

Two Decades of Rhizosphere Microbiome Research under Sewage Sludge Amendment: From Petri Dishes to High-Throughput Sequencing (2006–2026)

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Abstract

Recycling organic waste in agriculture is a pivotal process to maintain soil fertility, as it contributes to providing plants with nutrients, improving the physical structure and chemical properties of soils, and enhancing their biological activity, but the impact of these residues varies according to climate, soil type, and waste treatment, especially in light of the increase in environmental pollutants resulting from human activities such as industrial chemicals, pesticides, and sewage waste over the past two decades. In 2006, the original study relied on traditional planting and counting methods to detect six microbial groups in the roots of bean and winter wheat crops, grown in forest soils mixed with biomaterials of different proportions and concentrations. and metagenome, metatranscriptome) along with qPCR technology to measure the density of functional genes. Recent findings confirm the findings of a 2006 study on increasing the abundance and activity of microorganisms, while revealing new dimensions such as the presence of antibiotic resistance genes in sludge-treated soils. The study relied on the analysis of 28 peer-reviewed papers published between 2015 and 2026, according to specific criteria including the use of modern sequencing or qPCR techniques, the presence of control samples, and conducting experiments in realistic agricultural environments. The study aims to provide an objective comparison between traditional and modern methods, and to propose a practical framework that integrates them, in order to contribute to the development of safer and more sustainable sludge management, environmental protection, crop safety, and food security.

Keywords: Sewage sludge, biosolids, rhizosphere microbiome, 16S rRNA sequencing, metagenomics, plate counting, antibiotic resistance genes.

المخلص

يُعد تدوير المخلفات العضوية في الزراعة عمليةً محوريةً للحفاظ على خصوبة التربة، إذ يُسهم في تزويد النباتات بالعناصر الغذائية، وتحسين البنية الفيزيائية والخصائص الكيميائية للتربة، وتعزيز نشاطها البيولوجي، غير أن تأثير هذه المخلفات يتباين تبعاً للمناخ ونوع التربة وطريقة معالجة النفايات، لا سيما في ظل تزايد الملوثات البيئية الناتجة عن الأنشطة البشرية كالمواد الكيميائية الصناعية والمبيدات ومخلفات الصرف الصحي خلال العقود الماضية. تهدف هذه الدراسة إلى تتبع تطور فهم تأثير المواد الحيوية على ميكروبيوم جذور النباتات خلال الفترة من عام 2006 إلى 2026، ففي عام 2006، اعتمدت الدراسة الأصلية على أساليب التزريع والعد التقليدية لرصد ست مجموعات ميكروبية في جذور محصولي الفول والقمح الشتوي، المزروعين في تربة غابية ممزوجة بمواد حيوية بنسب وتركيزات مختلفة، ومع التطور التقني، تحولت الأدوات المستخدمة من العد التقليدي إلى استخلاص الحمض النووي مباشرة من عينات التربة، باستخدام تقنيات تسلسل متقدمة (16S/ITS)، والميتاجينوم، والميتاترانسكريبتوم) إلى جانب تقنية qPCR لقياس كثافة الجينات الوظيفية. أكدت النتائج الحديثة استنتاجات دراسة 2006 بشأن زيادة وفرة ونشاط الأحياء الدقيقة، مع الكشف عن أبعاد جديدة كوجود جينات مقاومة المضادات الحيوية في التربة المعالجة بالحمأة. اعتمدت الدراسة على تحليل 28 بحثاً محكماً نُشر بين عامي 2015 و2026، وفق معايير محددة تشمل استخدام تقنيات التسلسل الحديث أو qPCR، ووجود عينات ضابطة، وإجراء التجارب في بيئات زراعية واقعية. تهدف الدراسة إلى تقديم مقارنة موضوعية بين الأساليب التقليدية والحديثة، واقتراح إطار عملي يدمج بينهما، بما يسهم في تطوير إدارة أكثر أماناً واستدامة للحمأة، وحماية البيئة، وضمان سلامة المحاصيل، وتحقيق الأمن الغذائي.

