

Soil chemical and biological properties after long-term of spring barley monoculture under different agricultural practices

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Received: August 22nd, 2025; Accepted: January 20th, 2026; Published: April 2nd, 2026

Abstract. Sustainable agriculture supports soil health and the growth of microorganisms through various agricultural management practices. This multidisciplinary study connected agrotechnology, agrochemistry, and soil microbiology. A 29-year barley monoculture field trial was conducted, focusing on soil pH, soil organic carbon (C_{org}), dehydrogenase activity (DHA), and the abundance of bacteria, including Gram-positive (Gram+) and Gram-negative (Gram-) bacteria, *Actinobacteria*, and saprotrophic fungi. Three agrotechnological factors were considered: chemical plant protection (no protection (PP0), herbicides (H), herbicides, and fungicides (HF)); fertilization (only mineral fertilizer (NPK) and mineral fertilizer with cattle slurry addition (NPKS)); and tillage (autumn shallow mixing (AM), autumn ploughless tillage (APT), autumn plowing (AP), and spring plowing (SP)). All agrotechnological factors were found to have a significant effect on the measured variables, with fertilization having the strongest effect, followed by chemical plant protection and tillage practices. The application of cattle slurry six years ago with annual mineral fertilizer had a significant positive effect on all measured properties, except for saprotrophic fungi. The absence of chemical plant protection significantly increased C_{org} and DHA, Gram-positive bacteria, and *Actinobacteria*, whereas the lowest amounts of Gram-positive bacteria, Gram-negative bacteria, and *Actinobacteria* were found in treatments with only herbicide application, followed by herbicide and fungicide combined. Tillage is an important factor for Gram-positive and Gram-negative bacteria and fungal biomass. Tillage had the most significant effect on fungal biomass compared to plant protection and fertilization. Intensive tillage (deep plowing) in autumn had the strongest negative effect on soil microbiota.

Key words: chemical plant protection, fertilization, long-term field trial, tillage.

INTRODUCTION

Manuscript according to the EU Mission ‘A Soil Deal for Europe’, healthy soil is the foundation of life on earth. Conventional agriculture has an intense impact on the natural environment, particularly through the application of large amounts of mineral fertilizers and synthetic pesticides to the soil. The European Commission (European Commission 2019) has accepted the European Green Deal as an action plan for Europe

to become climate-neutral by 2050, including many sectoral strategies that directly impact agriculture. The climate, energy, and food crises have increased the importance of soil sustainability. Soil properties (biological, chemical) are affected by agricultural management practices both directly and through their impact on soil functions (Massaccesi et al., 2020). Agrotechnology, a multidisciplinary approach that includes crop rotation, soil management, fertilization, and plant protection, plays an important role in maintaining soil health.

The soil biota is an important driver in soils and is responsible for nutrient cycling, aggregate formation, soil processes, and food safety. Soil microorganisms are affected by various factors such as temperature, moisture, seasonality, soil type, and soil management practices (Morugán-Coronado et al., 2022). Organic or sustainable farming systems are usually considered to have a positive effect on soil microbial communities compared with conventional farming practices. To gain practical knowledge on soil health, there is an urgent need to evaluate the relationship between soil microorganisms and soil management practices.

Fertilization is one of the most essential factors in food production. Mineral fertilizers negatively affect soil microorganisms, whereas organic fertilizers (e.g., animal slurry and green manure) are known to have a positive effect (Francioli et al., 2016; Morugán-Coronado et al., 2022). A meta-analysis has shown that combined fertilization with mineral and organic amendments is the best practice for enhancing soil organic carbon, but the effect depends on environmental conditions, management practices, soil properties, and microbial diversity (Liu et al., 2023).

Pesticide use is common in conventional agriculture. For example, in 2022, approximately 322,000 tons of pesticides were sold in the European Union (Eurostat, 2024). Mostly fungicides and bactericides (43%) are used, followed by herbicides, harmful destructors, and moss killers (35%). The amount and type of chemical pesticides applied varies significantly from country to country. In Estonia, significantly lower quantities of chemical pesticides are used per hectare than the EU average (FAOSTAT, 2024). According to Estonian Statistics (2024), the total amount of pesticides sold in 2023 was 714 tons. Farmers primarily applied herbicides (72%, 516 tons), followed by fungicides and bactericides (16%, 118 tons). Therefore, although there is a general need to reduce the use of chemical pesticides, a country-specific approach is required.

In addition, soil mechanical management practices such as tillage have a significant impact on various soil properties. Conservation tillage (e.g., no-till and minimal tillage) is considered a sustainable soil management practice because of its positive effects on soil chemical properties (Lv et al., 2023). In contrast, conventional tillage methods, such as intensive tillage and moldboard plowing, are often associated with decreased soil health and fertility. For example, many studies have shown that conventional tillage negatively affects soil water-stable aggregation, total organic carbon (Karlen et al., 2013), and fungal and bacterial biomass compared with reduced tillage (Morugán-Coronado et al., 2022). However, the effect of tillage practices depends on soil type and climate conditions. Beneficial tillage practices are influenced by factors such as timing, soil properties, and climatic conditions (Gagnon et al., 2022). For example, conventional tillage has been found to enhance nitrogen uptake and yield in cool Atlantic climates (Brennan et al., 2015) and in northern latitudes with clay soils (Gagnon et al., 2022). Tillage is a major driver of soil biodiversity. The best way to select

an optimal tillage system to manage soil health and production capacity is to consider the local soil conditions for each case (van Capelle et al., 2012). Therefore, detailed studies that are specific to different geographical regions are required.

