A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Quantity and Quality Assessment of Soil Organic Matter: Humus Carbon and Nitrogen Dynamics under Diverse Land Uses, Amendments, and Long-Term Fertilizations

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KHOK PROS

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This undersigned certify that they have read, and recommended to the Graduate School for acceptance, a thesis entitled "Quantity and Quality Assessment of Soil Organic Matter: Humus Carbon and Nitrogen Dynamics under Diverse Land Uses, Amendments, and Long-Term Fertilizations" submitted by KHOK PROS in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Abbreviations

ANOVA	Analysis of variance
AYR	Alizarin yellow R
C	Carbon
CFU	Colony forming unit
DT	Degree of transformation (FA+HA)/HM
FA	Fulvic acid
FHA	Fulvic + humic acids
G	Greenhouse soil
HA	Humic acid
HI	Humification index (HA/FA)
HM	Humin
LSD	Least significant difference
N	Nitrogen
NHS	Non-humic substances
O	Orchard soil
P	Paddy soil
R	Reclaimed soil
RSD	Relative standard deviation
SOC	Total soil organic carbon
SOM	Soil organic matter
TN	Total nitrogen
U	Upland soil
V	Volcanic ash soil

Quantity and Quality Assessment of Soil Organic Matter: Humus Carbon and Nitrogen Dynamics under Diverse Land Uses, Amendments, and Long-Term Fertilizations

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Abstract

Soil organic matter (SOM) is a critical component of soil quality, influencing nutrient cycling, carbon (C) sequestration, and ecosystem resilience. The quantity of SOM reflects the total organic matter in the soil, encompassing C and nitrogen (N) stocks that contribute to soil fertility and ecosystem services. In contrast, SOM quality describes the chemical composition, nutrient availability, and stability of organic fractions, ranging from labile compounds to highly stabilized humus. Indicators such as carbon-to-nitrogen (C/N) ratios, humification indices, and the balance between labile and stable fractions are crucial for assessing SOM dynamics. Given that SOM is formed by complex biological, chemical, and physical processes and varies significantly across land-use types, amendments, and long-term management

practices, determining its composition may be more important than measuring its total concentration for assessing soil quality. The overall objectives of this study were to: (i) develop and optimize an alkaline persulfate digestion method for the simultaneous determination of total soil organic carbon (SOC) and total nitrogen (TN) in air-dried soils, ensuring precision, cost-effectiveness, and broad applicability; (ii) investigate the relative proportion of fulvic acid (FA), humic acid (HA), and humin (HM) in relation to the increase of SOC content in six different land uses sampled across the Korean peninsula; (iii) evaluate the effects of different amendments on CO_2 emissions, microbial populations, and SOM quality, with a focus on changes in humification indices and C/N ratios of SOM fractions (FA, HA, and HM); and (iv) assess the long-term impacts of fertilizations on SOC, TN, and humus stability in paddy soils, offering insights for sustainable soil management strategies. Soil samples collected from 6 land uses across the Korean peninsula, an incubation experiment, and a long-term experiment were fractionated into non-humic substances (NHS), debris, FA, HA, and HM. The results of the first chapter showed that the optimal oxidizing reagents, 0.4 M $Na_2S_2O_8$ and 0.6 M NaOH, along with the basic pH indicator solution of 15.5 mL of 0.1% (w/v) alizarin yellow R (AYR) in 1.0 M K_2CO_3, were ideal for the precise determination of SOC and TN in 0.1 g of ground air-dried soil samples. Optimized digestion conditions of 110°C for 1 h provided a cost-effective alternative to conventional methods. The method can be extended for measuring C and N in FA, HA, and HM fractions with slight modification. The second investigation assessed SOM quality in soils under six land uses in Korea. Based on the C/N ratio of FA, HA, and HM, the SOM quality in orchard, upland, and greenhouse soils was higher than that in paddy, volcanic ash, and reclaimed soils. In all land-use types, additional N is required to transform labile C forms into stable humus, particularly in paddy, volcanic ash, and reclaimed soils used for rice cultivation. The third study explored the impacts

of different amendments on SOM dynamics during a five-month incubation. Combined amendments, i.e., compost (CP) + rice straw (RS), significantly enhanced SOM stability by increasing the humification index (HI) and decreasing the degree of transformation (DT) compared to other treatments. In addition, the C/N ratio of HM in soil amended with CP+RS decreased as the HM content increased, suggesting that combined fertilization enhances SOM quality more effectively than applying amendments alone. This finding underscored the necessity of balanced organic C and N fertilization to enhance SOM quality and stability in agricultural soils. Lastly, a long-term experiment demonstrated that after 50 years of continuous cultivation of rice crop, SOC and TN increased by about 10% in CP-treated soil but decreased by about 8.5% in soils under NPK and Nil treatments. Long-term soil amendments with CP and NPK + CP increased C and N in the HA and HM fractions, whereas continuous inorganic fertilization or the absence of fertilization (Nil) led to SOM degradation. Collectively, these studies highlight the critical influence of N, land use, and fertilization strategies on SOM quality and stability, providing insights for sustainable soil management and improved soil health.

Keywords: C/N ratio, Degree of transformation, Humic substances, Humification index, Persulfate digestion method, Soil quality

CHAPTER 1

Introduction and literature review

1.1 Introduction

Soil plays a crucial role in food security, climate change mitigation, and other essential ecosystem services (Trigalet et al., 2017). Soil quality is the capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation (Karlen et al., 1997). Soil quality can be assessed by using a minimum data set comprising soil attributes such as texture, organic matter, pH, bulk density, and rooting depth (Gregorich et al., 1994). In other words, soil quality is generally assessed by evaluating soil property indicators, including physical, biological, chemical, and soil organic matter (SOM) (Doran et al., 1996; Guimaraes et al., 2013). Among these indicators, SOM is particularly significant because it is a critical component of terrestrial ecosystems, playing a pivotal role in maintaining soil quality, fertility, and ecosystem sustainability (Seybold et al., 1999). The quality of SOM is assessed through a comprehensive analysis of its attributes, including total organic carbon (SOC) and total nitrogen (TN), light fraction, particulate organic matter (POM), mineralizable C and N, microbial biomass, soil carbohydrates, enzymes (Gregorich et al., 1994; Trigalet et al., 2017), the C/N ratio, and humification indices (See et al., 2005; Ukalska-Jaruga et al., 2019). These attributes provide insights into essential soil processes, such as nutrient storage, biological activity, and structural stability, enabling the development of minimum data sets for SOM quality evaluation (Batjes, 1996; Gregorich et al., 1994; Marty et al., 2017).

SOC and TN, commonly measured and used as indicators of the quantity and quality of SOM, are often analyzed simultaneously from a single sample using an elemental analyzer (EA) by micro-dumas combustion on a CHN analyzer (Gibson et al., 2015; Gregorich et al., 1994). This method is fast and accurate for measuring both SOC and TN, but the instrument is expensive to purchase and maintain. SOC and TN are sometimes analyzed by two independent methods. SOC can be measured independently by dichromate oxidation procedures, but the digest process is temperature-sensitive, time-intensive, and generates concentrated acid wastes containing heavy metals (Angelova et al., 2019; Doyle and Schimel, 1998). TN can also be analyzed independently by the Kjeldahl method, but it is well known that this method does not convert nitrates, nitrites, nitroso, azo, and diazo compounds into ammonium ions (Craft et al., 1991). Persulfate ($S_2O_8^-$), when activated by heat, ultraviolet light, metal ions, or alkaline methods, produces sulfate free radicals ($SO4^{-\bullet}$) and hydroxyl radical ($OH^{\bullet}$) that can effectively oxidize organic matter to CO_2 and various by-products (Huang et al., 2005; Zhou et al., 2018). Alkaline persulfate methods have been successfully used for simultaneously determining dissolved organic C (DOC) and dissolved organic N (DON) in soil extracts (Doyle et al., 2004); particulate organic C (POC), particulate organic N (PON), and particulate organic P (POP) in seawater (Pujo-Pay and Raimbault, 1994); and total organic C (TOC), total organic N (TON), and total organic phosphorus (TOP) in zooplankton samples (Gibson et al., 2015). Although persulfate-based advanced oxidation methods are widely used, no method has been developed for analyzing both SOC and TN in air-dried soil samples.

The dynamics of SOM are profoundly influenced by land use, soil type, and management practices. Different land uses, such as paddy fields, uplands, orchards,

volcanic ash soils, and reclaimed coastal soils, significantly alter SOM content and composition. For example, volcanic ash soils (Andosols), which are developed from pyroclastic rocks, exhibit high SOC levels due to the protective effects of aluminum- and iron-humus complexes, as well as high allophane and imogolite mineral content (Pizarro et al., 2003; Takahashi et al., 2020). In contrast, common land uses (paddy, orchard, upland, and greenhouse) may have intermediate SOC content, as they are typically formed from acidic rocks (Yang et al., 2023). Conversely, reclaimed soils often exhibit the lowest SOC content because they are constructed in coastal areas with high salinity, elevated groundwater levels, and poor soil aggregation, all of which limit microbial activity (Park et al., 2022). Although SOC content differs among land uses, a comprehensive understanding of how humic fractions contribute to SOC accumulation is essential for elucidating SOM stabilization processes. This knowledge is critical for assessing overall SOM quality and its potential role in carbon sequestration.

In addition to land use, soil amendments such as organic and inorganic fertilizers significantly impact SOM quality. Organic amendments, particularly those rich in labile carbon compounds, often result in elevated CO_2 emissions during the initial stages of incubation. Yan et al. (2019) investigated the decomposition characteristics of rice straw and found that the loss of cellulose and hemicellulose as CO_2 was significantly greater than that of lignin, with a rapid release occurring in the first and second years after straw return. In addition to CO_2 emission, types of amendment applied significantly influence the microbial populations, including bacteria, fungi, and actinomycetes, which are key drivers of organic matter decomposition and nutrient cycling. Singh and Dhar (2011) demonstrated that organic farming in the rice-wheat-greengram cropping system, through the application of bio-inoculants, vermicompost,

blue-green algae, farmyard manure, and Azolla, whether applied alone or in combination, significantly increased bacterial, fungal, and actinomycetes populations over the years. The enhancement of microbial population growth and activity under organic or combined organic systems were also reported by many previous studies (Das and Dkhar, 2011, Meena et al., 2013, and Selvi et al., 2004). While several studies have explored the impact of organic amendments or combined fertilization on microbial populations in fields under crop growth, there is limited research comparing the effects of diverse amendments in plant-free conditions. This comparison is important for evaluating which amendments most effectively enhance microbial populations without the interaction or support from plant root exudates.

Given that SOC is formed through complex biological, chemical, and physical processes and varies significantly across land-use types, determining its composition may be more important than measuring its total concentration for assessing long-term or long-term soil quality (Asensio et al., 2014; Guimaraes et al.,2013; Ukalska-Jaruga et al., 2019). SOM can be chemically classified into various operationally defined forms: debris, non-humic and humic substances (Brady and Weil, 2010; Stevenson, 1994). Debris, known as free or non-complexed SOM, consists of identifiable organic matter, including plant roots, root hairs, undecomposed plant residue, and partially decomposed products (ranging in size from < 3 mm to 0.3 mm) occluded in macroaggregates, as well as tiny organic particles (e.g., plant roots or particulate organic matter) with fragment sizes around 0.03 mm, coated in microaggregates (Brady and Weil, 2010; Golchin et al., 1994a, 1994b). Non-humic substances (NHS) are also the chemically well-defined labile compounds that consist of low molecular weight aliphatic and aromatic acids, carbohydrates, amino acids, and their polymeric derivatives, such as polypeptides, proteins, polysaccharides, and waxes (Gregorich et

al., 1994; Guimaraes et al., 2013). These compounds have a relatively rapid turnover in soil and are used readily as substrates by soil microorganisms (Tan, 2014). Humic substances generally are dark-colored amorphous substances found in soil, resulting from the decomposition of plant and animal residues (Stevenson, 1994). It comprises about 60 to 80% of the SOC (Brady and Weil, 2010). Generally, humic substances were chemically fractionated into fulvic acid (FA), humic acid (HA), and humin (HM) (Stevenson, 1994). FA is soluble in both acid and alkali solution, while HA is soluble in alkali and precipitate in acidic solution, and HM is insoluble in any solution (Khalafalla et al., 2019). Since these fractions contribute greatly to SOC, the variation in these fractions can alter the quality and stability of SOM. Therefore, a deeper understanding of the changes in the chemical composition of SOC is essential for revealing the long-term dynamics of humus formation, SOM stabilization, and their implications for soil fertility under different fertilization practices.

Collectively, this dissertation integrates novel methodological advancements with empirical studies to address key challenges in SOM research. By bridging the gaps between SOM quantity and quality assessments, it provides actionable insights into soil management practices that enhance SOM stability, improve soil quality, and support sustainable agriculture. Therefore, the objectives of the study were to: (i) develop and optimize an alkaline persulfate digestion method for simultaneous determination of SOC and TN in air-dried soils, ensuring precision, cost-effectiveness, and applicability; (ii) investigate the composition and stabilization of SOM fractions, including non-humic substances, debris, FA, HA, and HM across diverse land uses, including orchard, paddy, upland, greenhouse, volcanic ash, and reclaimed soils; (iii) evaluate the effects of organic, inorganic, and combined amendments on SOM quality, CO_2 emissions, and microbial populations, focusing on changes in humification

indices and C/N ratios in SOM fractions; and (iv) examine the long-term impacts of fertilization practices on SOC, TN, and humus stability in paddy soils, providing insights into sustainable soil management strategies.