الكلمات المفتاحية: حمأة الصرف الصحي، المواد الحيوية الصلبة، ميكروبيوم الجذور، تسلسل 16S rRNA، علم الميتاجينوميكس، عد الصفائح، جينات مقاومة المضادات الحيوية.

1 Introduction

The use of organic waste in farming is a well-known method for maintaining soil health and fertility. It can improve the physical structure, change the chemistry, and boost the biological activity of the soil. However, in my view, we must be cautious. Not all waste products are identical. Each act differently depending on the climate and soil type. The type of waste added can significantly change the number and activity of microbes in soil. Many farmers, especially those with poor sandy soils, are now turning to sewage sludge as a cheap source of organic matter for soil amendments. However, there is a catch: we know very little about how useful this sludge is when the soil is already polluted with heavy metals. The global picture varies from country to country. For instance, consider Hungary. Significant political changes began in 1989–1990. Although they officially joined the EU much later (in 2004), legal work to match European standards began in the late 1990s. However, the situation is different in China. In China, the government set a target in 2005 to treat 40% of municipal sewage, which resulted in a significant increase in sludge production. Most of this sludge ends up in landfills because of heavy metal contamination. However, the potential for its use on farms remains promising. In southeastern

Australia, the numbers are huge— approximately 80% of urban sludge is intended to be reused on agricultural land. However, the presence of heavy metals in sludge is a significant concern. These metals are toxic to soil microbes. Therefore, if sludge is spread on fields, it can disrupt the natural breakdown of organic nitrogen in soil. This question requires further investigation. If we step back and look at history, the entire business of collecting and treating wastewater only began in the mid-to-late 1800s. Previously, people dumped waste into rivers and streams. As treatment technology improved, the pollution threat decreased because the water coming out was cleaner than before. However, there was a trade-off: the cleaning process left solids behind. Back then, we simply called this "sewage sludge." Nowadays, we have a friendlier term: "biosolids." This term is used for treated sludge that has passed strict safety tests and is considered safe for land applications. In contrast, sludge retains its original meaning of raw and unprocessed waste. Although the two terms are sometimes used interchangeably, biosolids always refer to safe, processed materials that meet specific criteria. Therefore, what is sewage sludge? They are the leftover solids from municipal wastewater treatment after steps have been taken to kill the pathogens. Previously, we disposed of this material by dumping it in the ocean, burying it in landfills, or burning it. However, this is expensive and harmful to the environment. Fortunately, sludge is rich in organic matter and nutrients. Bright and Healy (2003) pointed out that recycling through agriculture is a smart way to avoid disposal costs, and this point remains valid today.

Approximately 50% of the biosolid produced annually in the EU is used for land application, which is encouraged by the European Union Biosolids Directive (86/278/EEC) (Łukasiewicz et al., 2024; Collivignarelli et al., 2019). microbial communities response to sludge amendment is not uniform, however it is affected with environmental factors (soil organic matter, soil texture and soil pH) also biosolid (anaerobic digestion, composting, or chemical stabilization) these factors very important for bioavailability and sours of nutrients. The application of biosolids to agricultural land has been shown to have numerous benefits, such as increasing soil nutrients and improving soil quality, ultimately enhancing soil productivity. This is also a disposal method for sewage treatment facilities, ultimately reducing the amount of waste that must be incinerated or buried in landfills.

The challenges and concerns surrounding the land application of sewage sludge are complex social and environmental problems that directly result from the inadequate or excessive application of sewage sludge to agricultural lands. In order to curb the inappropriate application of sewage sludge, resource managers need an application that allows large amounts of spatial and attribute data to be incorporated. This technological evolution is best illustrated by contrasting the 'microbial counts' reported in a 2006 study (based on conventional plate cultivation) with the concept of the 'microbiome' – the full assembly of bacteria, fungi, archaea, viruses, and their collective genomes – which modern research investigates to capture sludge-induced shifts in community composition, functional potential, and interspecies interactions.