Long-term field trials provide an irreplaceable opportunity to evaluate multidisciplinary relationships between management options and soil properties under real conditions. Soil quality typically changes slowly. Previous studies have shown that the soil microbiota exhibits strong resilience, and long-term field experiments are needed to assess the significant changes associated with agricultural strategies (Pierantoni et al., 2024). Therefore, the results from long-term field trials are stable, indicating permanent equilibrium in the soil (Francioli et al., 2016) and enabling the determination of reliable connections between the measured parameters.

The aims of this study were to: (1) evaluate the long-term effects of different fertilization practices on soil chemical and biological properties, emphasizing the influence of mineral fertilizers and organic amendments, such as cattle slurry; (2) investigate the impact of chemical plant protection on soil health by assessing changes in soil organic carbon and soil microbiota in the presence and absence of chemical pesticides; (3) determine the influence of tillage depth and methods on soil microbiota; and (4) evaluate the relationships between agrotechnological practices, agrochemistry, and their impact on soil microbial communities, providing insights into optimal agricultural management strategies that promote soil health.

MATERIALS AND METHODS

Study site and weather conditions

A trial of monoculture spring barley (*Hordeum vulgare* L.) was set up in 1991 in Kuusiku, North-Estonia (58°58'46" N, 24°42'24" E, 53 m above sea level). The soil was characterized as *Calcaric cambisol* (WRB) with a sandy loam. At the beginning of the trial, the upper layer (0–22 cm) soil characteristics were as follows: pH_{KCl} 6.2 and C_{org} 1.95%. Spring barley variety 'Ida' was grown in the years 1991–1995, and variety 'Anni' in the years 1996–2019. Spring barley was sown at a rate of 450 seeds per square meter.

Weather data were obtained from a nearby weather station operated by the Estonian Environmental Agency (2024). The mean air temperature from September 2019 to March 2020 was 3.9 °C, which was 2.8 °C higher than the long-term average from 1991 to 2020. The accumulated sum of precipitation for that period was 459 mm, which also exceeded the long-term average of 409 mm. The period surrounding the sampling day (19th March 2020) was notably rainy, with monthly precipitation amounts of 88 mm in February and 52 mm in March 2020, compared to the 30-year averages of 43 mm and 40 mm, respectively.

Experimental design

The field trial was conducted using three plant growth techniques: plant protection, fertilization, and soil tillage, arranged in a criss-cross experimental design. In this layout, the management factors were crossed perpendicularly across the field to ensure that all treatment combinations were evenly represented. Each plot measured 11 m × 5 m (55 m²), with three replicates, totalling 72 plots. Two levels of fertilization were used in 2014: mineral fertilizer N₈₅P₃₉K₄₈ (NPK) and the same fertilizer with cattle slurry

addition at a rate of $40 \text{ m}^3 \text{ ha}^{-1}$ (NPKS). Cattle slurry chemical composition was as follows: dry matter content 7.5%, pH_{KCl} 7.7, N_{tot} 3.3 kg m^{-3} , $\text{NH}_4\text{-N}$ 1.6 kg m^{-3} , P_{tot} 0.63 kg m^{-3} . Mineral fertilizer was applied during seed sowing. After harvesting, the straw was returned to the soil. In the spring of 2014, cattle slurry was spread on the soil surface before sowing using a drag-hose spreader. The soil was mixed using a tillage cultivator for no later than one hour after spreading.

Plant protection was implemented at three levels: no plant protection (PP0), herbicide only (H), and herbicide + fungicide (HF). From 1991 to 2001, the herbicide Granstar 75DF was applied at a rate of 10 g ha^{-1} (active ingredient Tribenuron-methyl, 750 g kg^{-1}), which was later replaced by the herbicide Mustang Forte at a rate of 0.6 L ha^{-1} (active ingredients aminopyralid 10 g L^{-1} + florasulam 5 g L^{-1} + 2.4 D 180 g L^{-1}). For fungicide treatment, Tilt 250 EC was applied at a rate of 0.5 L ha^{-1} (active ingredient Propiconazole 250 g L^{-1}) from 1991 to 2001. Input 460 EC (active ingredients Prothioconazole 160 g L^{-1} + Spiroxamine 300 g L^{-1}) has been used at the same rate since 2002. The pesticides were applied as a mixture at a water rate of 300 L ha^{-1} . The water used for spreading was sourced from a deep well and tested by an accredited laboratory of the Republic of Estonia Health Board, revealing the following characteristics: pH of 7.5 and electrical conductivity (EC) of $94 \mu\text{S cm}^{-1}$ at $9 \text{ }^\circ\text{C}$.

Four tillage practices were used: autumn shallow mixing at 8–10 cm depth (AM), autumn ploughless tillage at 15–18 cm depth (APT), autumn plowing at 20–22 cm depth (AP), and spring plowing at 20–22 cm depth (SP). Deep plowing in autumn (AP) and spring (SP) represents conventional tillage practices, whereas shallow mixing (AM) and ploughless tillage (APT) are conservation tillage practices.

Soil sampling and analysis

To minimize the effects of the main crop, soil samples were collected on March 19, 2020, before spring tillage, seed sowing, and fertilization. Sixteen sub-samples from each plot were collected in a zigzag pattern at a depth of 0–20 cm using a 16 mm hand auger. The samples were combined and sieved through a 2-mm mesh.

The chemical properties of pH_{KCl} and C_{org} were determined in an accredited laboratory at the Agricultural Research Center in Estonia. Soil microbial activity, measured as dehydrogenase activity (DHA), was used as a bioindicator to assess the overall soil microbial activity, as it occurs only in living microbial cells (Tabatabai, 1982). The soil samples (5 g) were incubated at $30 \text{ }^\circ\text{C}$ for 24 h in the presence of triphenyl tetrazolium chloride (TTC), an alternative electron acceptor. The resulting red-tinted product, triphenylformazan (TPF), was extracted with acetone and measured using a spectrophotometer at 546 nm.

