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The overall and sequence-specific degradation of soil extracellular 16S rRNA genes across China: rates and influential factors

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eLife Assessment

This **valuable** study introduces an innovative experimental design to address a crucial and timely issue in microbial ecology: the potential bias in soil microbial community analyses caused by extracellular DNA degradation. While the evidence showing variable degradation rates of extracellular DNA is **convincing**, additional conceptual, methodological, and statistical clarifications could reinforce the claims and the study's contribution to the field. This research will appeal to microbial ecologists and researchers interested in using molecular techniques to evaluate microbial community structure.

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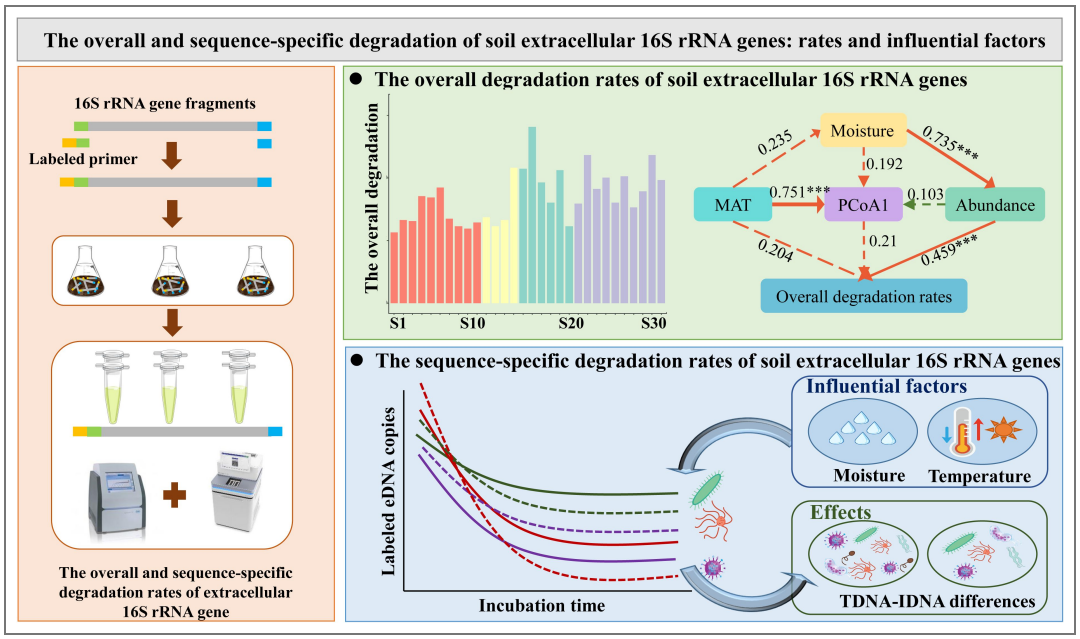
Abstract

While extracellular DNA persistence substantially influences soil microbiome investigations, its degradation kinetics remain poorly quantified. Here, we developed a primer-labeled DNA approach coupled with microcosm incubation to determine the overall and sequence-specific degradation rates of extracellular 16S rRNA genes across China. We observed substantial variations in the overall degradation rates of extracellular 16S rRNA genes among the study sites, with degradation rate constants ranging from 0.05 to 0.16 day^{-1}. The overall degradation rate constants showed significant correlations with soil moisture content, prokaryotic abundance, prokaryotic community profiles, and mean annual precipitation (MAP). The significant influences of moisture contents on the overall degradation rates were further verified by a moisture gradient microcosm experiment. The sequence-specific degradation rate constants were additionally correlated with pH, nitrogen content, and mean annual temperature (MAT). Furthermore, removing extracellular DNA significantly altered soil prokaryotic abundance, richness, and prokaryotic community profiles, and the sizes of sequence-specific extracellular 16S rRNA gene pools significant correlated with their respective degradation rates. This study developed a methodology for determining the overall and sequence-specific degradation rates of extracellular 16S rRNA genes, highlighting the profound influences of extracellular DNA on soil microbial research and informing the optimization of environmental DNA technologies.

Graphical abstract



1. Introduction

The investigation of soil microbial abundance and diversity heavily relies on DNA-based technologies, such as real-time PCR, metagenomic analysis, and amplicon high-throughput sequencing (Che et al., 2018; Du et al., 2020; Yang et al., 2023). Soil DNA originates from both living and relic microbial cells, with the DNA from deceased microbial cells commonly referred to as relic or extracellular DNA (eDNA) (Ye et al., 2022). eDNA serves as a critical vector for horizontal gene transfer (HGT), facilitating the cross-species spread of antibiotic resistance genes (Liu et al., 2024), and they also participates in soil biogeochemical cycling by releasing nutrients (*e.g.*, phosphorus and nitrogen) (Ye et al., 2022). Moreover, the prevailing paradigm of total DNA extraction in soil microbiome studies introduces eDNA as a critical noisy factor. Generally, its environmental persistence may lead to overestimation of microbial diversity in amplicon sequencing (Barnes et al., 2014; Wang et al., 2025; Xue et al., 2025) and distort qPCR-based quantification of marker genes (Carini et al., 2016; Sun and Ge, 2023; Wang et al., 2024a). A theoretical model simulation study showed that the extent of this influence is primarily determined by the abundance and sequence-specific degradation rates of extracellular DNA (Lennon et al., 2018). Therefore, determining the overall and sequence-specific degradation rates of soil extracellular DNA can provide crucial insights into evaluating the effects of extracellular DNA on soil microbial analysis and the reliability of the research based on environmental DNA technologies.

The overall degradation rates of soil extracellular DNA have received significant attention due to their crucial roles in nutrient cycling, signal transduction, and horizontal gene transfer (Nagler et al., 2018). In the late twentieth century, the degradation of extracellular DNA was mainly examined using PCR, DNA hybridization, radioisotope labeling, and competent cell transformation techniques (Paget et al., 1992; Zhang et al., 2020; Samuels et al., 2025). These investigations consistently demonstrated that extracellular DNA can persist in soils for months to years (Barnes et al., 2014; Pathan et al., 2020), maintaining its capacity to transform competent cells (Levy-Booth et al., 2007; Pietramellara et al., 2009). Most of these studies primarily concentrated on assessing the risks associated with transgenic technologies, narrowly examining the degradation dynamics of DNA sequences related to such technologies. Consequently, the insights they provided were usually qualitative or semiquantitative (Morrissey et al., 2015), limiting the comprehensive understanding of extracellular DNA degradation dynamics. In recent years, there has been a notable shift towards quantification, and the dynamics of soil extracellular DNA were quantified in several studies (Eichmiller et al., 2016; Wei et al., 2018). For instance, the degradation rates of soil extracellular DNA were quantified using real-time PCR with specific plasmid labels such as T7 and SP6 promoters (Ceccherini et al., 2009). Another innovative study simultaneously determined the dynamics of both soil microbial community and extracellular DNA by adding 16S rRNA gene primer labels to exogenous DNA (Sirois and Buckley, 2019). Moreover, stable isotopic probing was also utilized for determining the degradation rates of soil extracellular DNA (Morrissey et al., 2015). These studies consistently demonstrate that extracellular DNA can persist in soils for extended period. However, their scope has been restricted to single or highly specific DNA targets (*e.g.*, transgenic sequences), thereby overlooking critical variations in sequence-specific degradation of soil extracellular DNA (Pietramellara et al., 2009; Sirois and Buckley, 2019; Wang et al., 2019).