Our previous results, which are 20 years old, showed that the tools available for studying the soil microbiome have undergone a revolution. The 2006 study by Hamed (2006) study was a state-of-the-art investigation that relied on conventional plate counting to enumerate six functional groups of microorganisms in the rhizosphere of wheat and beans under different sludge doses. This method provided the first quantitative evidence that biosolid markedly increased the counts of total fungi and bacteria, actinomycetes, phosphate solubilizers, and cellulose decomposers, particularly in the legume rhizosphere. However, plate counting captures only a tiny fraction (<1%) of the total microbial community and provides almost no information on the community composition or functional potential.

The last ten years (2015–2026) have witnessed significant development towards heavy reliance on high-throughput sequencing (HTS) technologies, such as 16S/ITS rRNA sequencing, whole metagenomics, and metatranscriptomics, as well as quantitative PCR (qPCR) for the analysis of functional genes in the microbiome. These advanced methods have demonstrated the tangible effects of biosolid application on the structure of bacterial communities in the soil. They often decrease the ratio of oligotrophic to copiotrophic bacteria and increase alpha diversity. There was also stimulation of the growth of certain bacterial phyla, such as Proteobacteria and Gemmatimonadetes, whereas other phyla, such as Planctomycetes and Firmicutes, declined (Wołejko et al., 2021; D'Angelo, 2023). Additionally, studies based on genetic sequencing have confirmed the high capability of antibiotic resistance genes (ARGs) carried by sludge to persist within soil microbial communities, posing a potential and significant risk of horizontal gene transfer to human pathogens (Łukasiewicz et al., 2024; Wołejko et al., 2021).

Through this modest study, we attempted to find a temporal perspective to reframe the understanding of the root microbiome of sludge produced from wastewater. Therefore, given the rapid technological development, we aim to make an attempt based on a research methodology comparing results from plate count techniques in the 2006 study with those of recent studies and the research advancements that rely on sequencing techniques. The objectives of this study were as follows.

1. To find a way to integrate the results obtained in the 2006 study with advanced molecular techniques,
2. To examine, review, and evaluate the results obtained in 2006 as a historical reference.
3. We aimed to highlight the limits of agreement and disagreement regarding the different effects on the diversity, abundance, and multiple functions of the microbial communities.
4. A practical comparison was made between the methods used in previous studies and modern high-throughput sequencing methods.

5. This modest attempt benefits from advanced scientific research methods using old scientific results that can be developed and used for comparative studies.

2. Materials and Methods – The Comparison Framework

2.1. The original 2006 study (Hamed, 2006)

Soil samples of acidic sandy-brown forest soil with low humus content were collected from a non-cultivated area of Gödöllő, situated 30 km northeast of Budapest. Brown forest soil (sandy, acidic, pH = 5.31) was mixed with aerobically digested biosolids (sewage sludge) from Nyíregyháza (pH = 6.28) at five volumetric ratios: 0%, 30%, 50%, 70%, and 100%. Spring wheat (*Triticum vulgare* L.) and common bean (*Phaseolus vulgaris* L.) were cultivated for 50 d in 2 kg pots in a greenhouse. At harvest, rhizosphere soil was collected, and the following microbial groups were enumerated by plate counting on selective media:

- Total aerobic heterotrophic bacteria (nutrient agar).
- Aerobic spore-forming bacteria (heat-treated at 85 °C for 15 min).
- Actinomycetes (Jensen and Küsten–Williams agars).
- Fungi and yeasts (tryptone glucose yeast extract agar, Martin agar, malt agar).
- Aerobic phosphate-solubilizing bacteria (PSB) (medium of Pikovskaya, 1948; clear zone after five days).
- Aerobic cellulose-decomposing bacteria (cellulose Congo red agar; clear zones after 3–5 days).

All counts were expressed as colony-forming units (CFU) per gram of dry soil, and statistical analysis was performed using MANOVA with LSD_{0.05} (Hamed, 2006).

2.2. Search strategy and selection criteria for recent studies (2015–2026)

We conducted a database search using keywords such as ("sewage sludge" OR "biological organic materials") AND ("soil microbiome" OR "16S rRNA" OR "metagenome" OR "qPCR" OR "phosphate degradation" OR "cellulose degradation") AND ("rhizosphere" OR "wheat" OR "beans").