The microbial community composition was determined using phospholipid fatty acid (PLFA) extraction (Blight & Dyer, 1959), which is widely used as a sensitive bioindicator to study viable microbial communities in different soils. Approximately 4 g of freeze-dried soil was extracted, and phospholipids were separated using a solid-phase extraction column (Chromabond, Macherey-Nagel GmbH, Germany). Methylated fatty acid methyl esters (FAMES) were identified and quantified using an Agilent 7890A/5975C gas chromatograph/mass spectrometer (7890A GC System; Agilent Technologies, USA). A standard qualitative bacterial acid methyl ester

mix C4-C24 (Sigma-Aldrich) was used to identify FAMES based on retention time. The concentrations of individual FAMES were quantified relative to a known amount of methyl nonadecanoate (19:0), which was used as an internal standard (Sigma-Aldrich). The samples were injected in the splitless mode at 250 °C. FAMES were separated on an Agilent HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) using helium as a carrier gas. The number of Gram-positive bacteria was calculated as the sum of branched fatty acids i15:0, a15:0, i16:0, i17:0, and a17:0. For Gram-negative bacteria, it was calculated as the sum of cyclopropane fatty acids and monounsaturated fatty acids cy17:0, 16:1ω7, 18:1ω7, and cy19:0. The sum of the total bacteria represents the sum of Gram-positive and Gram-negative bacteria, and the 15:0 and 17:0. *Actinobacteria* were quantified as the sum of 10-methyl branched saturated fatty acids 10Me16:0, 10Me17:0, and 10Me18:0. The biomass of saprotrophic fungi was assessed using biomarker 18:2ω6 (Ameloot et al., 2015). The relative abundance of microbial groups (% nmol) was calculated to evaluate microbial community structure. The relative abundance of each microbial group was determined by dividing the sum of the specific microbial biomass (expressed as phospholipid fatty acids (PLFAs)) by the total PLFAs.

Statistical analyses

All statistical analyses were conducted using RStudio v1.3.1093 running R software v4.1.2 (R Core Team, 2020). All figures were generated using the ggplot2 package. To analyze the effect of plant protection, fertilization, and tillage on soil pH, soil organic carbon (C_{org}), and soil microbiota (PLFA and DHA), one-way analysis of variance (ANOVA) was performed using ‘*ov*’ function in the ‘stats’ package. Normality of data was assessed using the Shapiro-Wilk normality test with the ‘*shapiro.test*’ function ($P < 0.01$), and homogeneity of variance was tested using Levene’s test with the ‘*leveneTest*’ function in the ‘car’ package. To analyze the significant differences between treatments based on factors (plant protection, fertilization, and tillage), descriptive statistics were calculated. Tukey (HSD) test was used for post hoc comparisons. Pearson correlation analysis was conducted between variables, and significant correlations between soil chemical properties and microbiota were visualized using the ‘*corrplot*’ package (v 0.87). Principal Component Analysis (PCA) was used to illustrate the similarity between plots based on the microbial community, dehydrogenase activity (DHA), and soil chemical properties.

RESULTS AND DISCUSSION

After 29 years, the soil gradually became alkaline, with pH increasing from 6.2 to over 7.0 in all studied plots (Table 1). The soil pH was more neutral (pH 7.06) in the NPKS treatment, where the slurry was used. In plots receiving only mineral fertilizers (NPK), the pH was significantly higher (pH 7.24) (Tables 1, 2). Over 29 years, the percentage of C_{org} decreased in plots with mineral fertilization (NPK), whereas it slightly increased in plots with mineral fertilization and cattle slurry addition (NPKS). Overall, the C_{org} was significantly higher in the NPKS group ($C_{org} = 2.03\%$) than in the NPK group ($C_{org} = 1.85\%$).

Table 1. Mean values of soil pH_{KCl}, soil organic carbon (C_{org},%), dehydrogenase activity (DHA) (TPF, µg g⁻¹ h⁻¹ dry soil), abundances of Gram-positive bacteria (Gram+), Gram-negative bacteria (Gram-), *Actinobacteria*, saprotrophic fungi (PLFA, nmol g⁻¹ dry soil) by factors (plant protection, fertilization, tillage) and their significant differences between treatments

Factor	Variant	pH _{KCl}	C _{org}	DHA	Gram+	Gram-	<i>Actinobacteria</i>	Fungi
Fertilization	NPK	7.24 ^a	1.85 ^b	6.74 ^b	10.16 ^b	5.20 ^b	3.65 ^b	0.72
	NPKS	7.06 ^b	2.03 ^a	8.38 ^a	11.21 ^a	5.93 ^a	4.18 ^a	0.72
Plant protection	PP0	7.05 ^b	2.03 ^a	8.55 ^a	11.20 ^a	5.81	4.10 ^a	0.76
	H	7.23 ^a	1.89 ^b	7.10 ^b	10.32 ^b	5.41	3.78 ^b	0.71
	HF	7.18 ^a	1.89 ^b	7.03 ^b	10.51 ^b	5.45	3.85 ^{ab}	0.68
Tillage	AM	7.08	2.01	7.37	10.69 ^{ab}	5.67 ^{ab}	3.95	0.76 ^a
	APT	7.13	1.93	7.97	11.02 ^a	5.75 ^a	4.00	0.77 ^a
	AP	7.19	1.92	6.91	10.09 ^b	5.10 ^b	3.75	0.61 ^b
	SP	7.20	1.89	8.01	10.92 ^{ab}	5.72 ^a	3.95	0.75 ^a

C_{org}: soil organic carbon; DHA: dehydrogenase activity; NPK: mineral fertilizer; NPKS: mineral fertilizer with cattle slurry addition; PP0: no plant protection; H: herbicides only; HF: herbicides and fungicides together; AM: autumn shallow mixing at 8–10 cm depth; APT: autumn ploughless tillage at 15–18 cm depth; AP: autumn plowing at 20–22 cm depth; SP: spring plowing at 20–22 cm depth.