The potential variations in sequence-specific extracellular DNA degradation rates can be attributed to several factors. First, sequence-dependent enzymatic processes—such as restriction endonucleases targeting specific motifs—may preferentially degrade DNA fragments with compatible recognition sites. This can lead to differential degradation opportunities across microbial taxa, depending on their genomic signatures. Notably, terminal restriction fragment length polymorphism (T-RFLP) was developed based on this this property, and it used to be the most widely used methods for microbial diversity analysis (Schütte et al., 2008; Andronov et al., 2012). Second, the persistence of soil DNA can be mainly attributed to its adsorption and protection by minerals and humus in soils (Cai et al., 2006; Vuillemin et al., 2017; McKinney

and Dungan, 2020). Thus, variances in the absorbability of soil minerals and humus to different DNA bases may also contribute to the different degradation rates of various DNA sequences. Lastly, DNA sequences can also affect the high-level structure of DNA macromolecules, indirectly impacting their degradation rates. Consequently, multiple studies have proposed that the effects of DNA sequences on extracellular DNA degradation rates should be carefully considered (Paget et al., 1992; Pietramellara et al., 2009; Wood et al., 2020). However, despite the considerations mentioned above, a systematic investigation into the degradation rates of different DNA sequences has not been conducted.

Currently, 16S rRNA genes have been utilized to determine soil prokaryotic abundance and diversity in numerous studies, establishing their position as the most extensively used molecular marker (Knight et al., 2018; Du et al., 2025). Therefore, this study mainly aimed to investigate the overall and sequence-specific degradation rates of soil extracellular 16S rRNA genes and their influencing factors. Additionally, we explored the impacts of extracellular 16S rRNA genes on soil prokaryotic community analysis and their links with the overall and sequence-specific degradation rates. Soil samples were collected from 30 representative ecosystems across China. A new methodology, involving the addition of exogenous 16S rRNA gene labeled with specific primers, microcosm incubation, real-time PCR, and amplicon sequencing, was developed to determine the overall and sequence-specific degradation rates of extracellular soil 16S rRNA genes. Based on our current understanding, we proposed two hypotheses. First, the degradation rates of extracellular 16S rRNA genes were expected to be highly sequence-specific. Second, the sequence-specific degradation of extracellular 16S rRNA genes would have significant impacts on the analysis of prokaryotic abundance and diversity.

2. Results

2.1. The overall degradation rate constants of soil extracellular 16S rRNA genes

The GAPDH F-labeled 16S rRNA genes were consistently detectable throughout the 48-day incubation period, but their abundance rapaidly declined as the incubation progressed (Fig. 1a, $P < 0.05$). After a 48-day incubation period, only a small fraction (0.2–3.1%) of the GAPDH F-labeled 16S rRNA genes persisted in the soils (Fig. 1a). The degradation rate constances of soil exogenous extracellular 16S rRNA genes displayed considerable variability among the study sites, ranging from 0.05 to 0.16 day^{-1} (Fig. 1b). Random forest modeling revealed that soil moisture content, prokaryotic abundance, and prokaryotic community profiles were critical predictors for the overall degradation rate constants of extracellular 16S rRNA genes, and they all showed significant positive correlations (Figs. 1c and S1a–d). However, we did not observed significantly correlations between soil texture and the degradation rate constants (Fig S1e and f). The SEM analysis indicated that the overall degradation rate constants of 16S rRNA genes were mainly directly affected by prokaryotic abundance which was indirectly influenced by soil moisture (Fig. 1d). In the moisture gradient micrcosm experiemnt, we also observed strong positive correlation between soil moisture contents and the overall degradation rates of extracellular 16S rRNA gene (Fig S2).

2.2. The sequence-specific degradation rate constants of the extracellular 16S rRNA genes

The richness of the GAPDH F-labeled 16S rRNA genes significantly decreased during the incubation period, declining to approximately 60% of the initial values after 48 days (Fig. 2a). The prokaryotic community profiles based on the GAPDH F-labeled 16S rRNA genes also exhibited significant variations across different incubation time points (Figs. 2b and S3), with a notably higher decline in community profile similarities as the intervals of incubation time increased (Fig. S4). These findings suggest that the degradation rates of soil extracellular 16S rRNA genes are sequence-specific. Indeed, the degradation rate constants for different extracellular 16S rRNA

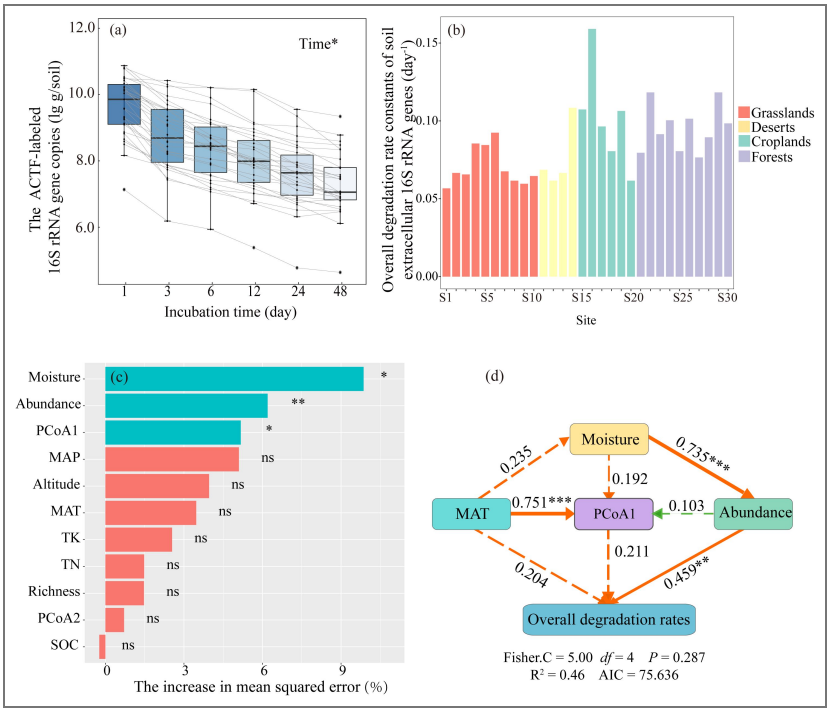


Fig. 1. The overall degradation rates of soil extracellular 16S rRNA genes and their influential factors.