1.2 Review of literature

1.2.1 Persulfate oxidation method

Persulfate-based advanced oxidation has emerged as a rapidly expanding approach not only for remediating groundwater and soil contaminated with organic pollutants, but also for determining C and N in microbial biomass extract, soil extract, as well asl zooplankton samples (Cabrera and Beare, 1993; Doyle et al., 2004; Gibson et al., 2015; Zhou et al., 2018). Peroxydisulfate ($S_2O_8^{2-}$), commonly known as persulfate, is the most recent and widely utilized oxidant in this field, demonstrating consistently effective outcomes. Since the discovery of persulfuric acid ($H_2S_2O_8$) by French chemist Marcelin Berthelot in 1878 (Kolthoff and Miller, 1951), this technology has shown significant promise as an efficient and cost-effective solution for environmental remediation and soil analysis. One key advantage of persulfate-based advanced oxidation is its eco-friendliness compared to other methods. Unlike traditional methods, it does not generate large quantities of hazardous sludge or simply shift contaminants between phases. Additionally, this technique effectively breaks down most organic compounds into biodegradable or non-toxic end products, further enhancing its environmental appeal.

1.2.2 Activation of persulfate

The persulfate ion ($S_2O_8^{2-}$) is a powerful oxidizing agent with a standard reduction potential of 2.1 V, capable of producing sulfate radicals ($SO_4^{\bullet-}$) under specific reaction

conditions, which exhibit an even higher reduction potential of 2.6 V (Tsitonaki et al., 2010). Due to its versatile reactivity, persulfate is extensively utilized across various industrial applications, including polymerization, metal surface treatment, and the synthesis of organic chemicals (Watts and Teel, 2006). Persulfate is typically available as sodium, potassium, or ammonium salts, with sodium persulfate being the most commonly used in in-situ chemical oxidation (ISCO) processes (Tsitonaki et al., 2010). This preference arises from its superior water solubility and environmentally benign byproducts. Activation methods such as heat, ultraviolet radiation, alkaline conditions, hydrogen peroxide, or transition metals are often employed to enhance the reactivity of persulfate by generating sulfate radicals ($SO_4^{\bullet-}$) and other reactive intermediates, collectively referred to as "*activated persulfate"*. The term "*persulfate"* encompasses both the persulfate ion, and the reactive intermediates derived from it, primarily sulfate radicals ($SO_4^{\bullet-}$) and hydroxyl radicals ($HO^{\bullet}$). *Activated persulfate* refers to the reactive intermediates produced through the use of an activation method, whereas "*non-activated persulfate"* denotes the direct use of the persulfate ion without any activation aid.

Persulfate generates sulfate free radicals when it is activated (eq 1). Under acidic conditions, formation of sulfate free radicals can be catalyzed by protons as shown in equation 2 and 3 (eq 2, 3). Once the sulfate free radicals are generated, it can be also transformed into sulfate ions and produce hydroxyl radicals ($OH^{\bullet}$) (eq 4). Under alkaline conditions, the hydroxyl radical is likely the dominant radical available for organic oxidation using alkaline activated persulfate (eq 5). Thus, the equation 4 and 5 show the potential coexistence of $SO_4^{\bullet-}$ and $OH^{\bullet}$; and the coexistence has been evidenced by electron spin resonance. When the pH is above 7, the conversion of $SO_4^{\bullet-}$ to $OH^{\bullet}$ (eq 5) becomes increasingly responsible for oxidation of organic compounds,

and one may predominate over the other depending on pH (Lee et al., 2012; Tsitonaki et al., 2010).

$S_2O_8^{2-}$ + heat or light → $2SO_4^{\bullet-}$ (1)

$S_2O_8^{2-} + H^+ \rightarrow HS_2O_8^-$ (2)

$HS_2O_8^- \rightarrow SO_4^{\bullet-} + SO_4^{2-} + H^+$ (3)

$SO_4^{\bullet-} + H_2O \rightarrow SO_4^{2-} + OH^{\bullet} + H^+$ (4)

$SO_4^{\bullet-} + OH^- \rightarrow SO_4^{2-} + OH^{\bullet}$ (5)

1.2.3 Effect of temperature

Persulfate oxidation depends on peroxydisulfate (S_2O_8) decomposition into the persulfate free radical, which is the active oxidizing agent. This decomposition follows an Arrhenius relationship between 50 and 130ºC (Kolthoff and Miller, 1951; Goulden and Anthony, 1978); persulfate has a half-life of about 30 sat 130ºC and 4 h at 75ºC. The decomposition is the rate-limiting step, and further oxidation steps are rapid relative to free radical initiation (Peyton, 1993). Under some conditions, higher temperature may decrease C recovery (Goulden and Anthony, 1978). Thus, high temperature may increase reaction rate but not necessarily completeness. Doyle et al. (2004) used alkaline persulfate to analyze DOC and DON in soil extracts and found that the results were consistent at temperatures ranging from as low as 80ºC to as high as 125ºC. The author concluded that oxidation efficiency was not temperature sensitive as long as adequate time was allowed for persulfate radicals to be generated. Goulden and Anthony, (1978) and Doyle et al. (2004) recommended digesting samples at a lower temperature to reduce leakage. For the determination of SOC and TN in air-dried soil samples, it is necessary to confirm the optimum digestion temperature at a given digestion duration.

1.2.4 Effect of pH

The pH of the solution plays a significant role in affecting oxidation reaction of organic compounds. Persulfate exhibits increased reactivity at pH levels above 10, a process known as alkaline activation. This method involves combining persulfate with sodium hydroxide (NaOH) or potassium hydroxide (KOH) to achieve a solution with a pH around 11.0 (Zhou et al., 2018). According to Solorzano and Sharp (1980), the persulfate oxidation must be carried out at a pH of 12.6 to 13.2. In the oxidizing reagent, NaOH serves to neutralize the H^+ ions generated during the oxidation process, thereby facilitating the reaction. However, excessive concentrations of NaOH can increase the risk of ammonia (NH_3) volatilization. Cabrera and Beare (1993), found that increasing the concentration of NaOH decreased N recoveries. It is necessary to review the persulfate to NaOH ratio to optimize the oxidizing reagent for a range of sample concentrations.

1.2.5 Persulfate to NaOH ratio

It was noticed that the compositions of the oxidizing reagent used by different researchers, i.e., the concentrations of potassium persulfate and NaOH, varied. For example, Cabrera and Beare (1993) used 5% $K_2S_2O_8$ dissolved in 0.375 M NaOH. Yu et al. (1994) used 1% $K_2S_2O_8$ in 0.075 M NaOH. Williams et al. (1995) adopted 1.34% $K_2S_2O_8$ in 0.3 M NaOH. When the persulfate oxidation method is employed to measure C and N in samples, an acidic final pH is required to convert all forms of N to NO_3^- and carbonate into CO_2. Therefore, acidification of the solution after the oxidation process is necessary for soil C and N analysis. Zhou et al. (2003) proposed using appropriate concentrations of $K_2S_2O_8$ to NaOH ratio (1:1.35) to ensure effective oxidation while maintaining an acidic pH after oxidation. This ratio allows for about 30 min of alkaline digestion followed by acidic digestion. By the completion of this

reaction, 0.22 M HSO_4^- is formed, neutralizing all the NaOH and creating a sulfate-buffered solution with a pH of ~1.9. Gibson et al. (2015) adopted this persulfate-to-NaOH ratio to analyze particulate organic C, N, and P in zooplankton samples, finding that the method demonstrated high recovery for standards and yielded C:N:P ratio values consistent with those obtained through other analytical approaches and reported in the literature. Striking a balance in the NaOH concentration is crucial for optimizing the recovery of not only C and N but also P in solution. However, when analyzing SOC and TN in air-dried soil samples, this ratio needs to be verified to ensure precise measurements.

1.2.6 Basic pH indicators

Acidic persulfate was first developed by Skeggs et al. (1960) to measure C in human blood plasma using a flow injection manifold (Fig. 1.1). The principle of the flow injection manifold introduced samples into the flow system through sample loop using a pump and mixed with an acidic diluent in a continuous stream. The acidified sample released CO_2, which became mixed with the air in the flow stream. A portion of the gaseous mixture, containing the liberated CO_2, was separated and introduced into a stream of a basic pH indicator containing Phenol red, phenolphthalein. The CO_2 reacted with the indicator solution, causing a reduction in its optical density proportional to the CO_2 concentration in the sample. A continuous record of the percentage transmittance was then obtained using a colorimeter, with the change in light intensity corresponding to the amount of CO_2 present in the sample. It was noted that acidic persulfate has been used to measure low molecular weight compounds, but it is not suitable for analyzing carbon in humic substances. Additionally, the system allows for the analysis of C but not N.

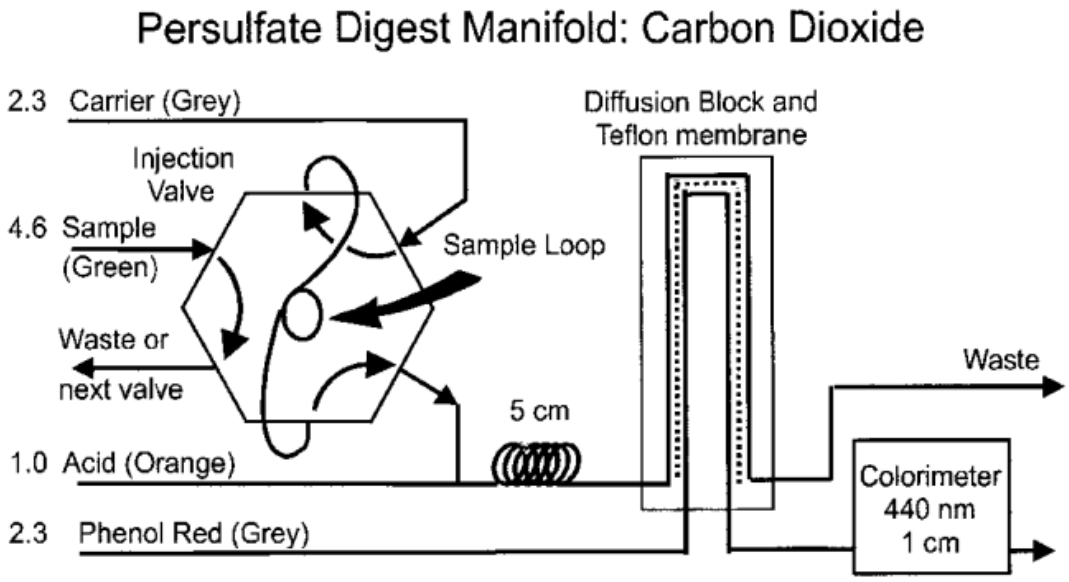

Fig. 1.1 Flow injection manifold for detecting CO_2 in persulfate digest, adopted from Doyle et al. (2004).

Alkaline persulfate was developed for the simultaneous determination of C and N, as well as P in microbial biomass, soil extract, particulate organic matters, freshwater, and seawater quality. However, previous studies typically oxidized samples in advance with persulfate, then used automated systems for flow injection analysis of CO_2 and NO_3^- (Doyle et al., 2004; Gibson et al., 2015; Pujo-Pay and Raimbault, 1994). This analysis utilizes expensive instruments and supplementary techniques for maintenance purposes.

An alternative approach is to use the dilute bicarbonate solution containing a pH indicator (a basic pH indicator) to absorb CO_2 diffused during soil oxidation inside a sealed vial. As CO_2 is trapped, it slightly acidifies the indicator solution, causing a

color change and creating a peak that can be measured spectrophotometrically. Rowell et al. (1995) compared five pH indicators, including cresol red, phenol red, thymol blue, bromthymol blue, and brilliant yellow to estimate soil biological activity (respiration, mineralization and biomass) using colorimetric method, and found that cresol red showed higher sensitivity than other indicators. Nazar et al., (2010) investigated the effect of CTAB on the absorption spectrum of alizarin yellow R (AYR) under different pH ranges and found that at pH values above 10, AYR exhibited a bathochromic shift from 373 to 493 nm (Fig. 1.2). AYR is a polyfunctional compound with pKa values of 5.0 and 11.0 (Nazar et al., 2010). In strongly acidic conditions (pH 1 or 2), the dye has limited water solubility, as its carboxylic acid and phenol groups remain un-ionized. At pH levels below 4.5, AYR exists in a mono-ionic form, while it exhibits di-anionic behavior at pH values above 10. Given that several pH indicators have been used to quantify CO_2 gas, those with low pH transition ranges may be less effective in bicarbonate solutions (pH > 10), as their sensitivity is often decreased when the pH remains high (Rowell et al., 1995). Among pH indicators, AYR has a higher pH transition range (10.0-12.0) (Sabnis, 2007) and exhibits stable absorbance bands above pH 10.0, making it a promising alternative for our research.