Letters behind the means (n = 3) indicate significant differences according to Tukey's test ($P > 0.05$).

Table 2. Soil chemical and microbial parameters of the one-way ANOVA analysis for the three factors (plant protection, fertilization, and tillage) and their interaction after a 29-years monoculture experiment

	Fertilization	Plant protection	Tillage
pH _{KCl}	F _(1,70) = 30.36***	F _(2,69) = 8.36***	F _(3,68) = 1.99
C _{org} ,%	F _(1,70) = 33.68***	F _(2,69) = 7.75***	F _(3,68) = 2.04
DHA (TPF µg g ⁻¹ h ⁻¹)	F _(1,70) = 16.97***	F _(2,69) = 5.71**	F _(3,68) = 1.45
Gram-positive bacteria (nmol g ⁻¹)	F _(1,69) = 23.82***	F _(2,68) = 5.27**	F _(3,67) = 3.10*
Gram-negative bacteria (nmol g ⁻¹)	F _(1,69) = 22.21***	F _(2,68) = 2.19	F _(3,67) = 3.44*
<i>Actinobacteria</i> (nmol g ⁻¹)	F _(1,69) = 52.14***	F _(2,68) = 4.57*	F _(3,67) = 1.28
Saprotrophic fungi (nmol g ⁻¹)	F _(1,69) = 0.02	F _(2,68) = 2.02	F _(3,67) = 6.02**

C_{org}: soil organic carbon; DHA: dehydrogenase activity; NPK: mineral fertilizer; NPKS: mineral fertilizer with cattle slurry addition; PP0: no plant protection; H: herbicides only; HF: herbicides and fungicides together; AM: autumn shallow mixing at 8–10 cm depth; APT: autumn ploughless tillage at 15–18 cm depth; AP: autumn plowing at 20–22 cm depth; SP: spring plowing at 20–22 cm depth.

Significance * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The total PLFA content ranged between 20.4 and 34.0 nmol g⁻¹ of dry soil. Significantly higher contents of Gram-positive and Gram-negative bacteria, *Actinobacteria*, and dehydrogenase activity (DHA) were observed in plots treated with NPKS (Table 1). No significant difference was found between the NPK and NPKS treatments on the level of saprotrophic fungi (Table 1). Correlation analysis revealed significant negative associations between soil pH and soil microbial enzyme activity (Table 3) in Gram-positive bacteria (Fig. 1, A), Gram-negative bacteria (Fig. 1, C), and *Actinobacteria* (Fig. 1, E). These findings suggest that soil microbial activity decreases with increasing pH in alkaline soil. Additionally, a negative correlation was observed between the soil pH and C_{org} (Table 3). Significant positive correlations were found between C_{org}% and microbial enzyme activity (Table 3) in Gram-positive bacteria (Fig. 1, B), Gram-negative bacteria (Fig. 1, D), and *Actinobacteria* (Fig. 1, F).

Table 3. Spearman's rank correlations (r) between tillage depth (cm), soil pH_{KCl}, soil organic carbon (C_{org}, %), dehydrogenase activity (DHA) (TPF, $\mu\text{g g}^{-1} \text{h}^{-1}$ dry soil), abundances of Gram-positive bacteria (Gram+), Gram-negative bacteria (Gram-), *Actinobacteria*, saprotrophic fungi (PLFA, nmol g^{-1} dry soil)

Parameter	pH	C _{org}	DHA	Gram+	Gram-	<i>Actinobacteria</i>	Fungi
Tillage depth	0.29*	-0.30*	-0.004	-0.11	-0.13	-0.14	-0.32*
pH		0.64***	-0.36**	-0.45**	0.38***	-0.55***	-0.02
C _{org}			0.44***	0.55***	0.46***	0.63***	0.17
DHA				0.66***	0.66***	0.63***	0.40***
Gram+					0.93***	0.94***	0.54***
Gram-						0.86***	0.57***
<i>Actinobacteria</i>							0.38**

Significance * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Furthermore, one-way ANOVA indicated a significant impact of all examined factors, with fertilization exerting the strongest effect (Table 2). Principal Component Analysis (PCA) revealed consistent trends in the abundance of different microbial groups (Gram-positive bacteria, Gram-negative bacteria, *Actinobacteria*, and fungi), DHA, and soil parameters (pH and C_{org}) (Fig. 2). The PCA results explained 70% of the variance (PC1, 54%; PC2, 16%). Most of the variation observed in the PCA was attributed to fertilization, with clear clustering according to this factor. Plots treated with only mineral fertilizer are predominantly located on the left side of the graph.

Plant protection influences soil pH, organic carbon content (C_{org}), soil dehydrogenase activity (DHA), and the abundance of Gram-positive bacteria and *Actinobacteria*. Significantly higher soil pH was observed in treatments involving herbicides (H), herbicides, and fungicides (HF). A lower soil pH was observed in treatments with no chemical plant protection (PP0) (Table 1). ANOVA indicated a significant effect of plant protection on C_{org}% (Table 2), with the highest organic carbon content observed in treatments without plant protection (Table 1). Soils treated with herbicide (H) and herbicide with fungicide (HF) showed similar levels of C_{org} % (Table 1). The long-term use of chemical pesticides (H and HF) negatively affects the soil microbiota. Soil microbial activity, as indicated by dehydrogenases, and the biomass of Gram-positive bacteria and *Actinobacteria* were significantly higher in the absence of pesticides (Tables 1, 2). Plant protection is an important factor that influences the abundance of Gram-positive bacteria and *Actinobacteria* (Table 2). Interestingly, plots treated with herbicides and fungicides (HF) showed higher biomass of bacterial groups (Gram-positive bacteria, Gram-negative bacteria, *Actinobacteria*) than those treated with herbicides only (H).