(a) The GAPDH F-labeled 16S rRNA gene copies at different incubation time points; (b) the degradation rates of soil extracellular 16S rRNA genes across the study sites; (c) the factors influencing extracellular 16S rRNA gene degradation rates; and (d) the influencing factors for the overall degradation rates of soil extracellular 16S rRNA genes revealed by structural equation modeling. Orange and green lines indicate positive and negative relationships, respectively. Solid and dashed lines indicate significant and non-significant relationships, respectively. Path coefficients are denoted by numbers adjacent to the arrows, with arrow width reflecting their strength. Significance levels are indicated as follows: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Moisture: soil moisture content; Abundance: prokaryotic abundance; NMDS1: the scores at the first axis of the NMDS ordination of prokaryotic community profiles; MAP: mean annual precipitation; Richness: soil prokaryotic richness; NMDS2: the scores at the second axis of the NMDS ordination of prokaryotic community profile; TK: soil total potassium contents; MAT: mean annual temperature; AP: soil available phosphorus content; TN: soil total nitrogen contents; and TOC: soil total organic carbon content.

genes were within 0.40 day^{-1}, with most were between 0.06 and 0.12 day^{-1} (Fig. 2c). For example, the degradation rate constants of many sequences idenfified as Proteobacteria were significantly higher than those beling to Acidobacteriota, and the degradation rate cosntants of Methylomirabilota were also relatively higher than those of Acidobacteriota but lower than those belongling to Proteobacteria (Fig. 3a). The sequence-specific degradation rate constants of the extracellular 16S rRNA genes also showed significant correlations with multiple environmental factors including soil moisture, mean annual temperature (MAT), mean annual precipitation (MAP), soil pH, as well as the contents of soil ammonium nitrogen ($NH4^{+}$-N) and nitrate nitrogen (NO_3^{-}-N) (Fig. 3b), but no significant relationships were found with the GC content of the represititve sequences (Fig. S5). Further analysis suggested that the degradation rate constants of extracellular 16S rRNA genes of the dominant phyla showed similar correlations with the environmental factors, and MAT was identified as the strongest predictors for them (Fig. S6).

2.3. The influences of extracellular 16S rRNA genes on soil prokaryotic community analysis and their links with the degradation rates of extracellular 16S rRNA genes

Based on the PMA treatment, we observed significant impacts of extracellular 16S rRNA genes on the analysis of soil prokaryotic abundance and diversity (Figs. 4 and 5). Specifically, intracellular prokaryotic abundance accounted for 40.3% of total prokaryotes, while richness accounted for 86.5% (Fig. 4a and b). Furthermore, significant differences were found between the profiles of total and living prokaryotic communities, especially in the forest and cropland ecosystems (Figs. 4c, 4d and S7). For instance, Abditibacteriota, Bacteroidota, Nitrospirota, Fibrobacterota, Entotheonellaeota, Elusimicrobiota, and Armatimonadota were significantly enriched in the total prokaryotic community, whereas Actinobacteriota and Planctomycetota showed opposite trends (Fig. 5a). However, many other microbial taxa, such as Proteobacteria, Acidobacteriota, and Chloroflexi showed no significant differences between the total and living prokaryotic communities (Fig. 5a). Additionally, the correlations with environmental factors were stronger for the structure of the total soil prokaryotic community structure than those for the living one (Fig. 5b). Total prokaryotic community structure was significantly correlated with soil pH, TK, NH^{+}-N, MAT, and MAP. However, the living prokaryotic community structure was only significantly correlated with soil pH, MAT, and MAP (Fig. 5b).

Interestingly, a significant correlation was observed between the total prokaryotic community structure and the degradation rates of extracellular 16S rRNA genes. However, the relationships were not observed for the living prokaryotic communities (Fig. 5b). The relationships between extracellular 16S rRNA gene pool sizes and degradation rates were further explored. We found a significant positive correlation between the overall degradation rates and the copies of soil extracellular 16S rRNA genes (Fig. S1c). Moreover, most study sites exhibited significant positive correlations between sequence-specific degradation rates and the contents of soil extracellular 16S rRNA genes (Fig. S1g). Additionally, there were significant correlations between the differences in relative abundance of taxa in the total and living prokaryotic community and the sequence-specific degradation rates; however, these relationships varied across the study sites (Fig. S1g).

3. Discussion

In this study, we found that extracellular 16S rRNA genes persisted in soils for at least several weeks and the range of the degradation constants is 0.05 to 0.16 day^{-1}. The degradation constants of soil extracellular DNA based on plasmid and stable isotope labelling micorbial genomes were usually 0.03-0.14 day^{-1} (Morrissey et al., 2015; Sirois and Buckley, 2019; Wang et al., 2019), which are consistent with our results. Therefore, the PCR-generated 16S rRNA fragments provide a standardized substrate for quantifying the degradation kinetics of added DNA, which can indicate some aspects of extracellular DNA turnover. Addtonally, substantial variations in the overall degradation rates of added exogenous extracellular 16S rRNA genes were also observed in this study, which can be mainly elucidated in the following ways (Fig. 1a). First, the degradation of

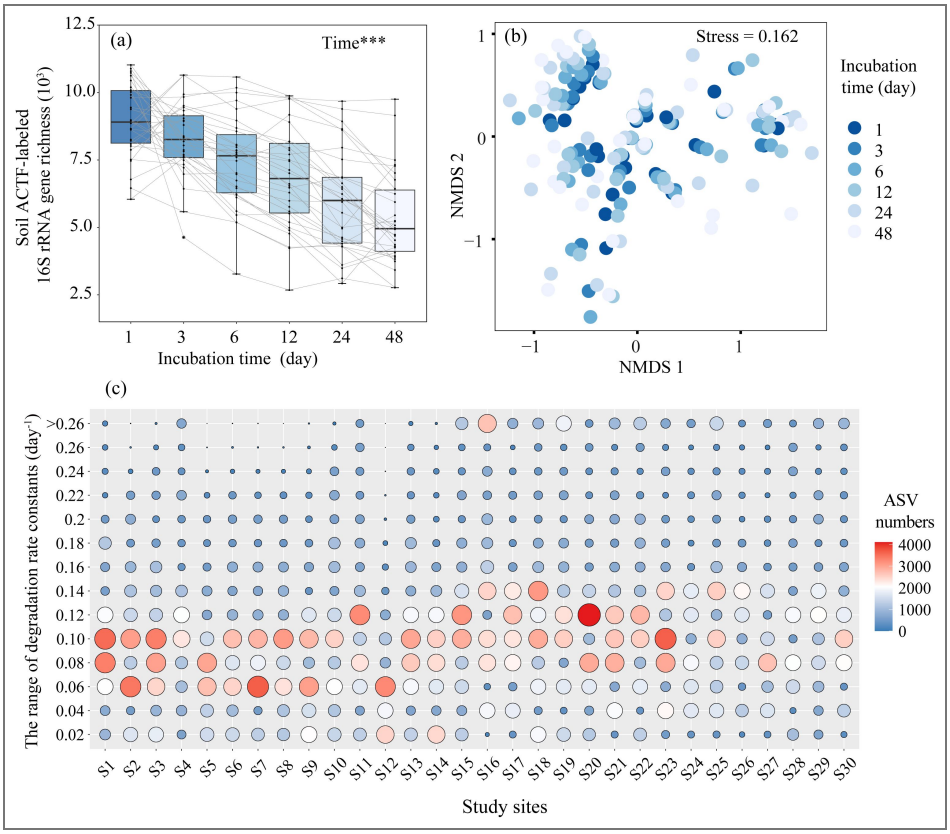


Fig. 2. The sequence-specific degradation rates of soil exogenous extracellular 16S rRNA genes.

