples being more distinct from one another when overall biomass is lower, an effect that is taken into account in our statistical analyses.

The relative abundances of numerous microbial lineages changed after the removal of relic DNA, but the taxa that changed and the direction of observed shifts varied across soils. For example, in a grassland soil from the northeastern USA (soil 25), Actinobacteria and α-Proteobacteria significantly increased in relative abundance after relic DNA was removed, but Verrucomicrobia decreased (Fig. 3). In many cases, the changes in estimated relative abundances after relic DNA removal approached or exceeded 25% (Fig. 3). The relative abundances of α-Proteobacteria were consistently greater after relic DNA removal (Mann–Whitney U two-tailed $P \leq 0.05$) (Supplementary Fig. 8a), suggesting that the relative abundances of viable α-Proteobacteria are underestimated in many soil studies. In contrast, agaricomycete fungi were significantly less abundant after relic DNA was removed (Mann–Whitney U two-tailed $P \leq 0.05$) (Supplementary Fig. 8b), suggesting that either Agaricomycetes are less abundant than commonly reported (or other fungal taxa are relatively more abundant after relic DNA removal), or viable Agaricomycetes are broadly permeable to PMA. These results highlight that the effects of relic DNA removal vary depending on the taxon in question, are not predictable *a priori*, and vary depending on the soil in question.

Relating the relative abundances of microbial taxa or protein-coding genes to soil biogeochemical process rates has been challenging, hindering attempts to link microbial communities to the ecosystem-level processes they can control[21]. Because the relative abundances of living microbial populations may be considerably higher or lower than estimated based on analyses of the total DNA pool, the presence of relic DNA probably obscures correlations between the relative abundances of individual microbial taxa and specific biogeochemical processes. For example, in several soils, relic DNA resulted in the misestimation of the relative abundances of ammonia-oxidizing archaea (AOA) and nitrite-oxidizing bacteria (NOB) by >25% (Supplementary Fig. 9a,b). We use AOA and NOB as examples because these taxa were relatively abundant across all soils, and several studies have attempted to link their abundances to nitrification rates (for example, ref. 22). As another example, we found that the measured abundances of Glomeraceae, a family of arbuscular mycorrhizal fungi important to plant health[23], increased by >25% in two soils and decreased by >25% in seven soils after removal of relic DNA (Supplementary Fig. 9c). Thus, failure to remove relic DNA before investigating relationships between specific soil processes and DNA-based quantification of abundances can obscure associations between microbial abundances and key soil processes.

Consistent with previous studies[12], we found that edaphic characteristics known to influence electrostatic interactions between DNA and soil particles were significant predictors of the presence of microbial relic DNA (Table 1). For example, soils with few exchangeable bases, especially K^+, were likely to contain relic DNA from both prokaryotic and fungal sources (Table 1 and Supplementary Fig. 10). Although soils with low pH, electrical conductivity and cation exchange capacity were more likely to harbour relic DNA, this pattern was stronger for prokaryotes than for fungi (Table 1). Interestingly, pH predicted the magnitude of the overall change in both prokaryotic and fungal community composition after relic DNA removal, as soils with higher pH were likely to have larger community differences after relic DNA was removed (Supplementary Fig. 11). However, the proportional abundance of relic DNA was not correlated with the community differences after PMA treatment (Supplementary Table 1). These results highlight that the effects of relic DNA are variable across different soil types. In particular, both prokaryotic and fungal relic DNA is more likely to be present in soils with few exchangeable base cations (below ~40 meq per 100 g; Table 1), but the microbial community differences apparent after relic DNA removal are more pronounced in higher pH soils (Supplementary Fig. 11).

Our finding that relic DNA can lead to significant overestimation of soil microbial diversity and reduce the ability to accurately quantify prokaryotic and fungal community structure has several important implications. First, it suggests that the actual microbial diversity in soil is lower than often reported (Fig. 1). Second, although the