The highest average pH was observed in the deep plowing systems at depths of 20–22 cm, SP (pH 7.20), and AP (pH 7.19), followed by APT (pH 7.13) and AM (pH 7.08) (Table 1). However, according to ANOVA, the differences in pH were not significant between the different tillage systems (Table 2). In addition, the effect of soil tillage did not reveal significant differences in soil C_{org} content. Compared to the plowing variants (AP and SP), non-plowing variants showed slightly higher soil C_{org} content, with shallow soil mixing at a depth of 8–10 cm (AM) and ploughless mixing at a depth of 15–18 cm (APT) (Table 1). However, no significant differences were observed between

the groups. Nevertheless, a significant positive correlation was found between tillage depth and soil pH (Fig. 3, A), and a negative correlation between depth and C_{org} (Fig. 3, B).

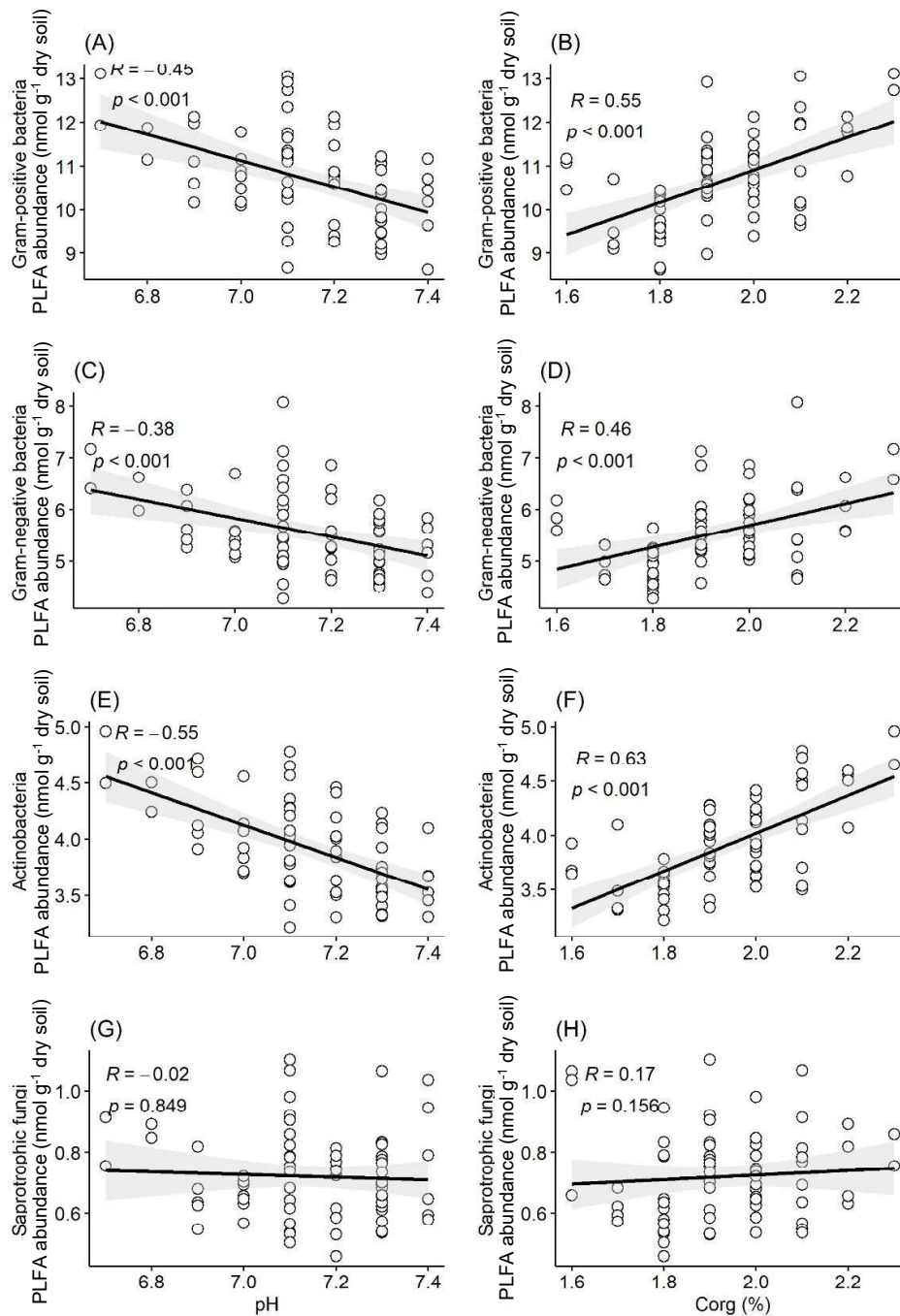


Figure 1. Spearman's rank correlation (r) between pH_{KCl} , soil organic carbon (C_{org} , %), abundances of Gram-positive, Gram-negative, *Actinobacteria*, and saprotrophic fungi (PLFA, nmol g⁻¹ dry soil).

Tillage systems affected the abundance of some microbial groups, with lower levels of Gram-positive bacteria, Gram-negative bacteria, and fungi observed in the autumn-plowing variant than in the other tillage systems (Table 1). *Actinobacteria* showed similar abundance regardless of tillage practices (Tables 1, 2). In addition, fungal biomass was significantly affected by tillage practices (Tables 1, 2), with the lowest values observed for AP tillage. Only a significant negative correlation was found between tillage depth and fungal abundance (Fig. 1, F).