- Studies must have utilized high-throughput sequencing (16S/ITS amplicon, shotgun metagenomics, metatranscriptomics) or quantitative PCR to characterize the microbiome/functional genes of sludge-amended soils.
- A clear, unamended control treatment should be included for comparisons.
- Studies had to be conducted in agricultural fields or controlled greenhouse settings with common crops (including, but not limited to, wheat and beans).

- The study reported sufficient methodological details to allow for the comparison of community composition and functional gene abundance.

2.3. Screening process and data extraction (PRISMA framework)

The screening process followed the updated PRISMA 2020 guidelines to ensure the transparency and reproducibility of the results. Initially, 1,245 records were identified from the four databases. We excluded 353 duplicate copies and tested 892 records based on their summaries and titles. We excluded 790 studies (non-agricultural settings and no sludge amendments). We then evaluated the remaining 102 full-text articles for eligibility and confirmation. We conducted a precise and specific screening and evaluation according to the inclusion criteria, excluding 74 articles (due to the lack of a control group, missing HTS/qPCR data, or studies focusing only on heavy metals). 28 studies met almost all the required and specified inclusion criteria, and we performed the necessary tests and highlighted the data.



Figure 1. PRISMA 2020 flow diagram showing the systematic and careful process of reviewing and selecting the study data for this review.

Supplementary Table S1 presents the main characteristics of the 28 studies, including the reference, soil type, crop type, level of sludge use, precise analytical technique used and microbial analysis results. Using this comprehensive method, we conducted a good comparative analysis linking the studies.

2.4. Integrative statistical framework for comparing old and modern data

We approximated the results between plate count data (CFU) and molecular data (qPCR copy numbers and relative HTS proportions) and established a workflow for meta-regression analysis. Wherever possible, we used available studies that measured both CFU and 16S rRNA gene copies from the same specific samples to obtain approximate conversion factors for the following: This was performed to obtain a more comprehensive comparison. All data were log-transformed to normalize and clarify the variance, and effect sizes (Hedges' g) were calculated to compare the effect sizes of the sludge across studies. We also used principal component analysis (PCA) to visualize community composition clusters reported in HTS studies, while acknowledging inherent methodological biases, so that a quantitative, precise, and accurate integration of data over 20 years could be achieved.

3. Results

3.1. What the 2006 plate counting study revealed

A 2006 study provided clear quantitative evidence that sewage sludge increases the abundance of all tested microbial groups in the rhizosphere, with the strongest effects observed at the highest sludge dose (100%) and in the legume (bean) rhizosphere. Table 1 summarizes the key findings.

Table 1. Microbial counts (CFU g^{-1} dry soil) in the rhizosphere of common bean and spring wheat after 50 days of growth in brown forest soil amended with different proportions of sewage sludge. Data are presented as the mean of three independent experiments. Asterisks indicate significant differences from the 0% control at $P < 0.05$ (MANOVA, $LSD_{0.05}$). Data from Hamed (2006).

Sludge dose	Plant	Aerobic bacteria ($\times 10^6$)	Spore formers ($\times 10^3$)	Actinomycetes ($\times 10^3$)	Fungi ($\times 10^4$)	PSB ($\times 10^2$)	Cellulose decomposers ($\times 10^3$)
0%	Bean	74	5.1	1.3	5.6	4.5	4.9
30%	Bean	136	11.1	3.5*	8.9*	7.3	9.1
50%	Bean	193*	18.4*	4.3*	10.7*	9.6*	12.2*
70%	Bean	253*	24.1*	5.2*	12.7*	13.2*	15.1*

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100%	Bean	381*	29.3*	6.3*	14.7*	19.7*	21.4*
0%	Wheat	85	6.5	1.4	6.4	4.4	4.1
30%	Wheat	221*	15.1*	4.4*	9.6*	7.7	8.1
50%	Wheat	332*	20.8*	6.2*	10.6*	9.6	11.2*
70%	Wheat	399*	24.7*	9.7*	12.7*	15.2*	15.3*
100%	Wheat	514*	28.6*	13.5*	17.7*	18.7*	22.4*

Table 1 shows a noticeable dose-related increase in all microbial groups. The numbers increased by approximately 3–6 times at the highest sludge concentration, and the rhizosphere of legumes had higher numbers than that of the wheat rhizosphere. This was evident for fungi, cellulose degraders, and phosphorus-containing bacteria.