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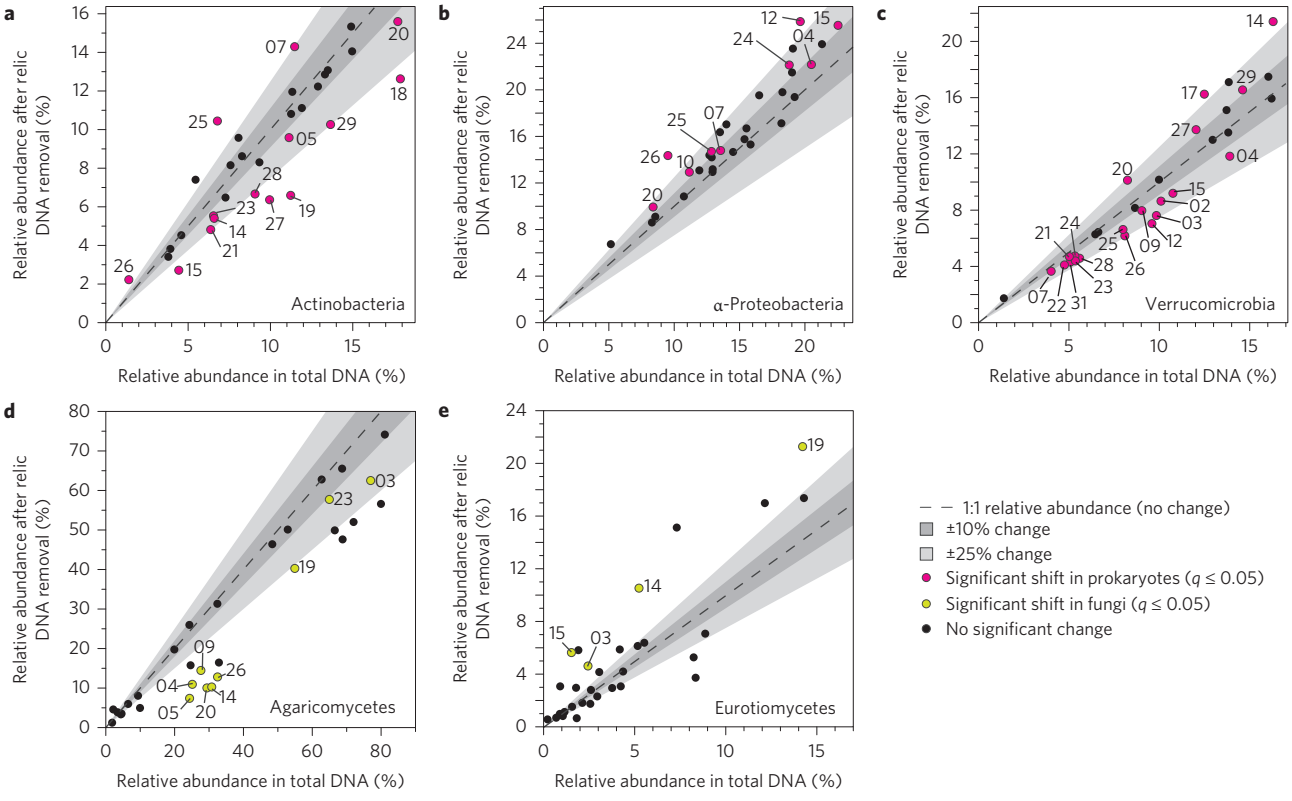

Figure 3 | The relative abundances of several microbial lineages change significantly in individual soils after removal of relic DNA. a–e, Points are the mean relative abundances of Actinobacteria (**a**), α-Proteobacteria (**b**), Verrucomicrobia (**c**), Agaricomycetes (**d**) and Eurotiomycetes (**e**) before ($n = 5$) and after ($n = 5$) relic DNA removal, as calculated from rarefied amplicon sequence data. The relative abundances of coloured points are significantly different after relic DNA removal (two-tailed Mann–Whitney U test $q \leq 0.05$). Numbers correspond to soil sample (Supplementary Table 1). These plots display all significant changes in taxa comprising ≥5% of the total prokaryotic and ≥3% of the fungal communities across all soils. In all plots, dashed lines represent no change in relative abundances (1:1 line). The dark grey shaded region represents ±10% change in relative abundance, and the light grey shaded region represents ±25% change in relative abundance. Supplementary Dataset 1 shows the mean relative abundances ± s.e. of each taxon in each soil.

presence of relic DNA is unlikely to obscure broad biogeographical patterns (for example, those reported in refs 24–26), relic DNA may obscure subtle spatiotemporal patterns or treatment effects in experimental manipulations of soil conditions. For example, shifts in soil microbial communities across seasons, or with plant species growing on a site, are often difficult to detect from DNA-based analyses[27,28]. Similarly, long-term soil transplant experiments investigating the effects of climate change on soil microbial communities have shown minimal change in microbial community composition[29]. We expect that the ability to detect meaningful shifts in soil microbial communities should increase if the 'noise' generated from non-living microbes is reduced by first removing relic DNA. Finally, the extreme diversity of soil microbial communities presents multiple computational problems for metagenomic assembly and analysis[30]. Because a significant portion of this diversity is coming from relic DNA pools, removal of relic DNA from samples before shotgun metagenomic analyses should result in more effective metagenomic assemblies and improve our ability to infer the genomic attributes of undescribed soil microbes[31].