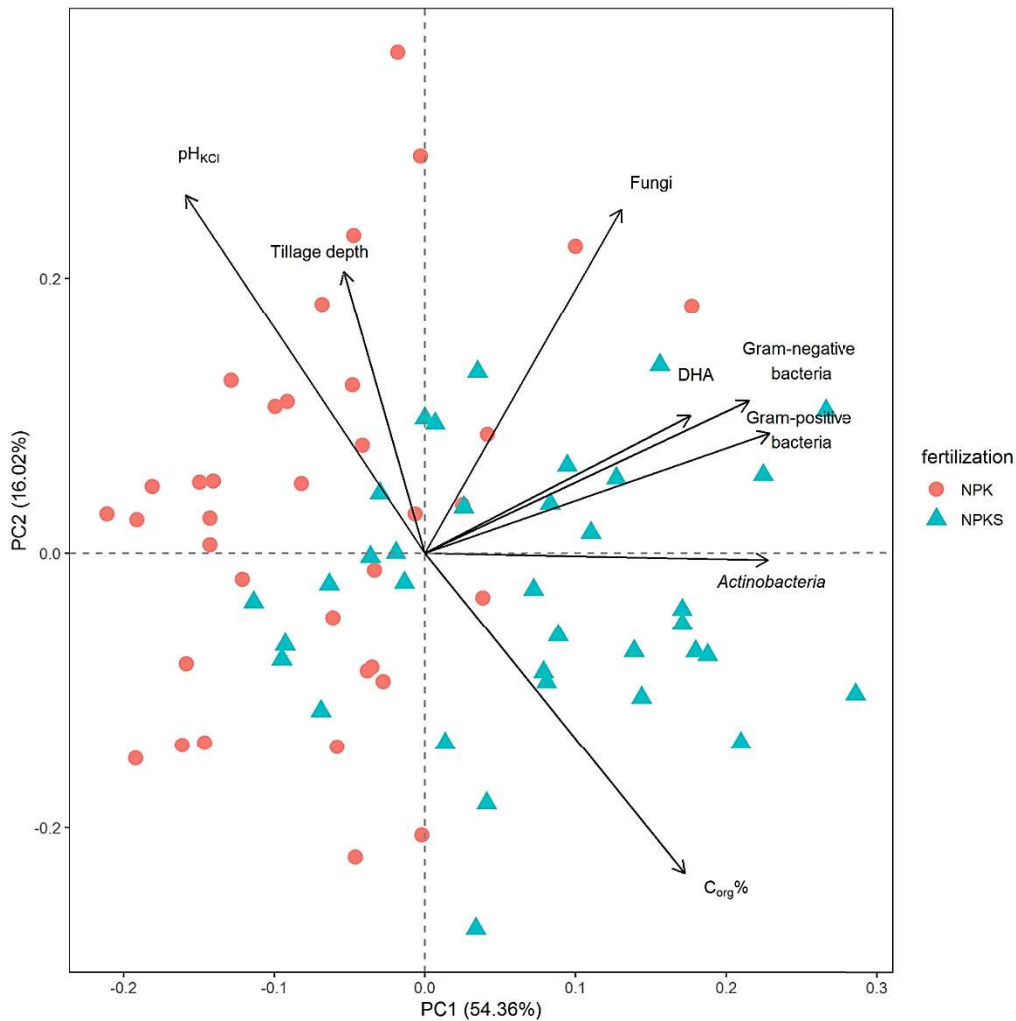


Figure 2. A biplot of principal component analyses (PCA) according to soil dehydrogenase activity (DHA) (TPF, $\mu\text{g g}^{-1} \text{h}^{-1}$ dry soil), abundances of Gram-positive, Gram-negative, *Actinobacteria* and saprotrophic fungi (PLFA, nmol g^{-1} dry soil), pH_{KCl} and soil organic carbon (C_{org} , %). Fertilization: mineral fertilizer $\text{N}_{85}\text{P}_{39}\text{K}_{48}$ (NPK) and the same fertilizer with cattle slurry addition (NPKS).