(a) Soil GAPDH F-labeled 16S rRNA gene richness; (b) the nonmetric multidimensional scaling (NMDS) ordination of the community profiles based on GAPDH F-labeled 16S rRNA genes at different incubation time points; (c) the number of GAPDH F-labeled 16S rRNA gene ASVs within different degradation rates range

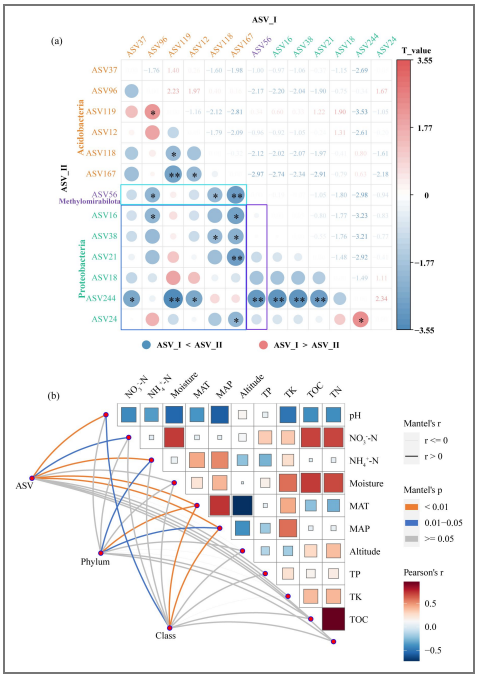


Fig. 3. The diffrence in sequence-specific degradation rates of soil exogenous extracellular 16S rRNA genes and their influencing factors.

(a) the paired comparison of degradation rates among different AVSs. Blue indicates that the degradation rate of the ASV I group is lower than that of the ASV II group, and the T value is negative; red indicates that the degradation rate of the ASV I group is higher than that of the ASV II group, and the T value is positive. Significance levels are indicated as follows: * $P <$ 0.05, ** $P < 0.01$, and *** $P < 0.001$. and (b) the relationships between the sequence-specific degradation rate profiles and environmental factors. NO $^{-}$-N: soil NO $^{-}$-N contents; NH $^{+}$-N: soil NH $^{+}$-N contents; TN: soil total N contents; TP: soil total P contents; TK: soil total K contents; AP: soil available P contents; TOC: soil total organic carbon content; MAT: mean annual temperature; and MAP: mean annual precipitation.

Fig. 4. The disparities in the abundance, richness, and community composition between total and living soil prokaryotes.

(a) The abundance of total and living soil prokaryotes. (b) The richness of total and living soil prokaryotes. (c) The differences between the relative abundance of total and intracellular ASVs. The red points represent the prokaryotic taxa exhibiting statistically significant differences. (d) The nonmetric multidimensional scaling (NMDS) ordination of total and living soil prokaryotes. The different colors represent samples from different sites.

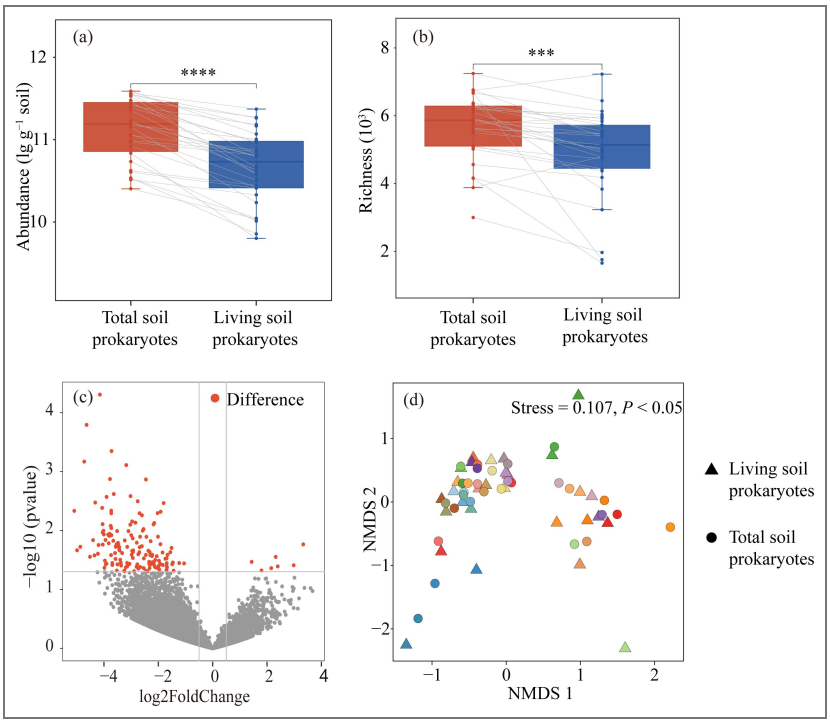
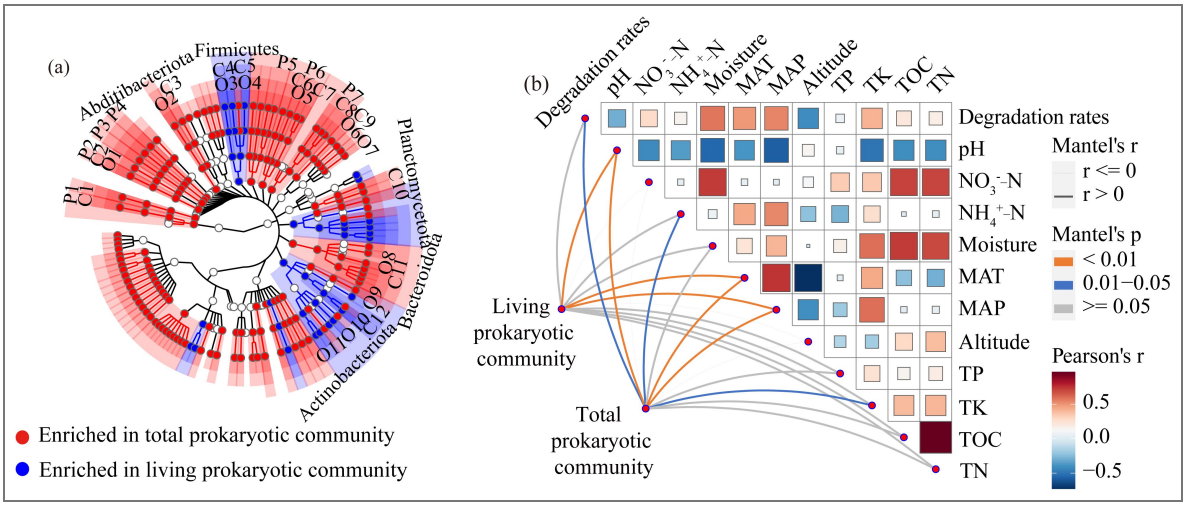


Fig. 5.

(a) The taxa with significant differences between total and living prokaryotic communities. P1: Thermoplasmatota; P2: Nitrospirota; P3:, Fibrobacterota; P4: Entotheonellaeota; P5: Elusimicrobiota; P6: Armatimonadota; P7: Myxococcota; C1: Thermoplasmata; C2: Nitrospiria; C3: Vampirivibrionia; C4: Clostridia; C5: Bacilli; C6: Armatimonadia; C7: Chthonomonadetes; C8: Polyangia; C9: Blastocatellia; C10: Parcubacteria; C11: Bacteroidia; C12: Thermoleophilia; O1: Nitrospirales; O2: Abditibacteriales; O3: Clostridiales; O4: Bacillales; O5: Armatimonadales; O6: Pyrinomonadales; O7: Bryobacterales; O8: Chitinophagales; O9: Rubrobacterales; O10: Propionibacteriales; and O11: Corynebacteriales; (b) the relationships between soil prokaryotic community profiles and environmental factors. NO $_3^-$-N: soil NO $_3^-$-N contents; NH $_4^+$-N: soil NH $_4^+$-N contents; Moisture: soil moisture content; MAT: mean annual temperature; MAP: mean annual precipitation; TP: soil total P contents; TK: soil total K contents; and TOC: soil total organic carbon contents.