3.2. What high throughput sequencing revealed (2015–2026)

Recent studies have shown that HTS has a stimulating effect of sewage sludge on the soil microbiome, but it has contributed significantly and beyond expectations in the finest details.

Table 2. Comparison of methodological approaches for studying the rhizosphere microbiome under sewage sludge amendment, highlighting capabilities and technical biases.

Feature	Plate counting (2006)	High-throughput sequencing (2015–2026)
Coverage of microbial community	Total cultured fraction only (typically <1% of total community)	It is comprehensive (targets DNA/RNA from all organisms) but is subject to biases (e.g., DNA extraction efficiency, primer mismatches, PCR amplification bias, and 16S rRNA copy number variation).
Taxonomic resolution	Morphology + simple biochemical tests: identified genera/groups only.	High resolution: Phylum to species/OTU/ASV level; full community composition but limited by database completeness.

Feature	Plate counting (2006)	High-throughput sequencing (2015–2026)
Diversity assessment	Not possible.	Quantitative assessment of alpha (Shannon, Simpson, and Chao1) and beta (Bray-Curtis and UniFrac) diversity.
Functional potential	Not possible directly; inferred from the physiological groups.	Direct assessment via qPCR of functional genes, shotgun metagenomics (gene catalogues), and metatranscriptomics (gene expression) represents potential rather than actual activity.
Detection of rare taxa	Not possible.	However, low-abundance taxa are affected by sequencing depth and may be obscured by noise.
Antibiotic resistance genes	Not possible.	The quantitative profiling of ARGs and mobile genetic elements is limited by the coverage of the reference databases.
Throughput	Low (several weeks for isolation and enumeration).	High (thousands to millions of sequences per sample; data processing is computationally intensive).
Cost	Low.	Moderate to high (sequencing reagents + bioinformatics analysis).

Table 2 clearly and precisely shows that high-throughput sequencing methods provide significantly superior taxonomic and functional depth, yet they are not far from major technical challenges. The plate count technique is considered a low-cost and simple method for measuring specific groups (such as cellulolytic organisms and phosphate-solubilizing bacteria), and it is difficult to infer these groups from marker gene data alone.

3.3. New Insights and Findings from HTS

3.3.1. Shifts in bacterial community composition

Many high-quality studies have shown that sludge use increases the abundance of bacterial species in the soil.

- *Proteobacteria* (especially Gamma- and Betaproteobacteria) often increase in sludge-amended soils, reflecting their copiotrophic lifestyle and ability to rapidly utilize labile organic carbon (Wolejko et al., 2021; D'Angelo, 2023).

- *Gemmatimonadetes* and *Chloroflexi* also became more abundant, partly because of the increased pH and phosphorus availability (Efthymiou et al. 2023).
- Conversely, *Planctomycetes* and *Firmicutes* are often depleted, indicating a shift from oligotrophic to copiotrophic dominance (Łukasiewicz et al., 2024; Wołejko et al., 2021).
- The oligotroph/copiotroph ratio (typically estimated from the relative abundance of *Acidobacteria/Proteobacteria*) decreases markedly, indicating a more productive and nutrient-rich soil environment (Łukasiewicz et al., 2024).

3.3.2. Metabolite cycles and functional genes

Studies related to qPCR and microbial genomics have shown that the use of sludge has a direct effect, leading to an increase in the abundance of genes involved in the major nutrient cycles.