Relic DNA dynamics may have important ramifications for understanding microbial communities in other ecosystems besides soil. For example, deep-sea sediments harbour large amounts of extracellular DNA[32], suggesting that removal of relic DNA may affect diversity estimates in the deep biosphere. Specific analyses of the relic DNA pool in deep biosphere samples may have particular utility as a 'fossil record' to distinguish extinct microbial taxa from living organisms and more accurately reconstruct the subsurface paleome[33,34]. More generally, relic DNA probably influences studies where DNA from dead organisms is abundant or where DNA is particularly resistant to degradation, including studies of the microbes found on aquatic particles, on mineral surfaces, or in the built environment.

Table 1 | *P* values for logistic regression models fitting edaphic characteristics to the presence (≥20% of total DNA for prokaryotes, ≥27% for fungi) or absence of relic DNA.

Soil characteristic	Prokaryotic	Fungal
MWD_w	0.062	NS
pH	0.048	NS
Electrical conductivity (mmhos cm^{-1})	0.042	NS
NO_3^--N (ppm)	0.014	0.025
K (ppm)	0.011	0.061
Exchangeable Ca^{2+} (meq per 100 g)	0.01	0.043
Exchangeable Mg^{2+} (meq per 100 g)	0.012	NS
Exchangeable Na^+ (meq per 100 g)	0.097	0.044
Exchangeable K^+ (meq per 100 g)	0.008	0.022
Total exchangeable bases (meq per 100 g)	0.008	0.036
Cation exchange capacity (meq per 100 g)	0.046	NS

All relationships are inverse, except mean weight diameter (MWD_w is a proxy for the amount of water-stable aggregates). NS, not significant at $P \leq 0.1$.

Methods

Soil collection and characteristics. A total of 31 surface soils (0–5 cm, mineral soils only) were collected from locations in Colorado, New Hampshire, Virginia and Kansas, USA, in August or September 2015 (Supplementary Table 1). Samples from outside Colorado (that is, non-local) were shipped, cool, overnight. Importantly, as a

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precaution against potential freezing-induced damage to cell membranes, soils were deliberately not frozen before PMA treatment. On arrival in the laboratory, soils were sieved to 2.0 mm, homogenized and stored at 4 °C until PMA treatment. The dates of collection and PMA treatment are detailed in Supplementary Table 1. Soils were chosen to capture a broad range of ecosystem types and edaphic factors (Supplementary Table 1). Standard soil characteristics were measured at the Colorado State University Soil Water and Plant Testing Laboratory using their standard protocols (Supplementary Table 1). These analyses included pH, electrical conductivity (mmhos cm^{-1}), percent organic matter, NO_3^--N (ppm), P (ppm), K (ppm), Zn (ppm), Fe (ppm), Mn (ppm), Cu (ppm), texture (percent sand, silt and clay), exchangeable bases (meq per 100 g) and cation exchange capacity (meq per 100 g). Percent moisture was determined gravimetrically on sieved soils before and after drying at 80 °C for 72 h. Mean weight diameter (MWD_W, a proxy for the amount of water-stable aggregates) was determined using the wet sieving method[35].

PMA treatment and DNA extraction. Ten replicate sub-samples of each soil (0.03 g) were homogenized and resuspended in 3.0 ml sterile PBS (pH 7.4) in transparent plastic tubes. PMA was added to five of the sub-samples (the PMA-treated) at a final concentration of 40 μM in a dark room. The concentration of 40 μM PMA was used because (1) it is in the range of concentrations used in other studies (for example, refs 4,16); (2) our own experiments showed 40 μM was sufficient to remove spikes of naked extracellular DNA in six soils (Supplementary Fig. 1); and (3) our own laboratory experiments also showed insignificant penetration into exponentially growing *E. coli* or *S. cerevesiae* (Supplementary Fig. 2). The five PMA-treated and the five untreated sub-samples were gently vortexed for 4 min in the dark at room temperature. After the incubation, both PMA-treated and untreated samples were exposed to a 650 W halogen lamp placed 20 cm from the sample tubes for four consecutive 30 s/30 s light/dark cycles, while continually vortexing. After light exposure, samples were frozen at −20 °C until DNA extraction. DNA was extracted from 960 μl of homogenized soil/PBS slurry using a MoBio PowerSoil DNA extraction kit, following the manufacturer's instructions, transferring 500 μl of the bead-beaten supernatant for DNA extraction. To enable comparisons of relic DNA amounts across samples, we treated each soil type identically.