soil extracellular DNA is highly determined by the availability of enzymes (Nihemaiti et al., 2020). As a large proportion of soil enzymes originate from microbes (Bhardwaj et al., 2024; Tan et al., 2025), differences in microbial abundance among study sites could make a substantial contribution to the variations in the overall degradation rates. Second, different soil microbial taxa play diverse roles in extracellular DNA degradation. In this study, there were considerable discrepancies in soil community profiles across study sites, providing another plausible explanation for the differing degradation rates of extracellular DNA. Third, moisture availability has long been recognized as the key factor governing enzymic reaction rates (He et al., 2024; Shah et al., 2024). Consequently, the disparities in soil moisture content among the study sites should also be critical factors contributing to varied overall extracellular DNA degradation rates. This can be further verified by the strong positive correlaitons between moisture contents and extracellular DNA degradation rates both in the sruvey and microcosm experiments (Figs. 1c ,d and S2). These findins underscore the vulnerability of dryland ecosystems to DNA persistence. Projected increases in aridity under climate change may prolong extracellular DNA persistence in dry soils, potentially exacerbating the overestimation of microbial abudance and diversity in arid regions (Carini et al., 2016; Lennon et al., 2018). This could mislead conservation efforts by inflating perceived biodiversity in ecosystems undergoing rapid environmental stress. For example, in the Loess Plateau of China—a region already experiencing severe soil erosion—relic DNA from historical microbial communities may obscure the true extent of biodiversity loss driven by land-use intensification, thereby delaying critical restoration measures (Wang et al., 2025).

Consistent with our first hypothesis, we observed that the degradation of soil extracellular 16S rRNA genes showed strong sequence-specific patterns (Fig. 2c). In particular, many sequences belonged to Proteobacteria exhibited a significant faster extracellular DNA (eDNA) degradation rate than those of Acidobacteria (Fig. 3a). The sequence-specific degradation of the extracellular 16S rRNA genes can be interpreted from multiple perspectives. First, the variation in the number and position of restriction enzyme cutting sites for different sequences could be a critical reason for the sequence-specific degradation rates of extracellular 16S rRNA genes (Brown, 2020). Second, sequence differences could also influence the spatial structure and mineral adsorption of 16S rRNA genes (Cleaves II et al., 2011; Buitrago et al., 2021), indirectly affecting the sequence-specific degradation rates. Third, microbes might preferentially degrade and recycle the abundant DNA sequences present in their habitats. Thus, the sequence-specific degradation rates could be also ascribed to the varied abundance of different extracellular 16S rRNA gene sequences. This assertion is further evidenced by the generally observed positive correlations between extracellular DNA abundance and degradation rates (Fig. S1). As observed in this study, the differential sequence-specific degradation rates also led to significant alterations in the community profiles based on the exogenous extracellular 16S rRNA genes (Fig. 2a and b). This can be one of the main reasons for the influences of extracellular DNA on the analysis of soil microbial community profiles (Lennon et al., 2018).

Accordingly, we further determined the influences of extracellular DNA on prokaryotic community analysis through PMA treatment, and the significant disparities were observed between the profiles of the total and living soil prokaryotic community profiles (Fig. 4c and d). As mentioned above, the disparities can be mainly ascribed to the differential sequence-specific degradation rates of extracellular 16S rRNA genes. Additionally, these differences are also related to several other factors. The primary reason should be the distinct source of extracellular and intracellular rDNA which are derived from dead and live microbes, respectively. The death of microorganisms is mainly caused by environmental selection or stochastic processes (Zhang et al., 2016). In terms of environmental selection, the dead microbial taxa should be less competitive than living ones (Upton et al., 2019; Chu et al., 2020). In contrast, the stochastic death should be correlated with the population size of each microbial species, but the varied stochastic mortality among different microbial taxa can still lead to different community profiles between dead and live microbes (Blazewicz et al., 2020). The significant correlation between total prokaryotic community structure and extracellular DNA degradation rates, in contrast to the lack of such relationships in living communities (Fig. 5b), highlights the divergent ecological roles of

extracellular DNA. The total community integrates both intracellular DNA from metabolically active cells and extracellular DNA derived from historical microbial residues (Lennon et al., 2018). This composite signal likely reflects legacy effects of past environmental conditions preserved in extracellular DNA pools (Wang et al., 2021), whereas the living community represents transient microbial activity under current selective pressures. Furthermore, this study revealed that extracellular 16S rRNA genes led to substantial overestimation of soil prokaryotic abundance and diversity (Figs. 4 and 5). These findings were supported by many recent studies (Carini et al., 2016; Sun and Ge, 2023; Du et al., 2025) and can be explained as follows. The overestimated prokaryotic abundance can be attributed to the long-term persistence of extracellular DNA (Sun and Ge, 2023). Similarly, the overestimated prokaryotic richness may arise from historically accumulated microbial taxonomic information stored in extracellular DNA pools (Deshpande and Fahrenfeld, 2023; Wang et al., 2024b).

Notably, we also observed significant positive correlations between sequence-specific degradation rates and the pool sizes of extracellular 16S rRNA genes at most of the study sites (Fig. S1g). This finding suggests that abundant extracellular DNA degrades at a faster rate compared to rare extracellular DNA. As mentioned earlier, this can be explained by the preference of soil microbes for degrading abundant DNA. However, inconsistent with our second hypothesis, the relationships between the sequence-specific degradation rates and the effect sizes of extracellular 16S rRNA genes varied across the study sites (Fig. S1g). The sequence-specific effect sizes of extracellular 16S rRNA genes are mainly determined by both their production and degradation rates (Levy-Booth et al., 2007; Pietramellara et al., 2009). These inconsistent correlations emphasize the critical role played by the production rates of extracellular 16S rRNA genes in influencing the analysis of prokaryotic communities. Therefore, future studies should systematically determine both the production and degradation rates of extracellular DNA. Additionally, this study essentially determined the effects of DNA sequences on the extracellular 16S rRNA gene degradation rates. However, actual sequence-specific degradation rates of extracellular 16S rRNA genes can be additionally influenced by the varying degrees of protection offered by cell residuals from different microbial taxa (Shi et al., 2024). Thus, the differences in the actual sequence-specific degradation rates of extracellular 16S rRNA genes should be even more significant than those observed in this study.

4. Materials and methods

4.1. Study sites and soil sampling

Soil samples were collected from 30 sites across China (Fig. S8). The selection of sampling sites was mainly based on National Soil Fertility and Fertilizer Effect Long-term Monitoring Network and the Chinese Ecosystem Research Network (CERN). Several other sampling sites were included, based on the systematical consideration of ecosystem typicality, soil types, and climates. The 30 sites spanned major climatic zones and land-use types in China, including 10 grasslands, 10 forests, 6 croplands, and 4 deserts, ensuring broad applicability of results to diverse environmental scenarios. The longitude of the sites ranged from 80.724 °E to 124.817 °E, while the latitude ranged from 21.917 °N to 50.168 °N. The altitude of the study sites varied between 13 m to 4397 m. The ranges of mean annual temperature (MAT) and precipitation (MAP) were -3.35–22.53℃ and 39–1809 mm, respectively. The geographic and climate information of study sites is detailed in Table S1. The heterogeneity of the study sites largely ensured the generalizability and reliability of this research.