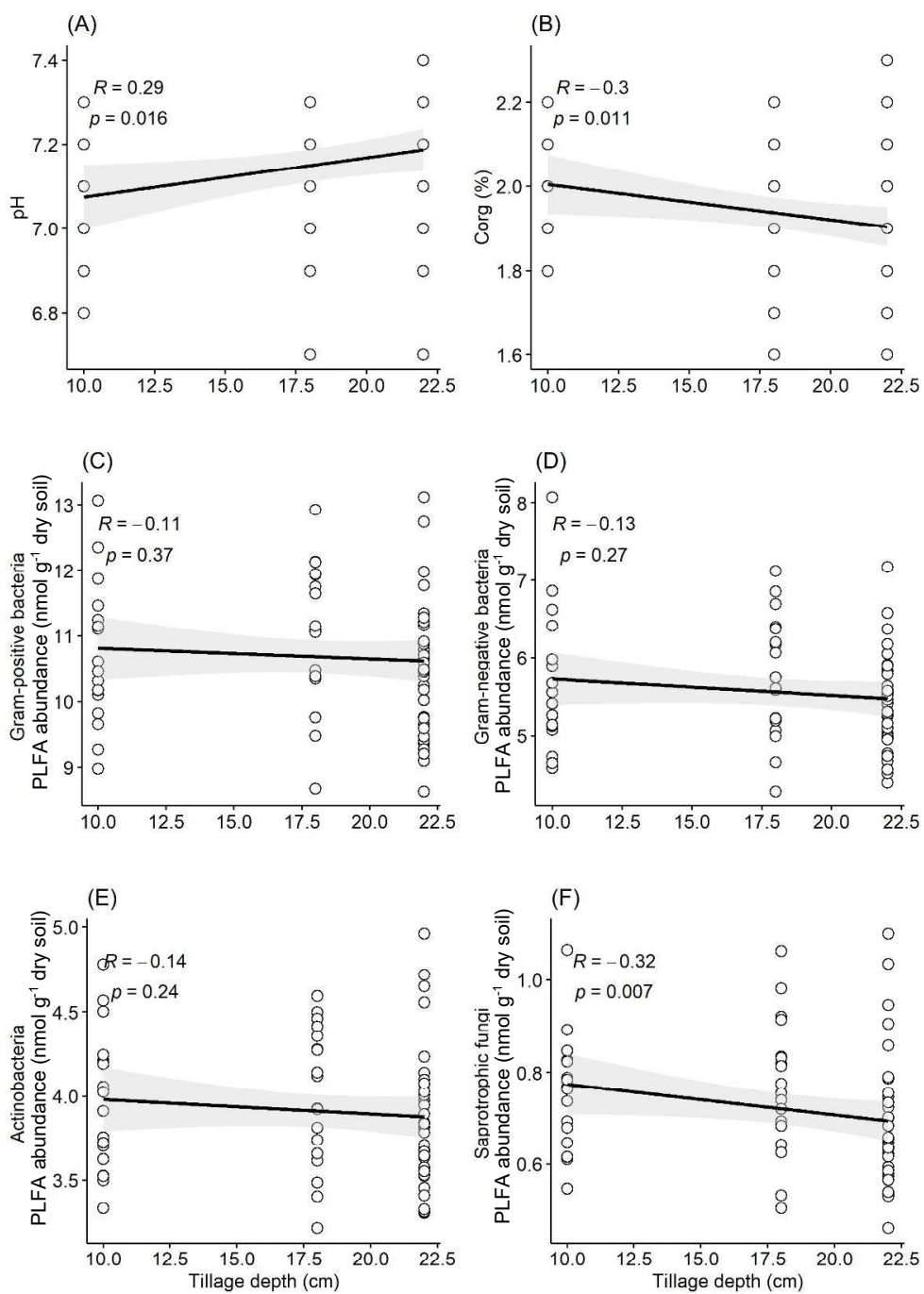


Figure 3. Spearman's rank correlation (r) between tillage depth (cm), pH_{KCl} , soil organic carbon (C_{org} , %), abundances of Gram-positive, Gram-negative, *Actinobacteria*, and saprotrophic fungi (PLFA, nmol g^{-1} dry soil).

Our study revealed long-term multifactorial agrochemical effects on soil pH, organic carbon content, and soil microbiology. All studied agrotechnological factors (fertilization, soil tillage, and plant protection) were found to have a significant effect, with fertilization exerting the strongest impact, followed by plant protection and tillage practices. Overall, pH increased during the long-term experiment. In our study, the strongest effect on pH was attributed to fertilization, with soils treated only with mineral fertilizer (NPK) showing a more alkaline pH than NPKS treatments. Long-term studies have shown that the use of mineral fertilizers can lower soil pH; however, the extent of acidification is directly related to the amount of N applied (Souza et al., 2023; Wang et al., 2023). The level of mineral fertilizer was $N_{85}P_{39}K_{48}$ and remained consistent across all treatments. The only exception was the NPKS treatment, in which $40\text{ m}^3\text{ ha}^{-1}$ cattle slurry was added in spring 2014. As the long-term annual addition of manure has been shown to lower soil pH (Chang et al., 1990; Feng et al., 2024), we hypothesized that the lower soil pH observed in our experiment could be attributed to the long-term effect of additional $\text{NH}_4\text{-N}$ from cattle manure. Second, fertilization in alkaline soils can result in different reactions. Zhang and co-workers (Zhang et al., 2021) came to the conclusion that long-term manure application in alkaline soils improved soil aggregate stability and increased aggregate-associated carbon by decreasing pH. Consistent with our study, their 13-year field experiment with manure addition lowered pH by 0.21-0.31 (Zhang et al., 2021).

Soil organic carbon (C_{org}) is primarily influenced by management practices but tends to remain relatively stable (Gubler et al., 2019). Organic amendments, such as cattle slurry, increased the C_{org} content in our field trial. While the long-term effects of slurry have been relatively understudied, positive effects of compost on soil organic carbon and soil physical quality have been detected even after 24 years (Atoloye et al., 2022). This aligns with our results, which demonstrate a significant effect of slurry on soil properties even six years after addition. In addition, organic amendments such as compost have had a positive effect on soil organic carbon pools, even after a single application (Atoloye et al., 2022). Moreover, several studies have demonstrated that the addition of manure and mineral fertilizers has a significant positive effect on soil microbiology and soil organic carbon (Lazcano et al., 2013; Abdalla et al., 2022). A large-scale meta-analysis showed a consistent trend, indicating that combined fertilization with mineral and organic fertilizers has the strongest positive effect on soil organic C compared to the use of mineral or organic fertilizers (Liu et al., 2023). However, the effect of fertilization on SOC also depends on environmental factors and management practices.

Most likely, the addition of cattle slurry increased the soil organic carbon content and the activity and biomass of the microbial communities. In our study, the greatest effect of fertilization was observed in *Actinobacteria*, followed by Gram-positive and Gram-negative bacteria. No significant differences due to fertilization were found for the saprotrophic fungi. Many previous studies have also shown that animal manure positively affects soil microorganisms (Gautam et al., 2020; Hou et al., 2022b). This result could be explained by several factors. First, manure addition increases the proportion of macroaggregates, thereby positively affecting the content and distribution of soil organic carbon and microbial biomass (Wang et al., 2017). In accordance with our results, even small amounts of manure addition have shown a positive effect on soil

bacteria, especially Gram-negative bacteria, whereas fungi exhibit a minimal response (Lazcano et al., 2013; Hou et al., 2022b). In our study, manure addition six years ago increased C_{org} and decreased soil pH to a more neutral level compared to plots using only mineral fertilizer. In addition, we found a negative correlation between soil microbiological parameters and pH. Soil microbial communities are strongly influenced by pH and organic carbon (Francioli et al., 2016). Positive relationships between pH and soil bacteria have been observed in soils with pH <7.0 (Hou et al., 2022a). But Sadeghi and co-workers (Sadeghi et al., 2023) showed a negative correlation between microbial parameters and pH.