- Phosphorus cycle: The *phoD* gene (encoding alkaline phosphatase) was consistently enriched, explaining the higher phosphate solubilizing activity observed in a 2006 study. da Silva et al. (2025) found that chemically stabilized sludge increased *phoD* abundance by up to 25% compared with thermally stabilized sludge.
- Nitrogen cycle: Sludge application enhances the abundance of *nifH* (nitrogen fixation), *amoA* (ammonia oxidation), and denitrification genes (*nirS*, *nirK*, *nosZ*) (D'Angelo, 2023; An nori et al., 2023). D'Angelo (2023) used metatranscriptomics to show that sludge amendment significantly upregulates the expression of catabolic glutamate dehydrogenase, nitrification, and denitrification enzymes, creating a dynamic organic N mineralization–nitrification–denitrification cycle near sludge particles.
- Carbon cycle: Genes encoding cellulases and hemicellulases (glycosyl hydrolase families) were more abundant in sludge-amended soils, consistent with the increased number of cellulose-decomposing bacteria in the 2006 study.

3.3.3. Genes that have the ability to resist antibiotics (ARGs)

A growing concern is that sewage sludge is a reservoir of ARGs, and its application to agricultural land can spread resistance in the soil microbiome. Łukasiewicz et al. (2024) used shotgun metagenomics to show that sludge-amended soils exhibit significantly greater diversity and richness of ARGs, especially those conferring resistance to tetracyclines, sulfonamides, and β -lactams, than non-amended soils. The ratio of oligotrophic to copiotrophic bacteria decreased substantially, and network analysis revealed that sludge application resulted in qualitative and quantitative changes in the bacterial taxonomic profile, with the most abundant phyla being depleted and replaced by *Proteobacteria* and *Spirochaetes* (Łukasiewicz et al., 2024). These findings highlight an emerging risk that was completely overlooked by the plate counting methods used in 2006.

3.3.4. Rhizosphere versus bulk soil

HTS studies have confirmed the 2006 observation that the rhizosphere microbiome responds more strongly to sludge than to bulk soil. Efthymiou et al. (2023) showed that sewage sludge biochar increased bacterial alpha diversity and induced taxon-specific shifts in both compartments; however, the effects were more pronounced in the rhizosphere, and the legume rhizosphere consistently exhibited higher activity than that of wheat, owing to the selective stimulation of phosphate-solubilizing *Penicillium* and other beneficial microorganisms.

4. Discussion

4.1. Enduring value of the 2006 dataset

The results of the 2006 plate count clearly confirmed the ability of *M. guilliermondii* to persist over time and its resilience to environmental changes. The main conclusion is that sludge can also stimulate the abundance of the main functional microbial groups in the rhizosphere, which has been confirmed by recent studies using high-throughput sequencing (Wolejko et al., 2021; Efthymiou et al., 2023; Annori et al., 2023; Łukasiewicz et al., 2024; D'Angelo, 2023). In terms of trend and effect size, what we found in our 2006 study is fully consistent with the meta-analysis by Charlton et al. (2016), which confirmed that using sludge results in a 30–50% increase in soil microbial biomass.

Therefore, the results of our 2006 study are considered an important bank of reliable and credible historical results on the effect of sanitation sludge on root zone abundance. This is an old and simple study, but its importance comes from its careful professional design, which is why it matters, as it provides insights that will last for decades to come. The results remain valuable for making time-based comparisons and determining the relationship between the number of colony-forming units (CFU) and the abundance of microbes tested using qPCR.

4.2. What HTS added that plate counting missed

Our study in 2006 showed and explained general activation, and in return, HTS revealed important hidden additions.

1. Community composition. The method of counting plates lumps all the bacteria under 'total bacteria' or 'germ products' without providing any information about which groups are actually interacting. High-throughput sequencing techniques have shown that sludge tends to favor species that thrive in nutrient-rich environments (Proteobacteria, Gemmatimonadetes, and Chloroflexi) while limiting the groups that live in nutrient-poor or depleted environments (Planctomycetes and Acidobacteria). This shift affects the retention and presence of carbon in the soil and ecosystem function.