At each sampling site, 10 subsampling points were randomly selected, with a minimum distance of 10 m between adjacent subsampling points. Soil samples (0–20 cm) from 10 subsampling points at each study site were collected, thoroughly homogenized, and sieved to ≤ 2 mm to form a composite sample. Subsequently, all the composite soil samples were divided into two sub-samples. The first sub-sample was air-dried for the determination of soil pH values, as well as the contents of total

organic carbon (TOC), total nitrogen (TN), total phosphorus (TP), and available phosphorus (AP). The second sub-sample was preserved at 4°C for the determination of moisture content, inorganic nitrogen content, as well as microcosm experiment and propidium monoazide (PMA) treatment.

4.2. Analysis of soil physicochemical properties

Soil moisture content was determined by drying the soils at 105 °C for 48 hours (Che et al., 2019). Soil pH values were measured using a pH meter (Mettler Toledo, Switzerland) with a soil-to-water ratio of 1:2.5 (Zhang et al., 2023a). TOC content of the soils was examined via a TOC analyzer (GBT 30, 740–2014) (Li et al., 2023). The TN content of the soils was determined using an automatic Kjeldahl apparatus (Liu et al., 2023). Additionally, the determination of soil TP concentration was performed using the Mo-Sb colorimetric method. The soil NH^+-N and NO^--N contents were measured by indophenol blue colorimetry and vanadium chloride spectrophotometry with a potassium chloride (KCl) extraction method, respectively (Zhang et al., 2019; Zhang et al., 2023b). The soil AP content was measured following the methods of Olsen (1954). Detailed physical and chemical properties of the soils are listed in Table S2.

4.3. Determination of 16S rRNA gene overall and sequence-specific degradation rate constants

The overall and sequence-specific degradation rate constants of added exogenous extracellular 16S rRNA genes were determined using a primer labeling method, combined with real-time PCR and amplicon sequencing (Fig. 6). Briefly, exogenous extracellular DNA was prepared by the PCR amplification with GAPDH F (5'-CAT TGG CAA TGA GCG GTT C-3')-labeled 515F (GAPDH F-515F: 5'-CAT TGG CAA TGA GCG GTT C-GTG CCA GCM GCC GCG GTA A-3') and 806R (5'-GGA CTA CHV GGG TWT CTA AT-3') (Caporaso et al., 2011; Walters et al., 2016). GAPDH F is a primer for a human housekeeping gene and it has no homologous sequences in soils. This primer was selected to avoid interference from the original soil sequences (Huang et al., 2014; Yang et al., 2021; Arvizu-Hernandez et al., 2025). Subsequently, the exogenous extracellular DNA was separately added to the corresponding fresh soils collected from the 30 study sites, and incubated for 0, 3, 6, 12, 24, and 48 days. Finally, the overall and sequence-specific degradation rate constants of 16S rRNA genes were calculated based on amplicon sequencing and real-time PCR. Detailed procedures are described as follows.

4.4. Preparation of exogenous extracellular DNA

Soil DNA was extracted from 0.5 g of soils using the DNeasy PowerSoil kit (Qiagen, Hilden, Germany) following the manufacturer's protocols (Li et al., 2023). Subsequently, GAPDH F was selected as the label primer based on two criteria. First, no detectable PCR amplification was observed for the primer set GAPDH F-806R across all the soil DNA samples included in this study. Second, the melting temperature (Tm) value of GAPDH F approximately matched that of 806R. The GAPDH F-labeled PCR products were generated using DNA extracted from the original soil sample as templates and amplified with GAPDH F-labeled 515F and 806R. The PCR mixture (50 μL) consisted of ExTaq buffer (10×, TaKaRa; 5 μL), dNTP Mix (2.5 mM; 4 μL), forward primer (10 μM; 1 μL), reverse primer (10 μM; 1 μL), template DNA (1 μL), ExTaq (0.25 μL), and DNase-free water (38 μL). The PCR protocol involved an initial denaturation at 95 °C for 10 min, followed by 32 PCR cycles consisting of 30 s at 95 °C, 30 s at 56 °C, and 40 s at 72 °C. Additionally, a final extension was performed at 72 °C for 4 min. Three technical replicates were conducted to amplify each DNA sample, followed by PCR product purification using the GeneJET GEL Extraction Kit (Thermo Scientific, Lithuania). Finally, the purified GAPDH F-labeled 16S rRNA gene fragments were utilized as exogenous extracellular 16S rRNA genes for subsequent experiments.

4.5. Microcosm experiment

The microcosm experiment was conducted using 30 g of soil for each sample. After pre-incubation at 20°C for one week, each soil was thoroughly mixed with the GAPDH F-labeled 16S rRNA genes originating from the corresponding soil in a one-to-one manner, and all the soils were further

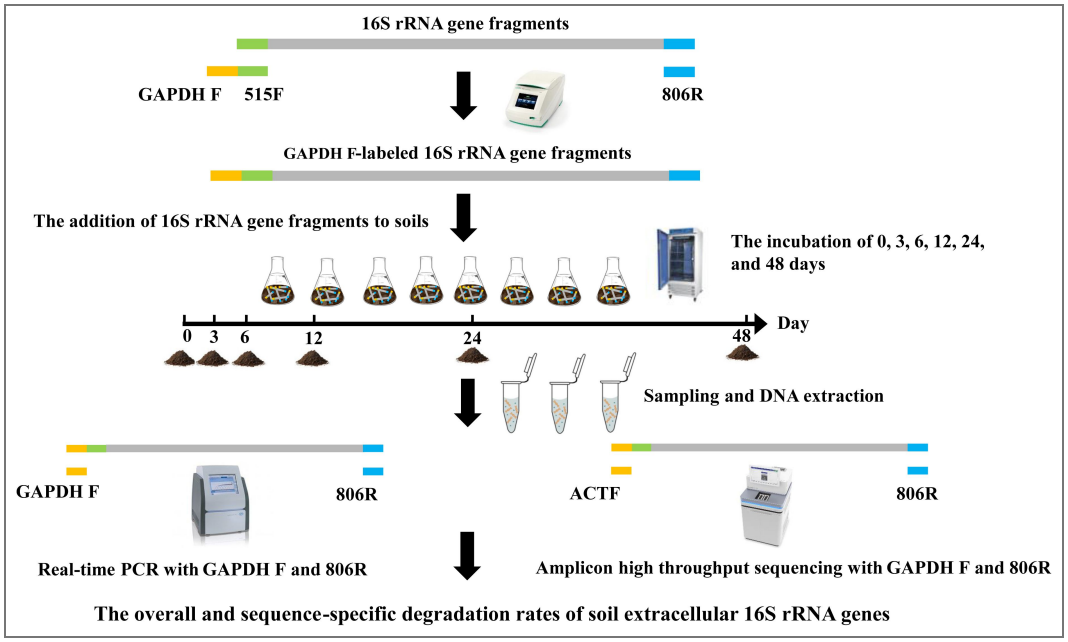


Fig. 6. The determination of soil extracellular 16S rRNA gene overall and sequence-specific degradation rates.

incubated at 20°C (Fig. S9). The exogenous DNA addition rates (1% of the natural soil DNA concentration) were selected to mimic natural extracellular DNA fluxes from microbial lysis (Table S2). Weekly water additions were performed to maintain the original moisture contents of the soils. Soil samples (5 g) were collected after incubation periods of 0, 3, 6, 12, 24, and 48 days with complete mixing prior to each collection. A total of 180 soil samples (30 study sites × 6 incubation times) were included in this study. We further explored the influence of soil moisture contents on the degradation rates of microbial extracellular 16S rRNA gene based on soils collected from Kaiyuan (KY) and Dashanbao (DSB) in the southwest China. The moisture content gradient included 100%, 75%, 50%, 25%, and 10% of soil water holding capacity. GAPDH F-labeled 16S rRNA genes originating from the corresponding soils were added to the soils based on the natural soil DNA concentration. The soils were harvested after 0, 1, 3, 6, 12, and 24 days of incubation.