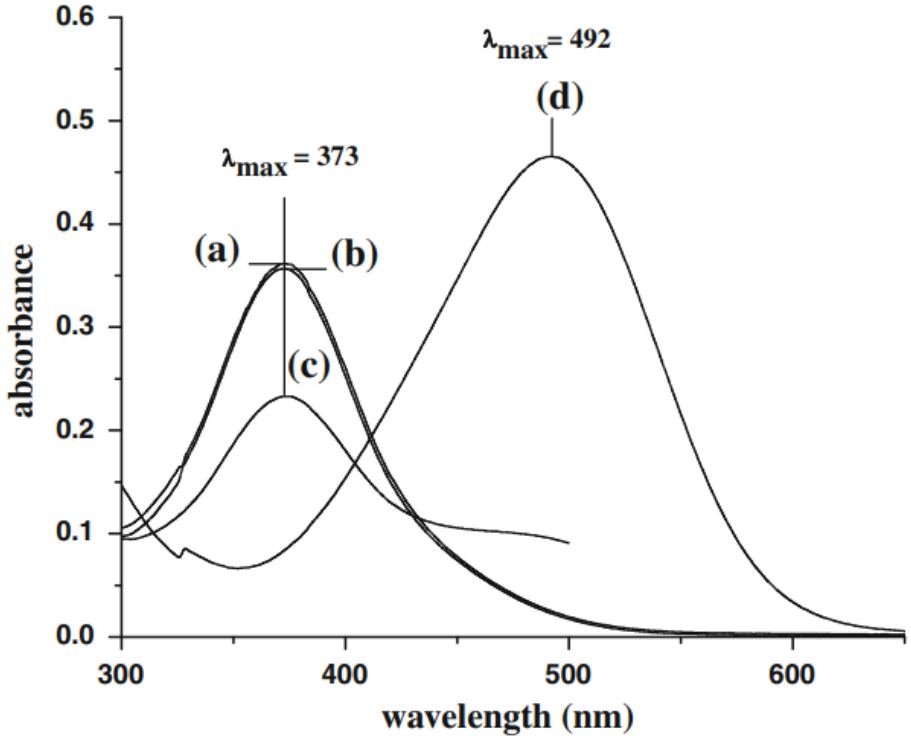

Fig. 1.2 Absorption spectra of AYR: *a* pH 4.0; *b* pH 6.6; *c* pH 10.0; and *d* pH 12.0, adopted from Nazar et al. (2010)

1.2.7 Fractions of SOM

SOM comprises a variety of humified and biologically active compounds, including readily decomposable materials, plant litter and roots, and dead and living organisms. According to Brady and Weil (2010) and Stevenson (1994), SOM can be chemically classified into several operationally defined forms, including debris, non-humic substances (NHS), and humic substances (Fig. 1.3).

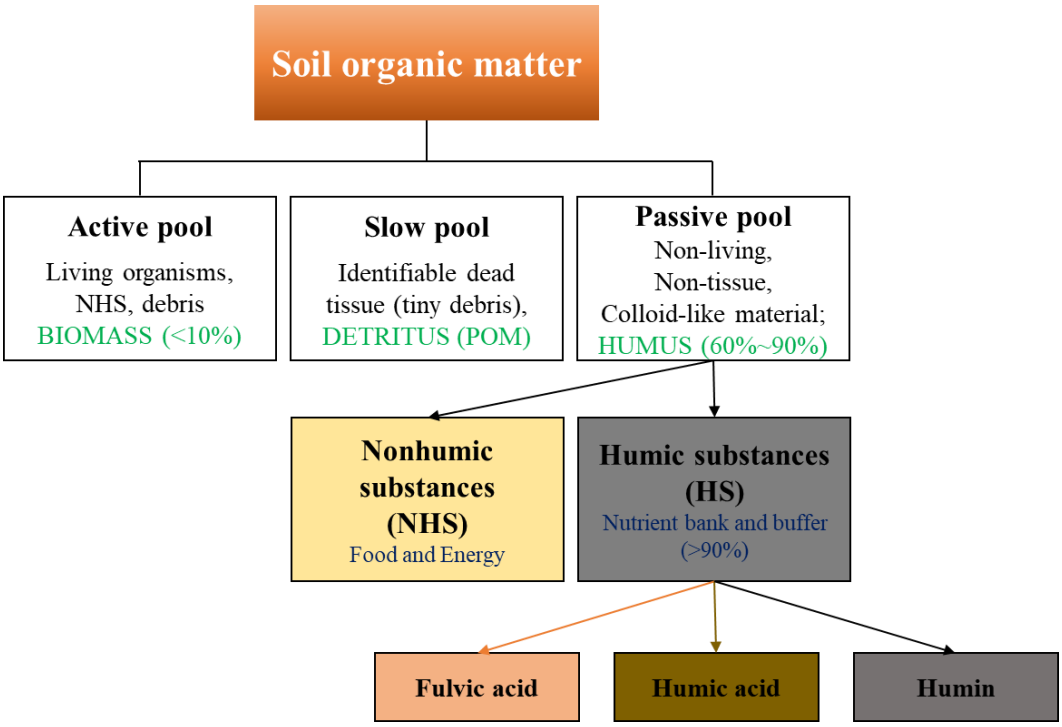

Fig. 1.3 Classification of soil organic matter components separable by chemical and physical criteria, adopted from Bardy and Weil (2010) and Stevenson (1994).

Debris, referred to as free or non-complexed SOM, consists of identifiable organic matter, including plant roots, root hairs, undecomposed plant residue, and partially decomposed products. These range in size from <3 mm to 0.3 mm and are often occluded in macroaggregates. Tiny organic particles, such as plant roots or particulate organic matter (POM) with fragment sizes around 0.03 mm, are coated in microaggregates (Brady and Weil, 2010; Golchin et al., 1994a, 1994b). Debris includes all fragments dispersible in high-density solutions and recoverable through filtration. Golchin et al. (1994a) applied a simple densimetric method to extract residual debris from soils and reported that partially decomposed root and plant

fragments comprised about 0.6–4.34% of dry weight and accounted for 7–31% of total C and 6–22% of total N. They also observed that the contribution of C and N decreased with decreasing fragment size.

Non-humic substances (NHS), on the other hand, are chemically well-defined, labile compounds, including low molecular weight aliphatic and aromatic acids, carbohydrates, amino acids, and their polymeric derivatives, such as polypeptides, proteins, polysaccharides, and waxes (Gregorich et al., 1994; Guimaraes et al., 2013). These compounds have a relatively rapid turnover in soil and serve as readily available substrates for soil microorganisms (Tan, 2014). NHS accounts for approximately 20–30% of soil humus (Brady and Weil, 2010). Their distribution decreases as SOM undergoes further decomposition and stabilization into more recalcitrant humic substances (Tan, 2014). Khalafalla et al. (2019) observed that NHS levels declined to below 5% compared to humic substances after 60 days of soil incubation, regardless of fertilization. Similarly, Raiesi et al. (2021) reported that non-humified fractions represented about 3–5% of total SOC in cultivated soils.

Humic substances generally are dark-colored amorphous substances found in soil, resulting from the decomposition of plant and animal residues (Stevenson, 1994). Humic substances are composed of huge molecules with variable structures characterized by aromatic rings, with molecular weight varying from 2000 to 300,000 g/mol (Brady and Weil, 2010). Because of their complexity, they are the organic materials most resistant to microbial attack and are very important in C sequestration and C cycling (Yang et al., 2004a and 2004b). Humic substances can improve soil buffering capacity, increase moisture retention, and supply plants with available micronutrients (Guimaraes et al., 2013). Humic substances comprise about 60 to 80%

of the SOM (Brady and Weil, 2010). Humic substances were chemically fractionated into fulvic acid (FA), humic acid (HA), and humin (HM) (Fig. 1.3 and 4) (Stevenson, 1994).

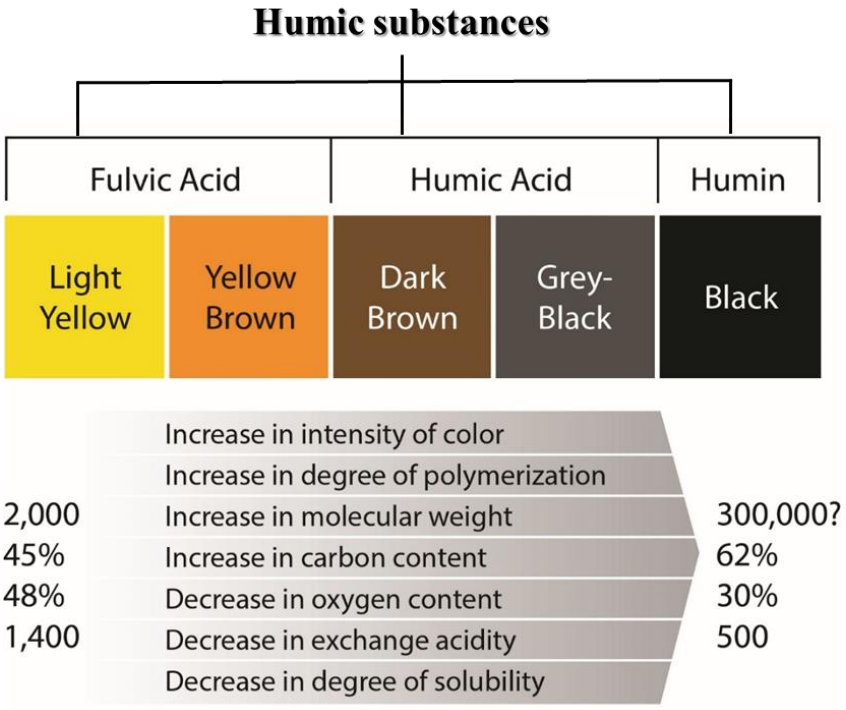

Fig. 1.4 Physical and chemical properties of humic substances, adopted from Stevenson (1994).

FA is soluble in both acid and alkali solution, while HA is soluble in alkali and precipitate in acidic solution, and HM is insoluble in any solution (Khalafalla et al., 2019). These humic fractions differ in quantity and quality depending largely on soil type, land-use types, tillage practices, cultivation duration, parent materials, climate, altitude, vegetation type, and soil management (Khalafalla et al., 2019; Raiesi et al., 2021). Guimaraes et al. (2013) compared humic fractions in soil cultivated with

different crops (conventional coconut, integrated coconut, citrus, and native forest) and found that native forest has significantly higher FA, but lower HA and HM than those of coconut and citrus crops. They also reported the distributions of FA, HA, and HM to the total SOC were about 19-32%, 15-25%, and 49-65%, respectively. Similarly, Ukalska-Jaruga et al. (2019) compared the humic fractions between grasslands and arable lands and found that the distribution of FA, HA, and HM to the total SOC was approximately 16.6-22%, 36.3-42%, and 35.6-42.6%, respectively. They also reported that arable lands had higher FA and HA but lower HM than grasslands.

1.2.8 Assessment of SOM quality

Several indexes can be used to assess the quality of humus fractions. The ratio between concentrations of humic and fulvic acids (HA/FA) indicates the potential mobility of C in the soil system. The proportions in relatively active and resistant HS fractions, expressed as the (HA + FA)/HM ratio, show the degree of HS transformation to the stable C form and SOC illuviation processes. Higher FA-C than HA-C indicates a higher mobility of organic matter with the labile HS fraction predominating, while higher FA-C + HA-C compared to HM-C indicates a lower intensity of organic matter humification associated with less transformation into stable SOM forms (Ukalska-Jaruga et al., 2019; You et al., 2014). Given that most long-term studies have examined the effects of N fertilizer rates on soil C and N contents and pools (Tong et al., 2009), the C/N ratio is commonly used as a parameter to estimate SOM quality. However, very few studies have evaluated the C/N ratio in FA, HA, and HM specifically in terms of SOM quality. Generally, a decrease in the C/N ratio in FA, HA, and HM is indicative of well-developed humic matter (Tan, 2014).

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CHAPTER 2

Rapid persulfate oxidation and simultaneous colorimetric analysis of soil organic carbon and total nitrogen in ground air-dried soil samples

2.1 Abstract

Alkaline persulfate digestion methods have been widely adopted for determining C and N simultaneously in soil extracts as well as in particulate matters. However, to our knowledge, no published study has attempted to use this method for the simultaneous determination of SOC and TN in air-dried soils (passed through a 150μm screen). In addition, no studies utilized alizarin yellow R (AYR) to quantify the CO_2 released during soil oxidation. We developed a new alkaline persulfate digestion method that allows the CO_2 released from soil oxidation to trap in a basic pH indicator solution inside a sealed serum vial, where the digestion mixture is analyzed for TN. This method used $Na_2S_2O_8$ and NaOH as oxidizing reagent and AYR with K_2CO_3 as a basic pH indicator to quantify C oxidized. In a basic solution (pH12.0-10.0), AYR experienced a bathochromic shift from red (490 nm, decrease) to yellow (375 nm, increase) as CO_2 absorption increased, but the changes in absorbance at these two wavelengths are not linear with increasing CO_2 absorption. However, using the Henderson-Hasselbalch equation, we established new linear calibration curve (R^2 > 0.99) based on the absorbance ratio at 375 nm to 490 nm (A375/A490), enabling precise determination of SOC. In addition, the salicylic acid nitration method also exhibited good linear calibration curve (R^2 > 0.99) for TN analysis. These results indicate the reliability of the methods, which can be used to determine SOC and TN analyses in soil digests. For safe and precise analysis, all digestion conditions were optimized. In a total digestion volume of 6 mL, 5 mL of 0.4 M $Na_2S_2O_8$ in 0.6 M NaOH (reaction concentration, 1:1.5 ratio), 1 mL of 1.0 M K_2CO_3 + 15 mL of 0.1

(w/v%) AYR, and 60-minute digestion duration at 110ºC, were found to be the optimal for a 0.1 g ground soil digestion system. These optimal conditions provide a digestion that is initially alkaline (pH > 12.0) but becomes acidic after about 30 min. Under these conditions, our method can measure SOC and TN up to 8% and 0.8%. Our confidence is based on good calibration curves, high precision, low detection limit, and strong concordance between the results from our method and those from elemental analyzers. To determine SOC and TN in air-dried soil samples simultaneously, our proposed method is simpler, faster, and more cost-effective than conventional methods.