Rationale for using PMA and method limitations. Several techniques have been developed to investigate microbial viability in different environments (reviewed in ref. 17). The advantages of the PMA-based approach we apply here include the following: (1) it is relatively easy to implement, meaning it can be used for high-throughput analyses; (2) the utility of PMA for the purposes of distinguishing extracellular DNA from DNA contained within cells is well documented and typically conservative, relative to ethidium monoazide based studies, because not all dead cells are permeable to PMA[4,16,18,36–38]; and (3) key assumptions in other approaches remain untested. Although rRNA-based approaches are thought to discriminate between metabolically active and inactive cells, this is not necessarily true[39]. Furthermore, the assumption that extracellular rRNA cannot persist in soil has not been explicitly tested. It is possible that, as with DNA, there could be substantial amounts of rRNA in soil coming from extracellular pools or cells that are no longer living, but additional studies are required to determine if this is the case.

In some laboratory studies of pure bacterial cultures grown on nutrient-rich medium, a fraction of the presumably viable population was found to be susceptible to PMA[36–38]. Thus, one potential limitation of this technique is that the effectiveness of PMA exclusion by intact cells has not been tested on the broad diversity of microbes found in soil. However, consistent with previous work (reviewed in refs 17,18), we observed that few taxa were consistently under- or over-estimated across soil samples after relic DNA was removed, suggesting that there are minimal systematic taxon-specific differences in the susceptibility of intact cells to PMA treatment (Supplementary Fig. 8). A second potential limitation is that in turbid sample preparations, such as soils, the effectiveness of PMA binding of relic DNA may be reduced because of PMA adsorption to soil particles or ineffective photoactivation of PMA (ref. 18 and references therein). In our experiments, PMA retained the capacity to completely remove naked DNA spikes in all but one soil sample (Supplementary Fig. 1). This suggests that, in this study, the photoactivation of PMA was sufficient and PMA adsorption to soil particles was negligible.

qPCR. qPCR reactions were conducted in 25 μl total volumes on a Eppendorf realplex^2 Mastercycler ep gradient S. The reaction mixture was as follows: 1.25 μl each of F and R primer (sequences follow), 12.5 μl 2X qPCR mix (Thermo Scientific Absolute qPCR SYBR Green Mix), 5 μl PCR-grade water and 5 μl template DNA. The following qPCR primers were used: prokaryotic 16S rRNA—515F 5′-GTGCCAGCMGCCGCGGTAA-3′ and 806R 5′-GGACTACHVGGGTWTCTAAT-3′; fungal ITS—5′-CTTGGTCATTTAGAGGAAGTAA-3′ and ITS2 5′-GCTGCGTTCTTCATCGATGC-3′. Cycling conditions: 95 °C for 15 min, followed by 40 cycles of (94 °C 45 s; 50 °C 60 s; 72 °C 90 s) and a final extension of 72 °C for 10 min. All reactions were run in triplicate and compared to standard curves containing purified *E. coli* DH10B genomic DNA for 16S rRNA gene quantification or purified *Aspergillus fumigatus* (ATCC MYA-4609D-2) genomic DNA for ITS amplicon quantification. 16S rRNA gene and ITS amplicon copies per g of soil were calculated for each sub-sample (Supplementary Dataset 1) using seven 16S rRNA gene copies per genome for *E. coli* DH10B[40] and 38 ITS region copies per genome for *A. fumigatus*[41]. Each qPCR reaction was calculated to contain DNA extracted from ~0.16 mg of soil. A qPCR 'no template' negative control was included with each qPCR run, none of which amplified successfully.