4.6. Soil DNA extraction, real-time PCR, and amplicon sequencing

The DNA was extracted from the microcosm experiment soils using the DNeasy PowerSoil kit (Qiagen, Hilden, Germany). The copy numbers of the GAPDH F-labeled 16S rRNA genes persisting in the soils were determined by employing a LightCyCler real-time PCR System (Roche, Mannheim, Germany) with primers GAPDH F and 806R. For each real-time PCR reaction mixture (20.0 μL), 1.0 μL of template DNA, 10.0 μL of TB Green™ Premix Ex Taq™ II (Takara, Japan), 0.5 μL of forward primer (20 μM), and 0.5 μL of reverse primer (20 μM) were mixed with 8.0 μL of DNase-free water. The real-time PCR protocol commenced with an initial denaturation at 95 °C for 40 s, followed by 40 cycles (5 s at 95 °C, 30 s at 56 °C, and 40 s at 72 °C). The standard curves exhibited fitted curve R^2 values higher than 0.99, with amplification efficiencies of approximate 80%. Each DNA sample quantification was performed in triplicate.

The community profiles of the GAPDH F-labeled 16S rRNA genes were determined using amplicon high throughput sequencing. Briefly, the GAPDH F-labeled 16S rRNA genes in the microcosm experiment soils were amplified with GAPDH F and barcode-labeled 806R primers. The PCR reaction system and parameters were similar to those mentioned above, except for increasing the number of PCR cycles to 35. Subsequently, the PCR products were purified with a GeneJET GEL Extraction Kit (Thermo Scientific, Lithuania), and all the purified PCR products were pooled in equal molarity. Finally, the purified PCR products underwent double-ended sequencing on the NovaSeq platform at MAGIGENE Co., Ltd. (Guangzhou, China).

The USEARCH (v11) with de-noising algorithm was used to analyze the raw sequences (Edgar, 2013). Briefly, the raw pair-end sequences were spliced using the USEARCH. The search_pcr2 script was utilized to remove primers (*i.e.*, GAPDH F-515F and 806R), and the sequences with primer mismatches greater than 2 were deleted. The fastq_filter script was employed to eliminate sequences with quality scores below 20. Redundancy sequences were removed using the fastx_uniques script. Amplified sequence variants (ASVs) were generated using the UNOISE3 non-clustering denoising algorithm, and ASVs with total sequence numbers less than 9 were removed. An ASV table was generated via mapping the ASVs to the clean raw reads using the otutab script at a similarity threshold of 97%. Taxonomic annotation of the ASVs was performed in QIIME2 with the Silva v138 database. A total of 89322 prokaryotic ASVs were obtained. The sequence numbers in each sample were rarefied to 53251 using the “rarefy” function from vegan package in R. The soil prokaryotic diversity (*i.e.*, richness) was assessed via the vegan package in R (Oksanen et al., 2021). All the raw sequencing data and analysis codes have been deposited in the NCBI Sequence Read Archive under BioProject PRJNA1141901 and GitHub repository (*https://github.com/lt916/16S-rRNA-genes-rates.git*), respectively.

4.7. Determination of soil extracellular 16S rRNA gene degradation rate constants

The overall degradation rate constants of the extracellular 16S rRNA genes were determined by examining the relationships between the copies of labeled 16S rRNA gene and incubation time, and they were mainly indicated by degradation rate constants. The relationships were fitted using first-order enzyme-catalyzed reaction kinetics, as described by the following equations.

$$\ln c = -kt + \ln c_0 \quad (1)$$

t: incubation time, day;

c: the copies of GAPDH F-labeled 16S rRNA genes at time t;

c_0: the initial copies of GAPDH F-labeled 16S rRNA genes;

k: the degradation rate constants of soil extracellular 16S rRNA genes, day^{-1};

The sequence-specific degradation rate constants of the exogenous extracellular 16S rRNA genes were determined using an approach similar to that used for the overall degradation rates of the extracellular 16S rRNA genes. The absolute abundance of each prokaryotic taxa was estimated by multiplying its relative abundance with the copies of total GAPDH F-labeled 16S rRNA genes. Subsequently, the reaction rate constants (k) were calculated by fitting the relationships between the sequence-specific 16S rRNA gene copies and incubation time. When comparing the rates of first-order reactions, the reaction rate constant (k) usually serves as the core indicator. Therefore, we use the degradation rate constant (k) to characterize the degradation dynamics of the added exogenous DNA, which serves as a proxy for how extracellular DNA may be processed in soils.

4.8. Impacts of extracellular DNA on prokaryotic abundance and diversity analysis

To further explore the implications of the overall and sequence-specific degradation rates of soil extracellular 16S rRNA genes, we also determined the impacts of extracellular 16S rRNA genes on soil prokaryotic community analysis and their links with the overall and sequence-specific degradation rates. The intracellular DNA in the soils was extracted based on PMA treatment. Upon photoactivation, extracellular DNA can form covalent bonds through cross-linking, leading to the inhibition of its PCR amplification. In contrast, viable cells with intact cell membranes can exclude PMA, and thus their DNA can still be normally extracted and amplified (Cangelosi and Meschke, 2014). The advantages of the PMA-based method include: 1) it is convenient to operate and exerts negligible influences on the subsequent analysis; and 2) the effectiveness and conservation of the PMA method can distinguish most extracellular DNA from intracellular DNA, and the probability of dead cells permeating PMA is relatively small (Nocker et al., 2007; Pinheiro et al., 2016). Therefore, currently, PMA treatment is the most widely used methods for extracting intracellular methods (Xue et al., 2023; Canini et al., 2024).

In this study, 0.50 g of soils were mixed with 0.5 mL of PMA-phosphate-buffered saline (PBS) solution (40 μM), while the control soil samples were mixed with PBS without PMA. Both the PMA-treated and control soil samples were gently vortexed for 10 min in the dark at room temperature. Following the incubation, both sets of samples were exposed to a 650 W halogen lamp placed at a distance of 20 cm from the tube, undergoing four consecutive cycles of alternating light (30 seconds) and darkness (30 seconds). Subsequently, the tubes were centrifuged at 10000 × g for 2 min, and the precipitates were retained. Finally, DNA was extracted from these precipitates using the DNeasy PowerSoil kit (Qiagen, Hilden, Germany) following manufacturer's protocols.

The copy numbers of 16S rRNA genes were determined using a LightCycler real-time PCR System (Roche, Germany). The universal primers set for the 16S rRNA gene amplification were 515F and 806R. The real-time PCR mixture and procedure followed the aforementioned protocol. The 20.0 μL real-time PCR reaction mixture included 1.0 μL of template DNA, 10.0 μL of TB Green™ Premix Ex Taq™ II (Takara, Japan), 0.5 μL of forward primer (20 μM), and 0.5 μL of reverse primer (20 μM) were mixed with 8.0 μL of DNase-free water. The real-time PCR protocol commenced with an initial denaturation at 95 °C for 40 s, followed by 40 cycles (5 s at 95 °C, 30 s at 56 °C, and 40 s at 72 °C). Additionally, prokaryotic 16S rRNA gene fragments were amplified via PCR with universal primers 515F-806R, following the same PCR system, procedure, and bioinformatics analysis as

described above. Ultimately, discrepancies in soil microbial properties between the control and PMA-treated samples were utilized to indicate the impact of extracellular 16S rRNA genes on soil prokaryotic community analysis.