2. Functional gene abundance: The 2006 experiment showed that the ability to break down phosphate was derived from parts of the filtration on Pikovskaya agar. In addition, high-throughput microscopic analysis showed that the *phoD* gene was present in large amounts, and its density matched the activity of the phosphatase enzyme in the wall region. Additionally, an increase in the number of cellulose-degrading microbes was more clearly reflected by the greater abundance of GH family genes.
3. Antibiotic resistance. There are many hazards related to HTS; when sludge is added, it results in an increase in a variety of antibiotic resistance genes (ARGs) in the soil, which may be a reason for the spread of some human pathogens (Łukasiewicz et al., 2024; Wolejko et al., 2021). Here, we highlight the importance of modern methods in detecting them and the inability of old methods (the plate count method) to detect them.
4. Rhizosphere specificity of bacteria. The results of a 2006 study showed that bean plants have a larger number of roots than wheat plants. In contrast, high-precision sequencing techniques confirmed that the difference is not just in quantity; the bacterial community composition is clearly different between the two plants, and sludge has a significant impact on these differences.

4.3. What explains the differences in dose-response patterns?

Our results in 2006 concluded that there was a linear increase across all microbial groups up to the highest sludge concentration (100%), while some recent high-throughput sequencing (HTS) studies have indicated a drop, even a slight decrease, at the highest concentrations (e.g., Charlton et al., 2016; An Nouri et al., 2023). Several factors may explain this discrepancy.

- Exposure period: The timeframe for the 2006 study was only 50 days, which allowed for direct observation of rapid microbial growth immediately after adding the sludge. Long-term studies (months to years) could reveal a more complex pattern, where the initial activation is followed by a gradual return to normal levels due to a decrease or severe lack of degradable carbon or an increase in heavy metal accumulation.
- Dose units: In 2006, we used volumetric ratios (v/v) in pots (limited volume), whereas most recent studies used absolute application rates (Mg ha^{-1}). Direct conversion is difficult, and the pot volumetric ratios (v/v) may correspond to approximately 100% of a relatively moderate-field rate.
- Sensitivity Analysis: The plate counting method tends to become saturated at high cell densities, which might hide the presence of a stationary phase that molecular methods could detect. Nevertheless, the general conclusion of a strong positive effect remains solid across both technological eras.

4.4. The effects associated with sludge treatment and stabilization methods.

A critical factor that is often overlooked in shaping the microbiome response, especially in short-term studies that rely on cultivation, is sewage sludge stabilization. HTS studies have demonstrated these effects. Heat-dried sludge or sludge digested under anaerobic conditions, which is rich in degradable carbon, tends to trigger stronger initial blooms of the phyla Proteobacteria and Bacteroidetes. On the other hand, chemically stabilized sludge (such as lime) often raises soil pH, which can mask the acidifying effects of nitrification and favor different types of actinobacteria. Additionally, advanced treatment processes, such as composting, significantly reduce the load of live pathogenic microbes and some antibiotic resistance genes, whereas thermal hydrolysis may increase the bioavailability of hard-to-remove compounds, affecting the functional gene expression related to the degradation of xenobiotics.

4.5. Fungi and viruses and their main role in the root zone treated with sludge

In 2006, we analyzed fungi as a single group in an experiment, and high-throughput sequencing techniques of the ITS region showed dynamic responses to the treatments. Usually, sludge amendment leads to an increase in decomposer Ascomycota fungi (such as *Penicillium* and *Aspergillus*), which play a key role in dissolving phosphates and breaking down complex organic polymers. In contrast, arbuscular mycorrhizal fungi (Glomeromycota) may respond differently. High doses of sludge can sometimes prevent colonization due to elevated phosphorus levels, which disrupt the desired interactions between fungi and plants.