Keywords: Air-dried soils, Alizarin Yellow R, Basic pH indicator, Oxidizing reagent, Salicylic acid nitration, Soil digestion.

2.2 Introduction

Soil organic matter (SOM), which is the largest pool of terrestrial C and N (Batjes, 1996; Marty et al., 2017), plays a central role in the soil's functional viability (Schoenholtz et al., 2000). Soil organic carbon (SOC) and total nitrogen (TN) are commonly measured and used as indicators of the quantity and quality of SOM (Gregorich et al., 1994). SOC and TN are often analyzed simultaneously from a single sample using an elemental analyzer (EA) by micro-dumas combustion on a CHN analyzer (Gibson et al., 2015). This method is fast and accurate for measuring both SOC and TN, but the instrument is expensive to purchase and maintain. SOC and TN are sometimes analyzed by two independent methods. SOC can be measured independently by dichromate oxidation procedures, but the digest process is temperature-sensitive, time-intensive, and generates concentrated acid wastes containing heavy metals (Angelova et al., 2019; Doyle and Schimel, 1998). TN can also be analyzed independently by the Kjeldahl method, but it is well known that this method does not convert nitrates, nitrites, nitroso, azo, and diazo compounds into ammonium ions (Craft et al., 1991). Regardless of their disadvantages, using two separate methods for SOC and TN requires lager sample amounts and additional analytical effort and costs.

One of the most common approaches for measuring C and N involves the decomposition of organic matter to CO_2 and NO_3^- ions. Persulfate ($S_2O_8^-$), when activated by heat, ultraviolet, metal ions, or alkaline methods (Johnson et al., 2008), generates sulfate free radicals ($SO_4^{-\bullet}$) and hydroxyl radicals ($OH^\bullet$) that can effectively oxidize organic compounds to CO_2 and various by-products (e.g., NO_3^-, PO_4^{-3}, SO_4^{2-}) (Huang et al., 2005; Zhou et al., 2018). Maher et al., (2002) demonstrated that persulfate activated by microwave, autoclave, or hot water bath is suitable for

measuring total N and P in lake and river waters containing < 150 mg L−1 of suspended sediments. Doyle et al. (2004) have also found that autoclave heat-activated persulfate, followed by automated flow injection and colorimetric procedure is effective for determining DOC and DON in soil extracts. Gibson et al. (2015) showed that the autoclave heat-activated persulfate method is suitable for analyzing organic C, N, and P simultaneously in plant samples. While persulfate-based advanced oxidation methods have been used widely, no method is developed for the simultaneous analysis of SOC and TN in air-dried soil samples without the need for expensive analyzers.

Persulfate has been reported to exhibit lower activation energies in acidic environments (100-116 kJ mol^{-1}) compared to basic ones (134-139 kJ mol^{-1}) (Zhou et al. 2018). In addition, hydroxyl radicals, which are critical oxidizing agents, are more predominant in alkaline persulfate environment than in acidic ones (Tsitonaki et al., 2010). Aiken et al. (2002) found that UV-activated acidic persulfate (e.g., Sievers 800) produced inaccurate measurements of DC in aqueous samples containing high-molecular-weight compounds, such as humic substances. Alkaline persulfate has proven effective for measuring POC, PON, and POP in seawater (Pujo-Pay and Raimbault, 1994), DOC and DON in soil extracts (Doyle et al., 2004), and TOC, TON, and TOP in plant samples (Gibson et al., 2015). This procedure allows for about 30 min of alkaline digestion to ensure complete oxidation of N, followed by an acidic digestion (pH < 2.0) to facilitate CO_2 diffusion (Cabrera and Beare, 1993; Zhou et al., 2003). At this low pH, carbonate is released as CO_2 in equilibrium with the partial pressure inside a sealed vial, while various N forms are oxidized to NO_3^- in the digestion mixture. It was noticed that previous studies typically oxidized samples in advance with persulfate, then used automated systems for flow injection analysis of CO_2 and to NO_3^- (Doyle et al., 2004; Gibson et al., 2015; Pujo-Pay and Raimbault,

1994). This analysis utilizes expensive instruments and supplementary techniques for maintenance purposes.

An alternative approach is to use the dilute bicarbonate solution containing a pH indicator (basic pH indicator) to absorb CO_2 diffused during soil oxidation inside a sealed vial. As CO_2 is trapped, it slightly acidifies the indicator solution, causing a color change and creating a peak that can be measured spectrophotometrically. Rowell et al. (1995) compared five pH indicators, including cresol red, phenol red, thymol blue, bromthymol blue, and brilliant yellow to estimate soil biological activity (respiration, mineralization and biomass) using colorimetric method, and found that cresol red showed higher sensitivity than other indicators. Similarly, Doyle et al., (2004) digested soil extracts in advance and introduced Phenol red indicator with a flow injection manifold to detect the diffused CO_2. Nazar et al., (2010) investigated the effect of CTAB on the absorption spectrum of alizarin yellow R (AYR) under different pH ranges and found that at pH values above 10, AYR exhibited a bathochromic shift from 373 to 493 nm. While several pH indicators have been used to quantify CO2 gas, those with low pH transition ranges may be less effective in bicarbonate solutions (pH > 10), as their sensitivity is often decreased when the pH remains high (Rowell et al., 1995). Among pH indicators, AYR has a higher pH transition range (10.0-12.0) (Sabnis, 2007) and exhibits stable absorbance bands above pH 10.0 (Nazar et al., 2010), making it a promising alternative for our research.

The objective of this study was to develop a new alkaline persulfate digestion method for the simultaneous determination of SOC and TN in air-dried soil samples. In this method, the CO_2 released during soil oxidation is trapped in a basic pH indicator solution inside a sealed vial. The trapped CO_2 was then measured

spectrophotometrically for SOC, while an aliquot of the digestion mixture was analyzed for TN by the Salicylic acid nitration method (Cataldo et al., 1975). To assess the reliability and validity of the method, we optimized oxidizing reagent and a basic pH indicator at a given digestion time and temperature and compared SOC and TN values obtained from various soils with those measured using an elemental analyzer.

2.3 Materials and Methods

2.3.1 Oxidizing reagent

Persulfate ($S_2O_8^{-}$), when activated by heat, ultraviolet, metal ions, or alkaline methods, generates sulfate free radicals ($SO_4^{-\bullet}$) and hydroxyl radicals ($OH^{\bullet}$) that can effectively oxidize organic compounds to CO_2 and various by-products (e.g., NO_3^{-}, PO_4^{-3}, SO_4^{2-}) (See in Fig. 2.1). We used an alkaline persulfate oxidizing reagent similar to those reported by Zhou et al. (2003), Suzumura et al. (2008), and Gibson et al. (2015). The oxidizing reagent concentration used in their studies was 0.11 M $K_2S_2O_8$ with a persulfate-to-NaOH of 1:1.36 ratio. This oxidizing reagent concentration allows for about 30 minutes of alkaline digestion (pH 12-13.0), followed by around 30 minutes of acidic digestion (pH > 2.0). However, to ensure complete oxidation of both C and N in a 0.1g ground soil digestion system, four soil samples with varying SOC contents were digested using 0.2, 0.3, 0.4, and 0.5 M $Na_2S_2O_8$ with a persulfate-to-NaOH ratio of 1:1.5. The digestion procedures and measurements of SOC and TN are described in sections *2.3.5* and *2.3.6*. Once the optimum persulfate concentation was determined, different persulfate-to-NaOH ratios, including 1:1, 1:1.25, 1:1.5, 1:1.75, and 1:2, were evaluated using the same digestion conditions as described above. The initial pH of the digestion mixture was about 13.0 and dropped

to below 2.0 by the end of digestion. The pH of the digestion mixture after digestion was recorded to assess the completeness of oxidaiton.

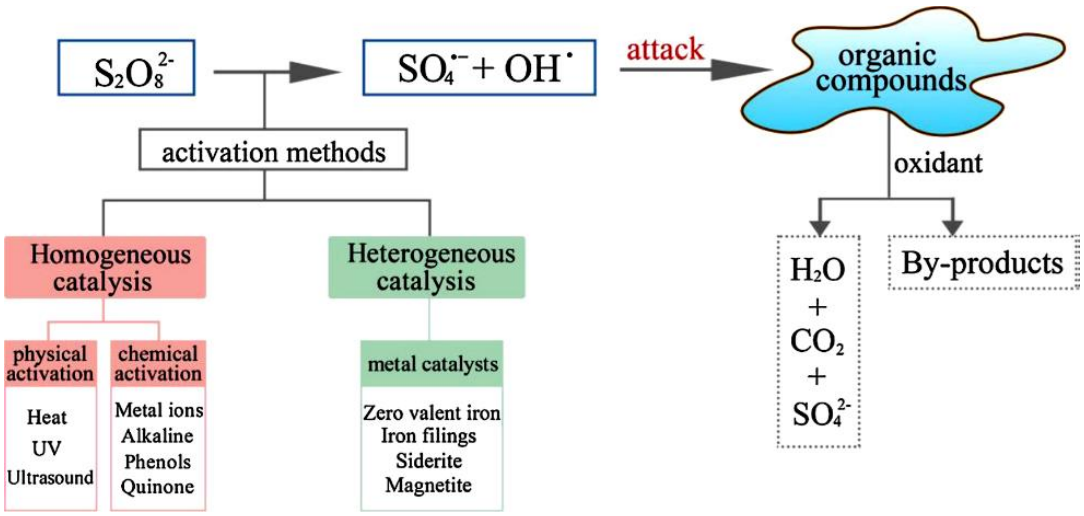


Fig. 2.1 Different methods to activate persulfate for the degradation of organic compounds, adopted from Zhou et al. (2018).

2.3.2 Basic pH indicator solution

When CO_2 released during soil oxidation by heat-activated persulfate is absorbed in a K_2CO_3 solution containing AYR, the following reaction takes place:

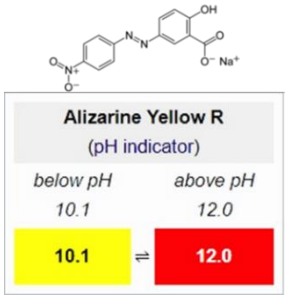
$K_2CO_3 \rightarrow 2K^+ + \mathbf{CO_3^{2-}}$

$CO_3^{2-} + H_2O \rightarrow HCO_3^- + \mathbf{OH^-}$

$CO_2 + OH- \rightarrow \mathbf{HCO_3^-}$

$HCO_3^- + OH^- \rightarrow CO_3^{2-} + H_2O$

$2H_2O \rightarrow OH^- + H_3O^+$



$CO_2 + K_2CO_3 + H_2O \rightarrow 2\mathbf{KHCO_3}$

The CO_2 released and absorbed occurs inside a sealed serum vial. When CO_2 is absorbed into a basic pH indicator solution, the pH of the solution systematically drops from 12.0 to 10.0 and the solution's color changes from red to yellow, as detected by the presense of AYR.

Based on the principle that CO_2 released during soil oxidation by heat-activated persulfate is absorbed in a basic indicator solution with a pH range of 10.0–12.0, K_2CO_3 and alizarin yellow R (AYR) (Jensei, CN 12535-0410) were selected as indicator to trap and quantify the amount of absorbed CO_2. AYR was selected because its pKa (11.0) is above the pKa of K_2CO_3 (10.25). The solution was prepared immediately before use by dissolving 138.90 g (1.0 M) of K_2CO_3 in 500 mL of distilled-deionized water (DDW), then mixing with 15 mL of 0.1% (w/v) AYR, and adjusting the volume to 1 L. The solution was stored in an amber bottle and purged with N_2 to minimize the absorption of atmospheric CO_2. To confirm the thermal stability of AYR, the basic pH indicator solution was autoclaved for 30, 60, 90, and 120 minutes at 110°C, respectively. The digestion procedures are described in sections *2.3.5* and *2.3.6*.

2.3.3 Digestion duration and temperature

Persulfate has a half-life of 4 hours at 75ºC, 45 minutes at 100ºC, and 30 seconds at 130ºC (Doyle at al., 2004; Goulden and Anthony, 1978). Based on the half-life of persulfate, four digestion durations (30, 60, 90, and 120 minutes) and three digestion temperatures (100, 110, and 120ºC) were examined, respectively. To evaluate the optimum digestion duration and temperature, 4 soil samples different in SOC levels

were used. The digestion procedures and measurements of SOC and TN are described in sections *2.3.5* and *2.3.6*.