4.9. Statistical analysis

The changes in GAPDH F-labeled 16S rRNA gene copies and richness across different incubation time points were determined using repeated-measurement analysis of variance (ANOVA). Prokaryotic community structure differences among the study sites and incubation time points were examined through non-metric multidimensional scaling analysis (NMDS) and permutation multivariate analysis of variance (PERMANOVA). Random forest modeling was conducted to assess the importance of environmental and soil variables in predicting the overall degradation rates of extracellular 16S rRNA genes. Structural equation modeling (SEM) was employed to further evaluate the direct and indirect effects of soil moisture, soil pH, MAP, and prokaryotic abundance on the overall degradation rates of extracellular 16S rRNA genes. The sequence-specific degradation rate constants of the exogenous extracellular 16S rRNA genes were visualized using a heatmap with corrplot and matlab packages. Only the abundant taxa, with the proportions exceeding 0.01%, and the degradation rate constants with fitted curve $R^2 > 0.5$ were included in the analysis. We also compared the degradation rates between different ASVs via paired *t-test*, but only the ASVs occurring in more than 90% study sites were included in the analysis. The relationships between the sequence-specific degradation rate profiles of soil extracellular 16S rRNA genes and environmental factors were analyzed by Mantel test.

The impacts of extracellular 16S rRNA genes on the abundance and richness of soil prokaryotic community were determined using the paired *t*-test. Differences in the relative abundance of microbial taxa between the total and living prokaryotic communities were assessed using Wilcoxon test and the taxa with significant differences were visualized with Graphlan (v1.1.3). Mantel test was employed to further investigate the relationships of living or total prokaryotic communities and environmental factors. Pearson correlation test was employed to examine the relationships between the pool sizes and degradation rates of sequence-specific soil extracellular 16S rRNA genes. Only the abundant taxa, with the proportions exceeding 0.01%, were included in the analysis. For each prokaryotic taxon, its extracellular 16S rRNA gene pool size was calculated as the total 16S rRNA gene copies × the relative abundance of the taxon × (1 – intracellular 16S rRNA gene copies of the taxon/total 16S rRNA gene copies of the taxon). Most of the aforementioned statistical analyses were conducted in R software with a range of packages including vegan, ggplot2, RandomForest, piecewiseSEM, corrplot, matlab, igraph, ggcor, ggpubr, Rmisc, and Hmisc (Breiman, 2001; Cutler et al., 2012; Lefcheck, 2016; Harrell and Dupont, 2020; Oksanen et al., 2021).

Data availability

All the raw sequencing data have been deposited in the NCBI Sequence Read Archive under BioProject PRJNA1141901.

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Additional information

Authors' Contributions

R.C. designed research. T.L. performed research. T.L., Z.W., S.Z. and Z.Z. analyzed data. R.C., D.L., X.C. and F.W. revised the paper. T.L. and R.C. wrote the paper. All authors contributed to the preparation of the manuscript. All the authors read and approved the final manuscript.

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Additional files

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Peer reviews

Reviewer #1 (Public review):

Summary:

This manuscript investigates the degradation dynamics of extracellular DNA in soils and its impact on estimates of microbial abundance and diversity. By combining a broad geographic sampling design with a primer-labeling strategy, qPCR quantification, amplicon sequencing, and PMA treatment, the authors aim to disentangle total versus intracellular DNA signals and explore sequence-specific degradation patterns. The topic is relevant, particularly given the increasing awareness of relic DNA as a confounding factor in microbial ecology. The experimental design is ambitious and potentially impactful. However, several conceptual inconsistencies, methodological ambiguities, and statistical limitations currently weaken the robustness of the conclusions. These issues need to be addressed.

Strengths:

The manuscript addresses a timely and important question in microbial ecology, particularly given the growing recognition that relic DNA can bias interpretations of community composition derived from amplicon sequencing. The study is ambitious in scope, incorporating a broad geographic sampling design across multiple soil types, which enhances the generalizability of the findings. The use of a controlled microcosm experiment combined with a primer-labeling strategy to track extracellular DNA dynamics is conceptually innovative and provides a structured framework to investigate degradation processes.

In addition, the integration of multiple approaches, including qPCR for absolute quantification, high-throughput sequencing for community profiling, and PMA treatment to differentiate extracellular from intracellular DNA, represents a comprehensive attempt to disentangle complex sources of bias in soil microbiome analyses. The effort to link degradation dynamics with environmental variables and to explore sequence-level patterns further demonstrates the authors' intent to move beyond descriptive analyses toward a mechanistic understanding.

Weaknesses:

Several conceptual and methodological issues currently limit confidence in the study's conclusions. Key terms such as "sequence-specific degradation" are not clearly defined or supported by a mechanistic or structural hypothesis, making it difficult to interpret the biological meaning of the results. In addition, the bioinformatic workflow presents inconsistencies, particularly the use of ASVs followed by clustering at 97% similarity, which undermines the resolution required to support sequence-level inferences. Statistical analyses are also insufficiently described, including unclear definitions of "T values," a lack of detail on pairing structure, and no indication of multiple testing correction.

Furthermore, important methodological details are missing or unclear, including primer design (e.g., GAPDH tag vs ACTF), Illumina library preparation (e.g., adapter and indexing strategy), and validation of PMA treatment efficiency. The interpretation of PMA-treated samples as representing "living communities" is likely overstated, given the known

limitations of the method in soil systems. Finally, typographical errors, inconsistent terminology, and unclear phrasing throughout the manuscript reduce readability and further complicate interpretation.

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Reviewer #2 (Public review):

Summary:

This manuscript describes the results of an interesting study examining the rate of degradation of extracellular DNA in soil ecosystems using a clever experimental approach. 16S ribosomal RNA genes were amplified from soil samples, and then purified PCR amplicons, containing a 5' linker sequence on the forward primer, were introduced to soils and monitored over time using real-time quantitative PCR and NGS amplicon sequencing. The study was able to measure rates of overall extracellular DNA degradation, but also sequence-specific degradation rates. I like the idea and execution of the study, and the results are interesting. The manuscript needs some help to improve the overall readability. Please see general and editorial comments below.

Strengths:

Innovative experimental design that is well deployed across a large number of soil types, revealing interesting variability in extracellular DNA degradation.

Weaknesses:

(1) The manuscript needs another review to improve the readability of the document.

(2) The authors have used 16S genes to look at sequence-specific degradation. But 16S rRNA genes are actually pretty well conserved, and there isn't as much genetic variation across this gene among organisms as there is for other genes. It might be more relevant to look at metagenomic DNA degradation from high AT, high GC organisms, etc. This would be more generalizable than 16S genes.

(3) Consideration of differential cell lysis during soil DNA extraction needs to be considered as well.

(4) It is not clear why the authors didn't put GAPDH linkers on the reverse primer as well. This would have given an easier amplicon to amplify (no degeneracies at all).

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