Recent comprehensive genetic and transcriptomic studies have revealed the presence of viral components (viromes) in soil. Sludge from wastewater introduces a variety of bacteriophages that can affect the bacterial community composition through lysis and horizontal gene transfer (HGT). Interestingly, phages have emerged as important reservoirs of antibiotic resistance genes, carrying beta-lactam and tetracycline resistance genes. The phage-carried resistance community can persist in the soil and contribute to spreading resistance genes among pathogenic and non-pathogenic bacteria, representing a hidden risk pathway that was completely invisible to both plates counting and standard 16S rRNA sequencing techniques

4.6. Reusing historical data with accuracy, integrity, and scientific commitment

In this study, we adhered to the highest standards of scientific integrity and ethical standards. We clearly laid out our study conducted in 2006 and emphasized the limitations of the 2006 study (short duration, relative doses), the temporal importance of this study, and our attempt to find a scientific framework for a systematic comparison. We also merged the 2006 study data with the results of modern high-resolution genetic analysis, none of which had been published before. We hope that this attempt serves as a model for how historical datasets can be ethically and productively reused while benefiting from technological advances and improving research methods.

5. Conclusions and Recommendations

5.1. Main conclusions

1. The study on plate counting from the 2006 experiments is still valid and can be further developed.

The final conclusion of this study is that sewage sludge activates and stimulates the main microbial functional groups in the rhizosphere, which has been proven several times through high-throughput sequencing studies. The 2006 study serves as a historical database that is reliably important for assessing long-term trends.

2. This change in the understanding of HTS is a revolutionary breakthrough in this field. Sequencing results confirmed that using sludge favors nutrient-loving bacterial filaments and boosts the abundance of functional genes in nutrient cycles (phoD, nifH, amoA, cellulases) and, more worryingly, introduces a variety of antibiotic resistance genes into the soil. However, we need to acknowledge the hidden biases inherent in high-throughput sequencing techniques (PCR, primers, DNA extraction), which means that these tools provide a very detailed picture, but it is still skewed and not completely accurate.
3. No competition exists between the two methods, as they complement each other. The plate counting method has proven its effectiveness over time and is useful for identifying and estimating certain functional groups (PSB and cellulolytic organisms) that are difficult to infer from marker gene data alone. Both methods complement one another.
4. Possibility of a decrease in microbial growth. While a 2006 study concluded that there is a linear increase of up to 100% from the sludge, long-term studies suggest that the positive effect might plateau or even decrease at very high doses due to the accumulation of heavy metals or depletion of degradable carbon. This detail was not captured in the short-term pot experiment, and our results from 2006 showed that increasing the amount of sludge had a positive effect. The key point here is the short duration of the experiment, unlike open-field experiments, which are long-term experiments.

5.2. Practical recommendations for researchers and those interested in this field are provided

- A recommendation for those interested in renewing scientific research and developing their data is to document their plate count data for comparison over time and for checking the relationship between the number of colony units (CFU) and the actual numbers measured by qPCR or HTS.

- For everyone interested in molecular biology: Continuously working on testing specific functional groups (such as PSB and cellulases) is not only about the abundance of marker genes but also about targeted experiments or activity assays; these tests support each other as evidence. Additionally, combining ITS methods with (viral) metagenomics helps obtain a complete picture of biodiversity and obtain effective results.
- Strong and useful policies for the safe use of sludge: The planting of antibiotic resistance genes (ARGs) into agricultural soil through sludge is a real challenge that cannot be detected by traditional microbiology. Regular monitoring of antibiotic resistance genes (through qPCR or metagenomics) should be part of sludge quality assessment, along with testing for heavy metals and pathogens, to obtain accurate and clear results.
- To ensure safe use in farming and protect the environment, it is useful and necessary to adopt balanced and long-term methods. There are many agricultural benefits to using treated sludge in farming, as it provides great advantages for crops (organic matter, nitrogen, phosphorus, potassium, and other essential nutrients for plant growth). However, these benefits should be balanced with long-term environmental consequences, especially the gradual accumulation of heavy metals in the soil (such as cadmium, zinc, copper, and lead) and increased soil resistance. Therefore, overuse should be avoided. Adaptive management strategies should be implemented, including regular assessments of soil health, covering physical and chemical properties, metal loads, and the abundance of resistance genes, to ensure the safe long-term use of sludge and avoid negative impacts on the environment.

6. References

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