2.3.4 C and N standards

The calibration standards for C and N were potassium hydrogen phthalate (KHP; Kanto, Tokyo, Japan) and glycine (Junsei, Tokyo, Japan). A stock solution containing 20 mg C L^{-1} (C/N ratio = 10) was prepared by dissolving 35.217 g of KHP and 10.719 g of Glycine in 500 mL of DDW, and diluting the volume to 1 L. The standard curve for C ranged from 1 to 8 mg C. For N, the calibration curve ranged from 0−133 ug N.

2.3.5 Digestion procedures

1 mL of standard solution or 0.1 g of ground soil sample, containing 0-8 mg C and 0-0.8 mg N, was transferred into a 30-mL serium vial with thread (WH.223743, 13 mm OD, ϕ37 x h63 mm, 20 mm seals, Wheaton, USA). Then, 5 mL of oxidizing solution was added, and the mixture was gently swirled. A glass tube (10 mm OD, 33 mm height, Wheaton, USA, assembled with teflon tube on the top 17 mm height) containing 1 mL of the basic pH indicator solution was carefully inserted vertically into the vial. Then, the vial containing both the mixture solution in the outer space and the basic pH indicator solution in the inner tube was immediately sealed with a silicone rubber stopper (13 mm ID x 20 mm OD, white color) and aluminum rings (WH.224191, 20 mm, solid-top type, Wheaton, USA), then crimp tightly using electronic crimper (CSR 6A20C0). The vials were autoclaved at 110°C for 1 h, which is analogous to the conditions used in previous studies (Zhou et al., 2003 and Suzumura et al., 2008). The CO_2 released during soil oxidation was trapped by the

basic pH indicator solution inside the digestion vial, while all forms of N was converted into NO_3^- concentrated in the digestion mixture.

2.3.6 Determination of C and N

After cooling to room temperature for 10 min, the digestion vials were refrigerated at 4°C for 1 h to facilitate CO_2 absorption. Prior to measurement, the caps and stoppers were carefully removed. An aliquot of the basic pH indicator solution was measured for C at 375 nm and 490 nm using a spectrophotometer (U3900, Hitachi, Tokyo, Japan). The color of the basic pH indicator solution gradually changed from red to yellow (pH ~12.0 to 10.0) as CO_2 absorption increased, which in turn caused the absorbance at 490 nm to decrease and the absorbance at 375 nm to increase. The changes in absorbance at 490 nm and 375 nm did not follow a linear relationship with increasing C contents. Based on the Henderson-Hasselbach equation (pH dependence of the conjugate acid and base ratio) (Harris, 2000), the absorbance ratio between 375 nm and 490 nm (A375/A490) was utilized for the determination of SOC content.

An aliquot of the digestion mixture was analyzed for N by the salicylic acid-nitration method (Cataldo et al., 1975). Briefly, 200 μL of the aliquot was mixed with 1 mL of 5% (w/v) salicylic acid dissolved in 10 mL of concentrated H_2SO_4. After standing for 10 min, it was alkalized with 9 mL of 4.5 N NaOH, and the absorbance at 414 nm was measured. Soil particles in the aliquot were removed beforehand by centrifugation at 16000 g for 3 min.

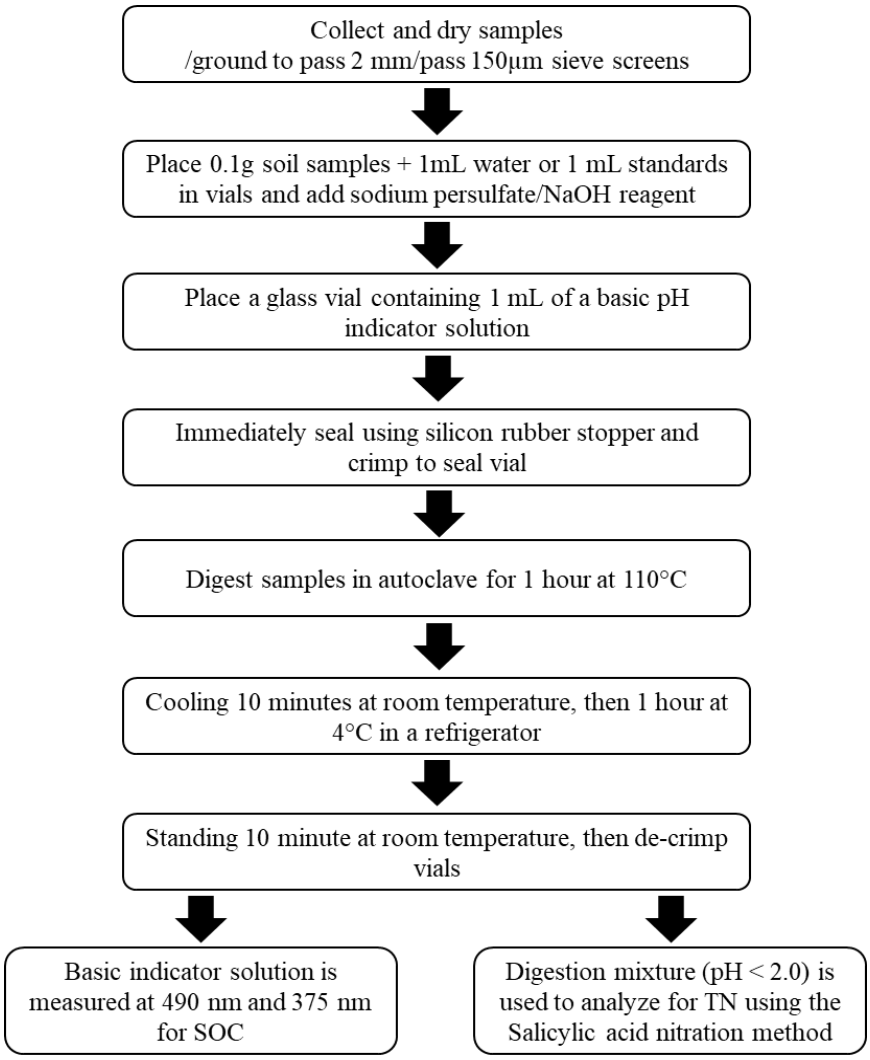


Fig. 2.2 Flow chart of the proposed alkaline persulfate oxidation method.

2.3.7 Comparison with an elemental analyzer

Twenty-two soil samples (0–30 cm depth) with varying SOC contents were collected in 2018 from various land uses across Korea, including 4 samples each from paddy fields, uplands, orchards, and greenhouses, and 6 samples from volcanic ash

soils. The samples were air-dried, sieved through a 2-mm screen, and ground to pass a 150-µm sieve. For SOC and TN comparision, three replicate samples were analyzed by our proposed method, and a single replicate sample was analyzed by the elemental analyzer (Skalar, SNC Analyzer, Primacs series, 1.0.44.x, 0401063A, USA). Tyrosine ($C_9H_{11}NO_3$, C/N 7.72) was used as CN standard reagent because it has a higher C/N ration then EDTA ($C_{10}H_{16}N_2O_8$, C/N ratio 4.287).

2.3.8 Statistical analysis

To assess the proposed alkaline persulfate digestion method, we determined the detection limit (DL) and relative standard deviation (RSD) for C and N standards. The DL was calculated by multiplying the standard deviation (SD) of the blank by 3 and dividing it by the slope of the calibration curve. The RSD was computed as the ratio of the SD to the mean value, expressed as a percentage. For method validation, we conducted a paired t-test (Harris, 2010) to compare the SOC and TN derived from the persulfate digestion method with those obtained from an elemental analyzer (EA). All the statistical analysis was performed using Microsoft Excel.

2.4 Results and Discussion

2.4.1 AYR's spectral response to the amount of C oxidized

The absorption spectrum of AYR shifted systematically from red (490 nm) to yellow (375 nm) in response to the amount of CO_2 absorbed (Fig. 2.3). This shift corresponds to the indicator's transition range (pH 12.0 to 10.0) and aligns with the findings of Nazar et al. (2010), who reported a bathochromic shift of AYR from 493 to 373 nm at pH levels above 10.0. The concentration and volume of the indicator solution (1.0 M K_2CO_3, 1 mL) were appropriate for effectively facilitating the pH

change resulting from the oxidation of up to 8 mg C in the sample (corresponding to a maximum of 8% SOC) and the subsequent absorption of CO_2. The concentration of AYR (0.1 w/v%) was also found to be optimal for providing maximum variations in absorbance. The absorbance at 490 nm decreased by approximately 0.63 (from 0.93 to 0.30), while the absorbance at 375 nm increased by 0.45 (from 0.45 to 0.90). Under the conditions outlined in this study, the pH of the indicator solution was observed to change from 11.3 to 10.1. In previous studies, including substrate-induced soil respiration (Rowell et al., 1994) and wet oxidation of soil extracts (Doyle et al., 2004), various pH indicators such as cresol red (pH 7.0–8.8), phenol red (pH 6.8–8.4), bromothymol blue (pH 6.0–7.6), and brilliant yellow (pH 6.4–8.0) were employed to measure CO_2 spectroscopically. These indicators, with near-neutral transition ranges, typically utilized bicarbonate-carbonate buffer solutions (pH 7.5–8.2). In contrast, the AYR system in this study offers a distinct advantage for CO_2 trapping due to its higher pH range, facilitating more effective absorption. However, the absorbance changes (at both 490 nm and 375 nm) with increasing C were inconsistent; they were higher at low carbon levels and decreased as carbon content increased, indicating a more accurate calibration method is needed.

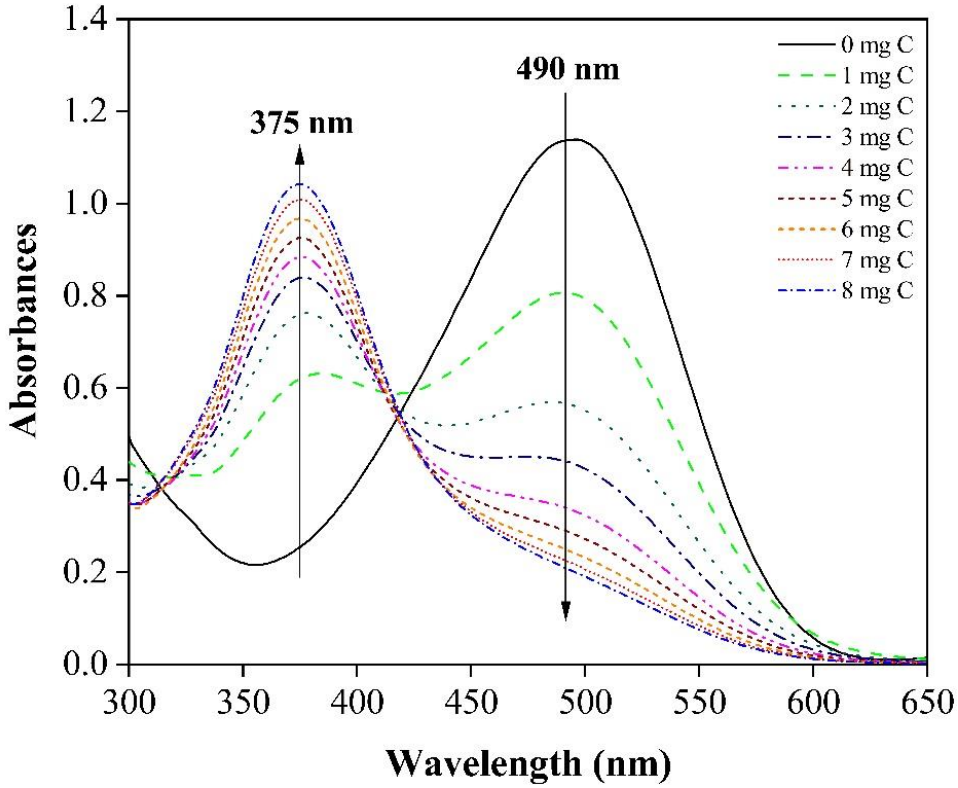


Fig. 2.3 Changes in absorption spectra of AYR in response to the amount of C levels. Standard solution ranged from 0 – 8 mg C (C/N ratio = 10). The outer solution consisted of 1mL of standard solution in 5 mL of oxidizing reagent (0.4 M $Na_2S_2O_8$ in 0.6 M NaOH) (pH > 13.0), while the inner tube contained 1 mL of basic pH indicator solution (0.3 M K_2CO_3 + 15 mL of 0.1% (w/v) AYR. The outer solution and indicator solution contained within a sealed vial was then autoclaved at 110°C for 1h. After cooling 1 h at 4°C, an aliquot of the basic pH indicator solution was measured for C at 375 nm and 490 nm using a spectrophotometer. AYR exhibits a bathochromic shift at 490 and 375 nm. The absorbance decreases at peak 1 (490 nm), while it increases at peak 2 (375 nm) with increasing C contents.