Amplicon sequencing and analytical methods. For sequence-based analyses of 16S rRNA and ITS gene regions, we used approaches described previously[42]. Briefly, we amplified each of the 310 DNA samples in duplicate in 25 μl PCR reactions containing 12.5 μl of Promega GoTaq Hot Start Colorless Master Mix; 0.5 μl of each barcoded primer (bacterial 16S: 515F 5′-GTGCCAGCMGCCGCGGTAA-3′ and 806R 5′-GGACTACHVGGGTWTCTAAT-3′; fungal ITS: 5′-CTTGGTCATTTAGAGGAAGTAA-3′ and ITS2 5′-GCTGCGTTCTTCATCGATGC-3′); 10.5 μl water; 1 μl of template DNA. Program: 94 °C for 5 min, followed by 35 cycles of (94 °C 45 s; 50 °C 60 s; 72 °C 90 s) and a final extension at 72 °C 10 min. Several negative controls, including 'no template' controls and 'DNA extraction kit' controls, were included alongside the soil DNA samples and sequenced. Duplicate reactions were pooled, cleaned and normalized using the ThermoFisher Scientific SequalPrep Normalization Plate kit. Cleaned and normalized amplicons were pooled, spiked with 15% phiX and sequenced on an Illumina MiSeq using v2 500-cycle paired end kits. Reads were processed as described previously[42]. Briefly, raw amplicon sequences were demultiplexed with the associated barcodes and processed with the UPARSE pipeline[43]. A database of ≥97% similar sequence clusters was constructed in USEARCH (Version 8)[44] after merging paired end reads, using a 'maxee' value of 0.5 when quality filtering sequences, dereplicating identical sequences, removing singleton sequences, clustering sequences after singleton removal and filtering out representative sequences that were not ≥75% similar to any sequence in Greengenes (for prokaryotes; Version 13_8)[45] or UNITE (for fungi)[46] databases. Demultiplexed sequences were mapped against the *de novo* constructed databases to generate counts of sequences matching clusters (that is, taxa) for each sample. Taxonomy was assigned to each taxon using the RDP classifier with a threshold of 0.5 (ref. 47) and trained on the Greengenes or UNITE databases. To normalize the sequencing depth across samples, samples were rarefied to 6,000 and 1,100 sequences per sample for the 16S rRNA and ITS analyses, respectively.

Statistical analyses. The percent relic DNA was calculated by comparing the mean qPCR abundances ($n = 5$ sub-samples) of 16S rRNA genes or ITS amplicons from untreated soil to the abundances of the amplicons from each individual PMA-treated sub-sample ($n = 5$) by subtracting the abundance of the targeted gene in each PMA-treated sub-sample from the mean abundance in untreated samples and then dividing this difference by the mean abundance in untreated samples. The percent decrease in microbial richness after relic DNA removal was calculated from rarefied amplicon data by comparing the mean prokaryotic or fungal richness determined from sub-samples ($n = 5$) of untreated soil to the prokaryotic or fungal richness determined from each individual PMA-treated sub-sample ($n = 5$) by subtracting the richness (prokaryotic or fungal) in each PMA-treated sub-sample from the mean richness (prokaryotic or fungal) in untreated samples divided by the mean richness (prokaryotic or fungal) in untreated samples. Because the replicates are sub-samples of a given soil and thus unlikely to harbour identical communities, we focused on overall differences in richness and did not specifically identify which taxa were exclusively found in the relic DNA pool. Microbial richness and Bray–Curtis dissimilarities were calculated in the mctoolsr R package[48]. Bray–Curtis dissimilarities were calculated on square root transformed taxon relative abundances. Where appropriate, statistical tests were corrected for multiple comparisons using the qvalue R package[49] using a significance threshold of q value ≤ 0.05. Spearman correlation coefficients and associated P values were calculated with the Hmisc R package[50]. Logistic regression models were used to identify which edaphic characteristics predicted the presence of relic DNA. Our rationale for using logistic regression for this analysis is that, although we obtained negative median relic DNA percentages (Fig. 1), there cannot be less than 0% relic DNA. It is likely that our estimates of negative relic DNA amounts are the result of small-scale heterogeneity in the proportional abundance of relic DNA across sub-samples of a given soil. This heterogeneity probably obscures our ability to accurately quantify the precise amount of relic DNA when the amounts are near zero. For these logistic analyses, we defined relic DNA as being present in soils with >20% prokaryotic relic DNA and >27% fungal relic DNA. These cutoffs were chosen because they are ± the most negative median percent relic DNA value across all soils.

Data availability. Raw sequence data are available in the NCBI Sequence Read Archive (project accession no. SRP070563). The full data set for Fig. 1, mean percent of each taxa per soil, per treatment and amplicon copies per replicate per gram of soil are provided in Supplementary Dataset 1.

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Author contributions

P.C. and N.F. conceived the project and wrote the manuscript. P.C., P.J.M. and E.E.M. performed experiments. P.C., P.J.M., N.F. and M.S.S. collected samples. P.C., J.W.L. and M.S.S. analysed data.

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Correspondence and requests for materials should be addressed to P.C. and N.F.

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Competing interests

The authors declare no competing financial interests.

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