Contrary to expectations, we found no correlation between fungi biomass and pH or C_{org} . Fungi appear to be more resistant to environmental factors such as pH (Hou et al., 2022b) and moisture (Sun et al., 2020). Therefore, according to our results from DHA and PLFA analyses, the addition of cattle slurry 6 years ago continues to have a positive effect on the soil microbial community.

All plots with chemical plant protection (H and HF) exhibited higher pH levels than the control (PP0). The alkalinity in treatments with chemical plant protection may be attributed to location-specific factors. It is possible that the use of alkaline deep-well water to spread these chemicals over the 29-year period contributed to this result. The spreading rate of the chemical mixture was 300 L ha⁻¹.

Lower levels of C_{org} were observed in treatments using chemical pesticides (H and HF) than in the control (PP0). The potential negative effects of synthetic pesticides on the soil have been extensively discussed in various studies (Rasool et al., 2022; Sim et al., 2022). Sim et al. (2022) directly showed that the application of different pesticides disrupted soil organic carbon formation and stabilization. Another reason could be the weed-killing effect of the herbicides. The absence of herbicide treatment (PP0) allowed additional weed biomass (both aboveground parts and roots) to contribute positively to C_{org} compared to the biomass contributed solely by the main crop (straw and roots).

In addition, plots treated with herbicides and fungicides (HF) showed a higher biomass of bacterial groups than plots treated with herbicides only (H). This could be because fungicides affect soil fungi, allowing for more space and resources for bacteria. Thus, the use of fungicides decreases the abundance of fungi, which may lead to an increased abundance of soil bacteria owing to reduced competition. Consequently, fungi exhibited the lowest values in plots treated with herbicides and fungicides (HF), likely because of the impact of the fungicides. However, the abundance of fungi in the HF treatment was the lowest, although the differences with other treatments were not significant.

The long-term effects of our experiment underscore a significant aspect: soil tillage practices influence soil conditions, particularly the tillage depth. By tillage, the upper soil layer (8–18 cm) plant residues are effectively mixed, thereby aerating the soil and creating favorable conditions for aerobic decomposition of plant residues by fungi and bacteria. Deep soil plowing, as conducted in our experiment over 29 years, transformed the aerobic upper layer into an anaerobic environment during each tillage operation. Our experiment revealed that fungi are the group of microorganisms that are most affected by tillage. Previous studies have shown that tillage affects fungi more significantly than it affects bacterial communities. For example, Li and co-workers (Li et al., 2024) results

showed that rotary tillage and plough tillage decrease the fungal richness compared to no-tillage, but no significant differences were observed in bacterial communities. Lower intensity management and reduced tillage are positively associated with soil fungi (Mann et al., 2019). Typically, fungi prefer aerobic soil conditions (Sun et al., 2020). Additionally, it became apparent that in the cultivation of carbonate-rich thin soil, plowing to a depth of 20–22 cm resulted in limestone fragments from the lower layer of soil. This process begins to increase the soil pH, thereby negatively affecting microbial groups. A negative correlation between microbial parameters and pH has been reported previously (Sadeghi et al., 2023). Several previous studies have concluded that soil pH is more strongly influenced by soil type, weather, and other management practices than tillage (Busari et al., 2015). The pH of the soil in this study could also be influenced by the fact that the field trials were located in a Calcaric cambisol (WRB), where the soil contains limestone fragments that may be brought up to the topsoil by tillage.

In addition, the soil tillage depth was negatively correlated with soil C_{org} content. Deeper tillage, such as plowing to a depth of 20 cm, buried the organic-rich layer of plant residues deeper in the soil, whereas shallow tillage variants, such as ploughless tillage and plant residues, were mixed only within the upper 8–15 cm layer. This result is consistent with previous studies where conventional tillage has been shown to reduce soil organic carbon compared to conservational tillage practices (Gagnon et al., 2022). This may be because deep plowing activates anaerobic soil microorganisms in the deeper layers, thereby decreasing C_{org} content. Soil pH was negatively correlated with soil C_{org} content, indicating that in more alkaline soils, soil C_{org} content decreases because of the potentially slower decomposition of plant residues in an alkaline environment. In particular, significant effects on fungal biomass were found only with tillage, whereas fertilization and chemical plant protection showed no significant differences. These results are consistent with those of a grassland study (Barreiro et al., 2022) across the European climate transect, suggesting that the soil bacterial community structure is primarily influenced by climate and soil properties, whereas soil fungal groups are affected more by management intensity. Similar to our results, a meta-analysis concluded that conservation tillage practices typically have a positive effect on fungi and bacteria, and conventional tillage with deep plowing often shows the lowest microbial abundance (Morugán-Coronado et al., 2022).

CONCLUSIONS

This study highlights the need for long-term field trials assessing various agricultural management practices to elucidate the connections between agrotechnology, agrochemistry, and soil microorganisms. Fertilization had the most significant influence on soil chemical and microbial parameters. The absence of pesticides increased C_{org} and the abundance of soil microbial groups. The impact of tillage was primarily related to cultivation depth.

ACKNOWLEDGEMENTS. This research received no specific grants from any funding agency, commercial sector, or not-for-profit sector. This study was supported by the Centre of Estonian Rural Research and Knowledge.

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