2.4.2 Thermal stability of AYR

Given that the persulfate digestion is thermally activated (typically at around 100°C), it is essential that the indicator maintains chemical stability under these severe conditions. As shown in Fig. 2.4, AYR exhibited notable stability for up to 120 min at 110°C, with the characteristic absorbance peaks at 490 nm and 375 nm remaining unchanged. In addition, AYR responded well to the different mount of C absorbed (3 and 7 mg C). This finding indicates that AYR is not only thermally stable but also contributes significantly to CO_2 dissolution in the basic buffer solution, making it a suitable indicator for CO_2 detection at high temperatures (e.g., 110°C). The stability and solubility of AYR is mainly attributed to the basicity of the buffer solution to maintain its intra-molecular H-bounding, forming di-anionic species (AYR to AYR^{2-}) (Nazar et al., 2010). AYR, salicylic acid, 5-(p-nitrophenylazo), is a water-soluble anionic dye having polycyclic aromatic hydrocarbons (PAH) in its structure (Sabnis, 2007). In a basic pH solution (pH 10.0-12.0), both carboxylic (-COOH) and hydroxyl (-OH) groups lose proton (H^+), resulting in the formation of two negative charges on the molecule. The presence of these negative charges enhances charge delocalization, which is important for maintaining AYR's structural integrity and optical properties in alkaline environments (Nazar et al., 2010).

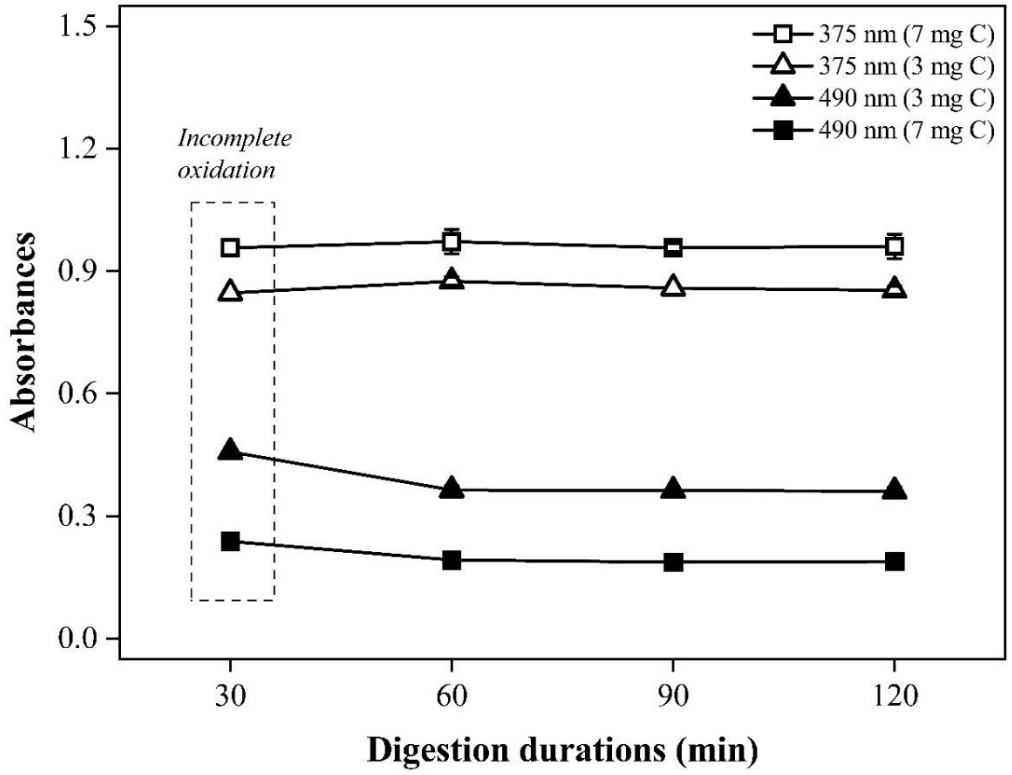


Fig. 2.4 Thermal stability of AYR digested for different durations (30, 60, 90, 120 min) at 110°C. The oxidizing reagent and a basic pH indicator solution were prepared as described in Fig. 2.3. Except for the 30 min, the variations in absorbance were very small, typically falling below 0.005 at 490 nm and 0.007 at 375 nm. This indicated that AYR is thermally stable and can be used as pH indicator for quantifying absorbed CO_2. For our digestion procedures, 30 min autoclaving is not sufficient to completely oxidize soil C. Error bars represent standard errors (n = 4) but are not clearly visible when smaller than symbol size.

2.4.3 Calibration curves for C

Figure 3 shows that the amount of C oxidized was well linearly correlated with the ratio of absorbance at 375 nm and 490 nm (A375/A490). In contrast, the absorbance at both wavelengths did not change linearly with the C content (Fig. 2.5). Previous studies using pH indicators, such as phenol red and phenolphthalein, to measure CO_2 found that single-wavelength absorbance often led to nonlinear relationships between absorbance and CO_2 levels (Doyle et al. 2004; Rowell et al. 1994; Skeggs et al. 1960). This nonlinearity in single-wavelength measurements can be attributed to the buffering effect of the indicator solution, which readily equilibrates the acid and base forms of the indicator. However, in the case of AYR, the absorbance at 375 nm and 490 nm directly represents the concentrations of the acid and base forms of the indicator, respectively. Therefore, by analyzing using the Henderson-Hasselbalch equation, the A375/A490 ratio can be directly related to the proton concentration in the solution, which is proportional to the amount of CO_2 absorbed. Utilizing this absorbance ratio, we can obtain a good calibration curve ($R^2 > 0.99$), high precision (RSD < 3.53%; n = 4), and low detection limit (0.012 mg C). These results indicate that the proposed persulfate digestion method, which utilizes the absorbance ratio of a basic pH indicator, provides a robust and reliable technique for accurately quantifying SOC in air-dried soil samples.

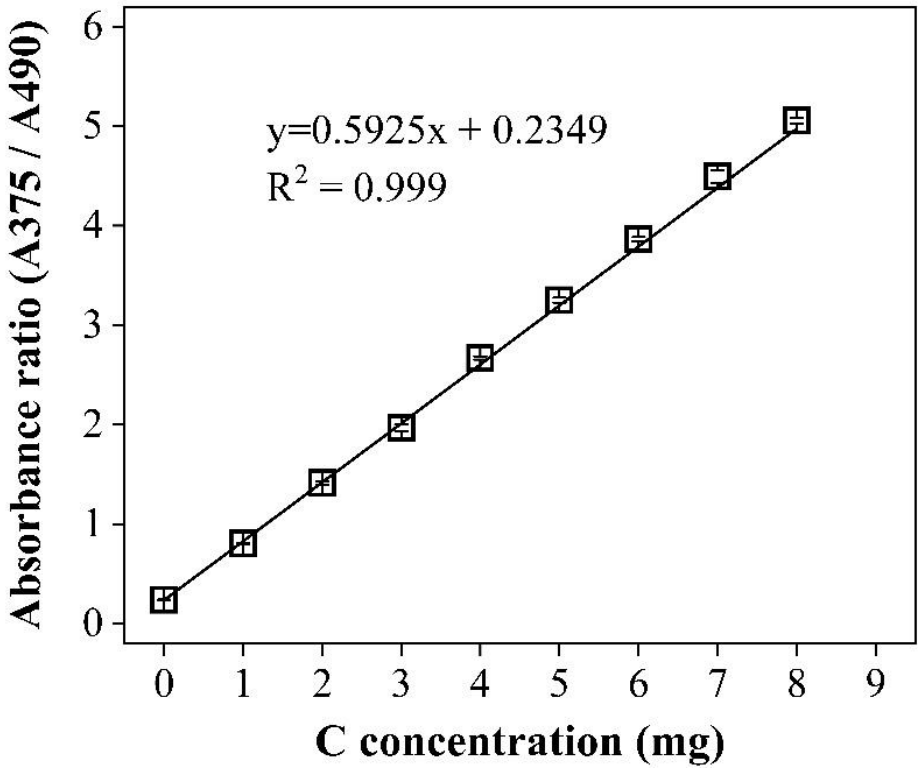


Fig. 2.5 Standard curve for C measured by the proposed alkaline persulfate digestion method. The digestion conditions followed those described in Fig. 2.3. Error bars represent standard errors (n = 4) but are not clearly visible when smaller than symbol size.

2.4.4 Calibration curve for N

We obtained a strong linear relationship between the measured amount of N and the known amounts in the standards (Fig. 2.6). Previous studies analyzed N in the digestion mixture using Griess-Ilosvay procedures (Doyle et al. 2004; Gibson et al.2015; Pujo-Pay and Raimbault, 1994). This approach requires a cadmium reduction column (to reduce nitrate to nitrite) and relies on an automated system for flow injection analysis. Since the aliquot of the digestion mixture is acidic, using the salicylic acid nitration method (Cataldo et al., 1975) can be an effective alternative for N analysis. This method relies on sulfuric acid to absorb water from the sample and protonate it to form nitronium, which then binds to the benzene ring of salicylic acid. Through this method, we can also obtain a good calibration curve (R^2 > 0.995), high precision (RSD < 2.23%; n = 4), and lower detection limit (1.27 ug N). We noted that the absorbances of the blank were always around 0.25, which is probably affected by the large addition of oxidizing reagent (Bronk et al., 2000) and some oxidation of atmospheric N_2 gas in the vial headspace (Hagedorn and Schleppi, 2000). However, the detection limit of the blank absorbances was calculated to be 1.27 ug N, while the lowest N standard concentration was 16.67 ug N. These findings suggest that the salicylic acid nitration method is a reliable alternative for measuring N in acidic digestion mixtures. Compared to existing methods, this technique is simpler, faster, and more cost-effective regarding instrumentation and maintenance.

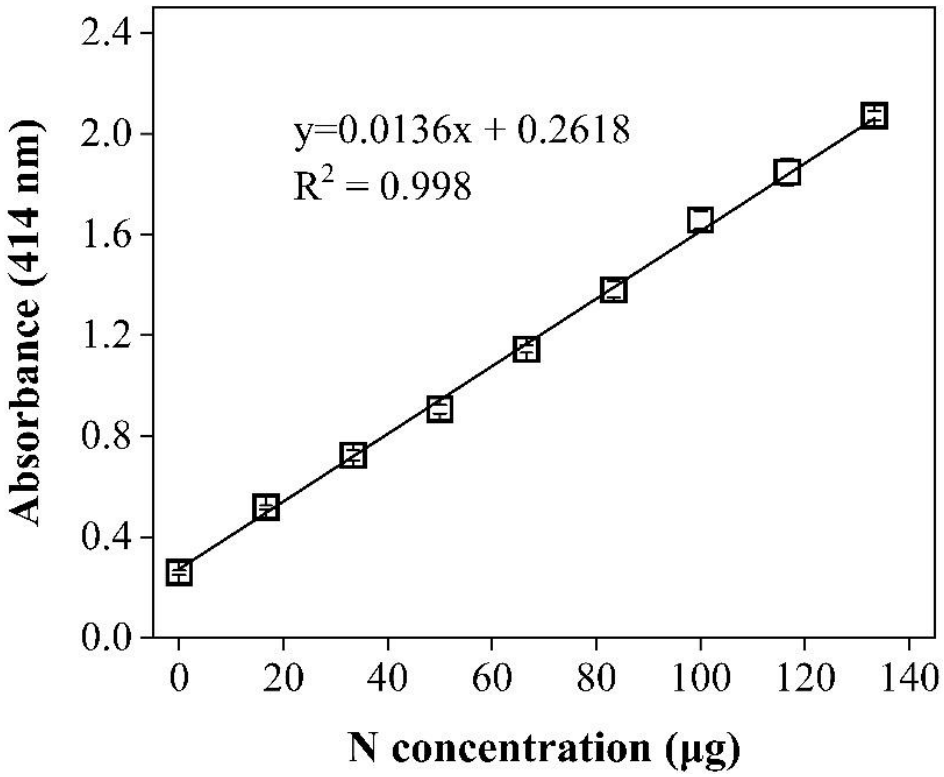


Fig. 2.6 Standard curve for N measured by the proposed alkaline persulfate digestion method. Digestion conditions followed those described in Fig. 2.3. Error bars represent standard errors (n = 4) but are not clearly visible when smaller than symbol size.

2.4.5 Optimum persulfate concentration

The optimum persulfate concentration and volume are critical for a precise estimation of SOC and TN in ground soil samples. For the digestion system, a 30 mL serum vial was used because it allowed us to autoclave more than a hundredth samples per trial. In addition, to prevent contamination between the outer solution (0.1g ground soil or 1 mL standard samples + oxidizer reagent) and the basic pH indicator solution (AYR + K_2CO_3) in the inner tubes, which are sealed inside a single vial, we decided to use 5 mL of oxidizing reagent for safety. Our results showed that soil samples digested with lower persulfate concentrations (e.g., 0.2 and 0.3M persulfate) yielded significantly lower SOC and TN compared to those digested with higher persulfate concentration, particularly for soils with high SOC and TN contents (Fig. 2.7a and 7b). Soil samples digested with 0.4 and 0.5M persulfate produced similar SOC and TN results. It is important to note that using higher persulfate concentrations may lead to not only solubility issues and increased N contamination (Bronk et al., 2000), but also increase oxygen amount produced by peroxydisulfate ion ($S_2O_8^{2-}$) in the vial headspace that can cause gas leakage (Goulden and Anthony, 1978). Therefore, 5 mL of 0.4 M $Na_2S_2O_8$ is the optimum concentration for oxidizing C and N in the 0.1g ground soil digestion system.

Previous studies utilized $K_2S_2O_8$ as oxidizer agent at different concentrations to oxidize extractable or particulate C, N, as well as P in soil extracts, fresh water, or aquatic matters. For example, Doyle et al. (2004) used maximum $K_2S_2O_8$ (50g L^{-1}) to determine DOC and DON in soil extracts. Gibson et al. (2015) utilized 0.11 M $K_2S_2O_8$ (~30g L^{-1}) to measure particulate OC, ON, and OP of seston, algae, and small zooplankton in fresh waters. The use of $K_2SO_2O_8$, with the solubility of 4.49 g in 100 mL at 20°C, is feasible for samples with low C and N contents or small sample amount.

It is impossible to use it in a ground soil digestion system unless large volume and maximum concentration were used. If this is the case, it could result in significant errors in the estimation of SOC and TN in the air-dried soil sample. In our study, therefore, we used $Na_2S_2O_8$ as an oxidizer because its solubility (55.6 g in 100 mL, 20°C) is approximately 14 times higher than that of $K_2S_2O_8$.

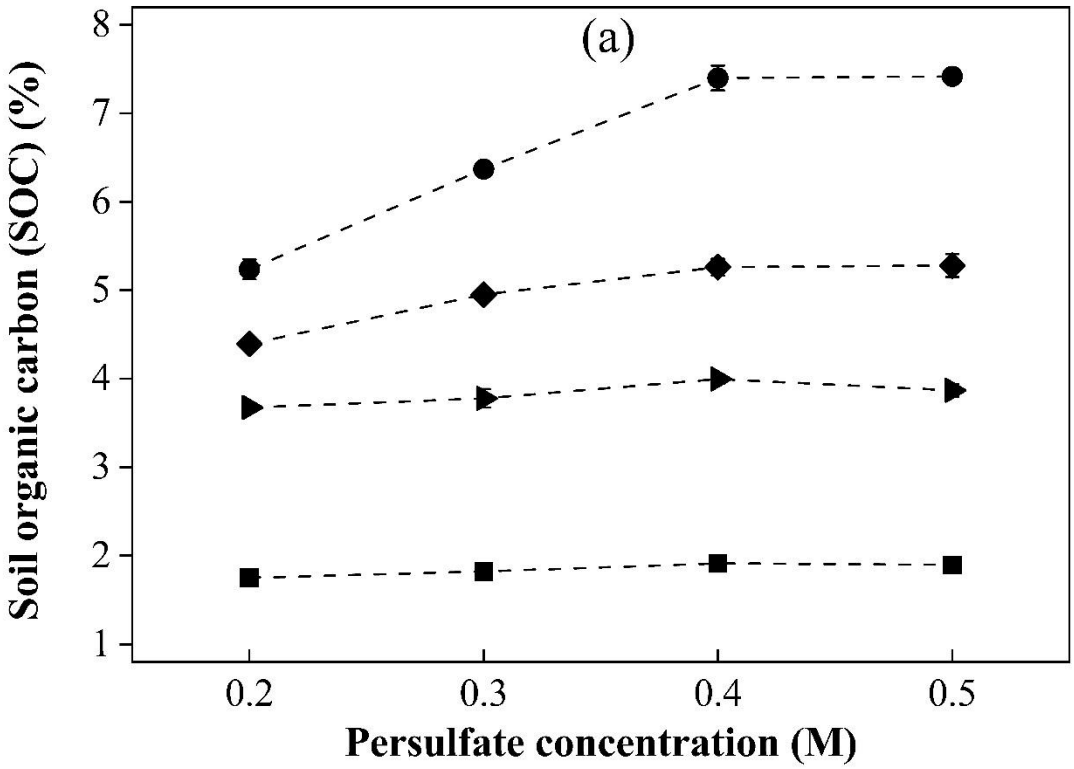


Fig. 2.7a Comparison of persulfate concentrations for SOC analysis. 0.1g of ground soil samples with different SOC levels, collected from various land uses (volcanic, paddy, greenhouse, and orchard), were digested using $Na_2S_2O_8$ concentration of 0.2, 0.3, 0.4, and 0.5M, respectively. The basic pH indicator solution, digestion duration, and temperature were maintained as described in Fig. 2.3. Each point represents the mean of three replications, with error bars representing standard errors (n = 4).

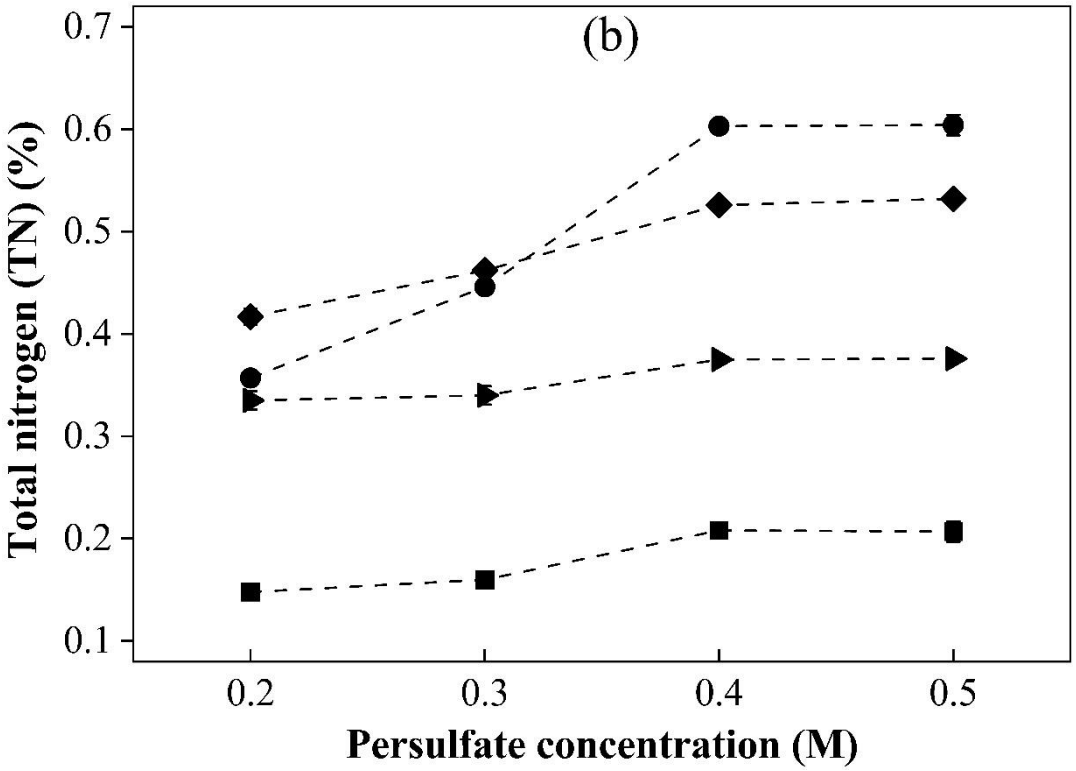


Fig. 2.7b Comparison of persulfate concentrations for TN analysis. 0.1g of ground soil samples with different SOC levels, collected from various land uses (volcanic, paddy, greenhouse, and orchard), were digested using $Na_2S_2O_8$ concentration of 0.2, 0.3, 0.4, and 0.5M, respectively. The basic pH indicator solution, digestion duration, and temperature were maintained as described in Fig. 2.3. Each point represents the mean of three replications, with error bars representing standard errors (n = 4).

2.4.6 Optimum persulfate-to-NaOH ratio

Obviously, the amount of SOC and TN produced depends largely not only on the concentration of persulfate but also NaOH. As can be seen from Fig. 2.8a, SOC content remained consistent at the concentration of persulfate-to-NaOH ratios ranging from 1:1 to 1:1.75 but significantly decreased when the NaOH was doubled relative to that of persulfate. In contrast, the amount of TN increased as the persulfate-to-NaOH ratio increased from 1:1 to 1:1.5 and then gradually decreased as the ratio increased from 1:1.75 to 1:2 (Fig. 2.8b). Our findings agree with Cabrera and Beare, (1993) who found that increase NaOH concentrations decreased N recovery. Therefore, the optimum persulfate-to-NaOH for our digestion procedure was 1:1.5 ratio, corresponding to a reaction concentration of 0.4M $Na_2S_2O_8$ and 0.6M NaOH. This optimal ratio (1:1.5) is similar to the ratio (1:1.36) used by Gibson et al. (2015), Suzumura et al. (2008), and Zhou et al. 2003), which corresponds to 0.111M $K_2S_2O_8$ in 0.15M NaOH and agree with the finding of Furman et al. (2011) and Block et al. (2004), who found that persulfate is highly reactive in basic system. However, when persulfate is high and NaOH is low, the oxidizing efficiency decreases rapidly because HSO_4^- produced as a by-product during the reaction neutralized all the NaOH (Furman et al. 2010; Gibson et al. 2015), resulting in lower SOC and TN production. Conversely, when NaOH is high and persulfate is low, C is not fully oxidized to CO_2, and all N forms are not completely converted to NO_3^- but instead volatilize as NH_3 (Cabrera and Beare, 1993; Zhou et al., 2003). Therefore, the oxidation reaction should begin under alkaline conditions (pH > 10.0) for about 30 min to oxidize organic matter into by-products (CO_2, NH_4^+, NO_3^-, PO_4^{-3}), followed by an acidic digestion phase (pH < 2.0) for another 30 min to completely convert carbonate to CO_2 and all forms of N to NO_3^- (Gibson et al. 2015).

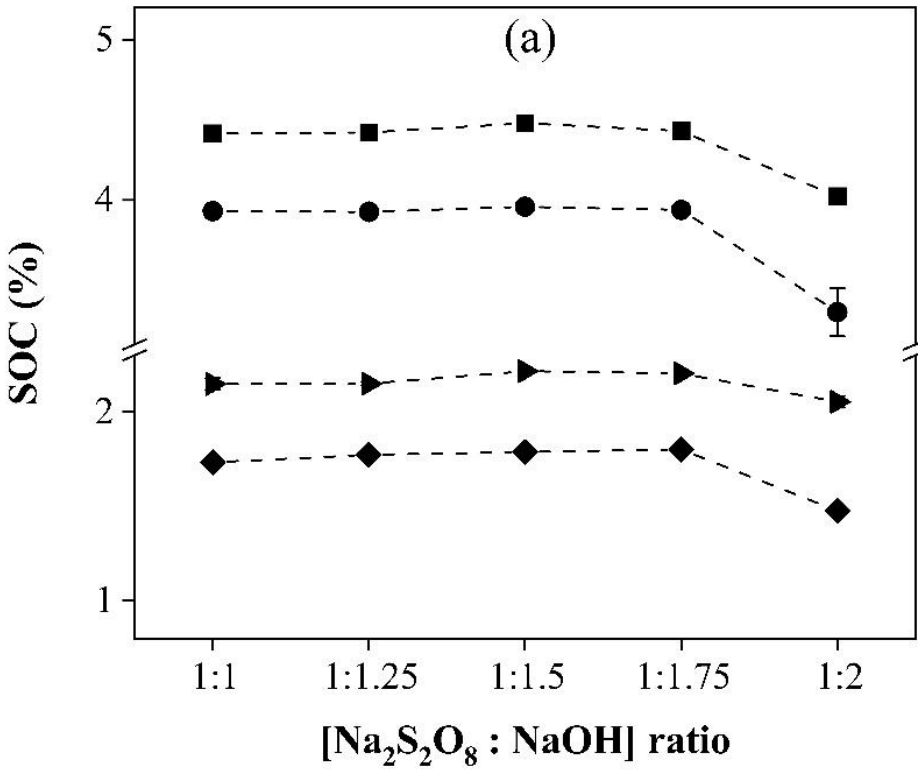


Fig. 2.8a Comparison of [$Na_2S_2O_8$: NaOH] ratios for SOC analysis. Four soil samples with different SOC contents, collected from various land uses (volcanic, paddy, greenhouse, and orchard), were digested with 1:1, 1:1.25; 1:1.5, 1:1.75, and 1:2 ratios of [Na2S2O8 : NaOH], respectively. The persulfate concentration (reaction) used in this test was 0.4 M. The basic pH indicator solution, digestion duration, and temperature were maintained as described in Fig. 2.3. Each point represents the mean of three replications, with error bars representing standard errors (n = 4).

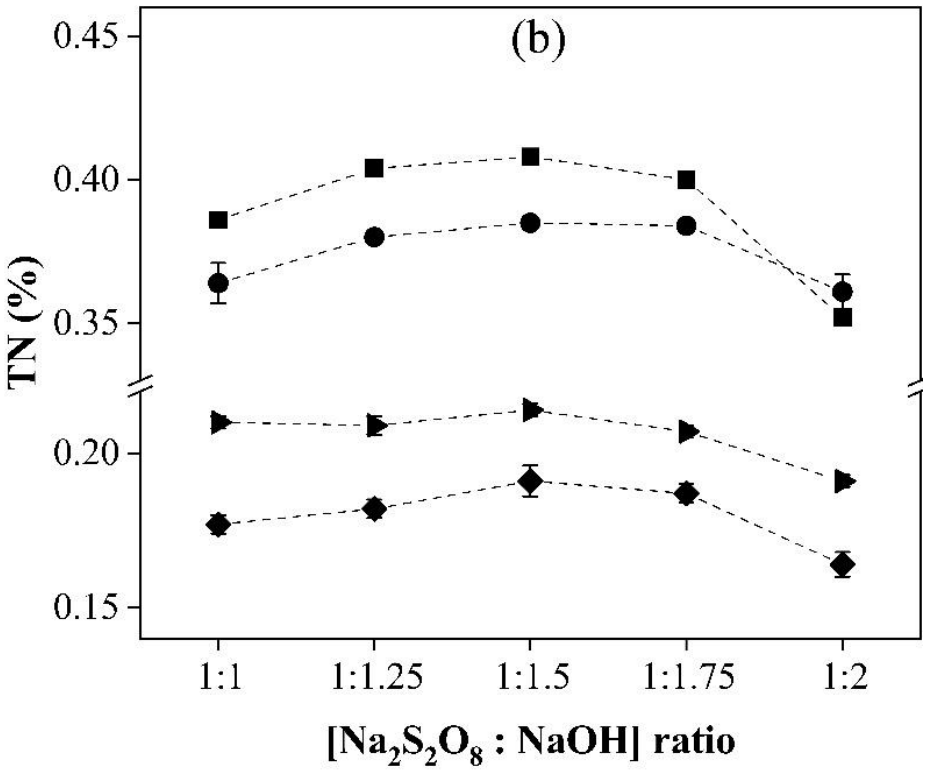


Fig. 2.8b Comparison of [$Na_2S_2O_8$: NaOH] ratios for TN analysis. Four soil samples with different SOC contents, collected from various land uses (volcanic, paddy, greenhouse, and orchard), were digested with 1:1, 1:1.25; 1:1.5, 1:1.75, and 1:2 ratios of [Na2S2O8 : NaOH], respectively. The persulfate concentration (reaction) used in this test was 0.4 M. The basic pH indicator solution, digestion duration, and temperature were maintained as described in Fig. 2.3. Each point represents the mean of three replications, with error bars representing standard errors (n = 4).

2.4.7 The pH changes before and after the course of oxidation

In the alkaline persulfate oxidation method, pH is crucial for determining whether the digestion process is complete. In general, the digestion system allows for about 30 min of alkaline digestion to ensure complete oxidation of N, followed by an acidic digestion (pH < 2.0) to facilitate CO_2 diffusion (Cabrera and Beare, 1993; Zhou et al., 2003). At this low pH, carbonate is released as CO_2 in equilibrium with the partial pressure inside a sealed vial, while various forms of N are oxidized to NO_3^- in the digestion mixture. As can be seen from Fig. 2.8a and 8b above, the critical role of the oxidizing reagent is to provide the optimal persulfate-to-NaOH ratio capable of achieving complete oxidation. We observed that the ratios from 1:1 to 1:1.75, the pH of the digestion mixture dropped from 13.0 to below 2.0 after digestion finished, indicating complete oxidation (Fig. 2.9). In contrast, the ratios above 1:1.75, the pH dropped from 13.0 to around 6–7, indicating incomplete oxidation. These results suggest that persulfate-to-NaOH ratio of 1:1.75 and 1:2 is not efficient for the complete oxidation of C and N. According to the amounts of SOC and TN produced, as well as the pH values at different persulfate-to-NaOH ratios, a ratio of 1:1.5 was found to be an optimal digestion condition in this study. However, the ideal pH after digestion can vary depending on whether the measurement is done using digestion mixture to measure C and N or using gaseous form of CO_2 to measure C and digestion mixture to measure N. For example, Doyle et al. (2004), who used an aliquot of the digestion mixture to analyze C with a continue flow injection manifold and adopted Griess-Ilosvay to measure N, achieved complete recovery of C and N at a pH of 9–10. In contrast, Gibson et al. (2015), who measured C in the presence of CO_2 gas and analyzed N from an aliquot of the digestion mixture using automated systems for flow injection (i.e., GC-MS and IC), found that complete recovery could only be achieved when the pH after digestion decreased below 2.0.

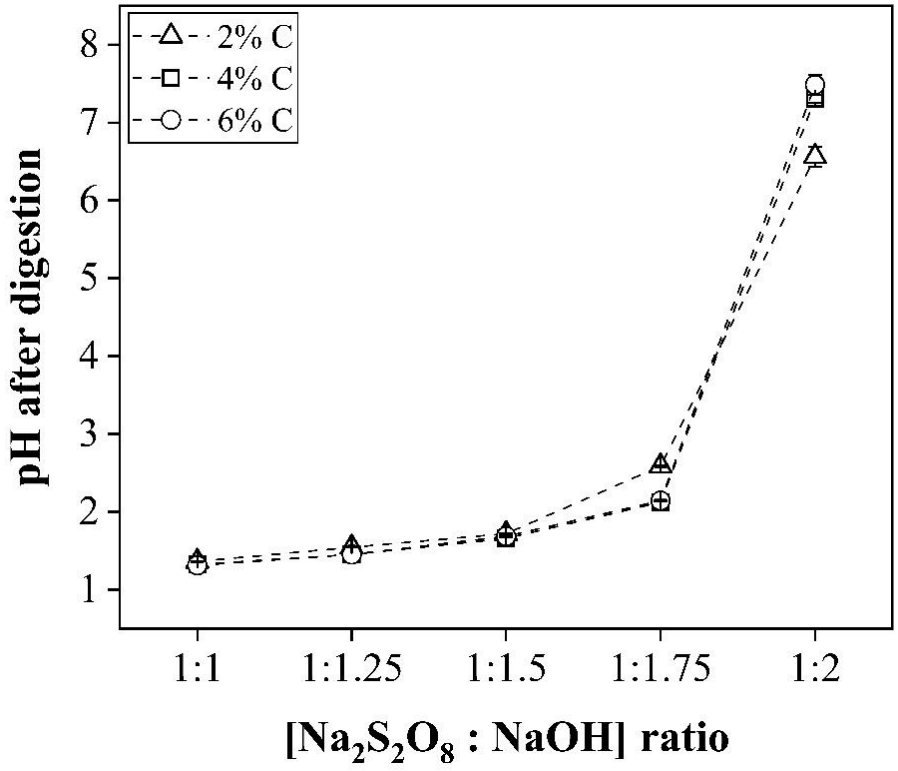


Fig. 2.9 The pH of the digestion mixture from soils digested with different [$Na_2S_2O_8$: NaOH] ratios. Three soil samples with different SOC contents, collected from volcanic, paddy, and upland, were digested with 1:1, 1:1.25; 1:1.5, 1:1.75, and 1:2 ratios of [Na2S2O8 : NaOH], respectively. The persulfate concentration (reaction) used in this test was 0.4 M. The basic pH indicator solution, digestion duration, and temperature were maintained as described in Fig. 2.3. Each point represents the mean of three replications, with error bars representing standard errors (n = 4).

2.4.8 Optimum digestion duration

For the additional assessment of the efficiency of our proposed method, we digested soil samples that varied in SOC contents with different digestion durations. Among the four digestion durations we investigated, SOC and TN contents obtained from 30-min digestion were significantly lower than those of 60-, 90-, and 120-min digestion durations (Fig. 2.10a and 10b). This indicated that a 30-min digestion duration is not sufficient to complete oxidation of soil C and N. The differences in SOC and TN between the 60-, 90-, and 120-min digestion durations were minimal, suggesting that one among the 3 could be chosen. Since our digestion system used heat-activated persulfate and sealed vials to facilitate soil oxidation and CO_2 absorption, a shorter digestion duration that adequately oxidizes C and N would be the best alternative. A shorter but sufficient digestion time can prevent gas leakage, improve safety, and reduce time consumption. Digestion duration is closely related to the decomposition and reaction of persulfate at a given time and temperature. Peyton et al. (1993) stated that long-time digestion at high temperatures may have little impact on the sulfate radical reaction once the persulfate is dissolved and the pH of the outer solution (digestion mixture) decreased around 2.0 (Zhou et al. 2003; Gibson et al. 2015). Based on the pH of the digestion mixture (Fig. 2.9) and the SOC and TN contents observed herein, we can conclude that 60 minutes is a safe minimum digestion duration for precise determination of SOC and TN from the same soil digest.

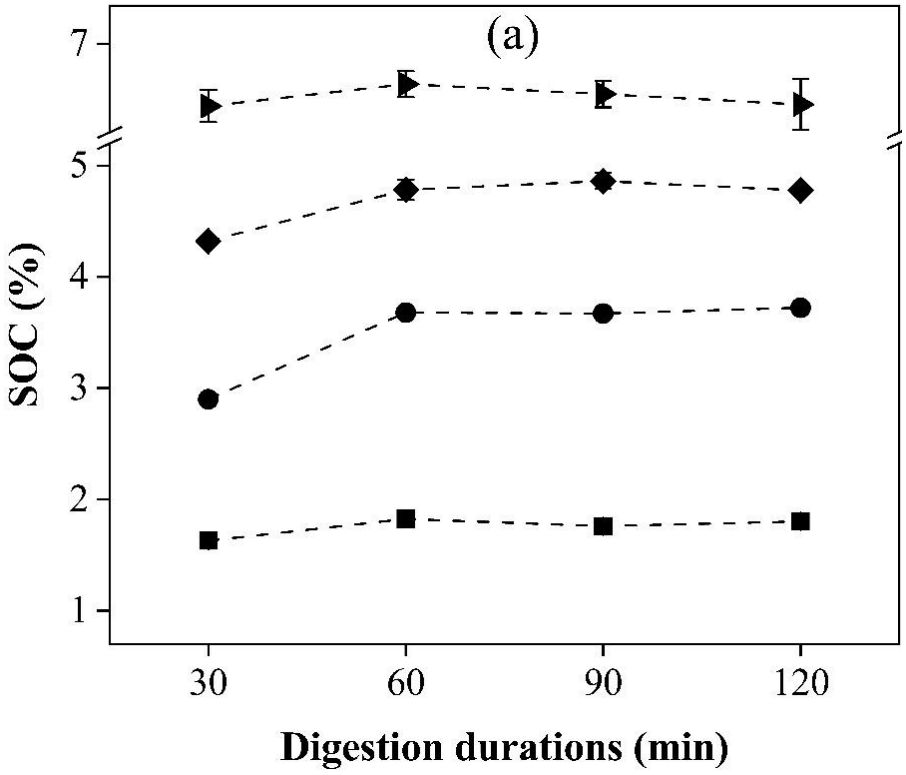


Fig. 2.10a Comparison of digestion durations for SOC analysis. Four soil samples with different SOC contents, collected from various land uses (volcanic, paddy, greenhouse, and orchard), were digested for 30, 60, 90, and 120 minutes, respectively. Digestion conditions were maintained as described in Fig. 1. 3. Each point represents the mean of three replications, with error bars representing standard errors (n = 4).

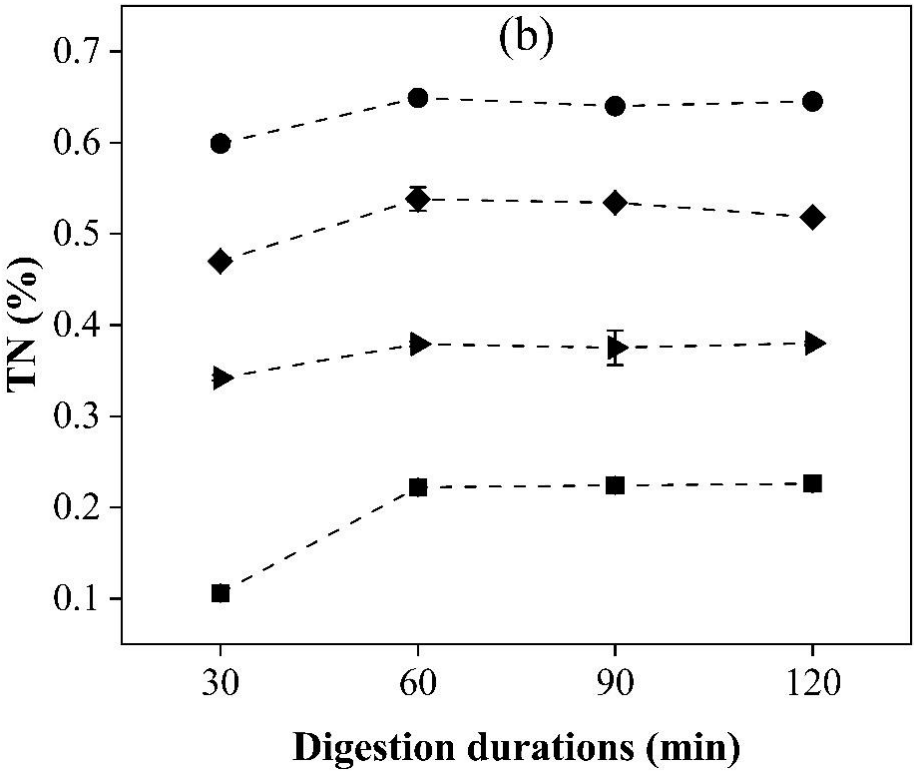


Fig. 2.10b Comparison of digestion durations for TN analysis. Four soil samples with different SOC contents, collected from various land uses (volcanic, paddy, greenhouse, and orchard), were digested for 30, 60, 90, and 120 minutes, respectively. Digestion conditions were maintained as described in Fig. 1. 3. Each point represents the mean of three replications, with error bars representing standard errors (n = 4).

2.4.9 Optimum digestion temperature

As in the case of digestion duration, we digested soil samples that varied in SOC contents with three different digestion temperatures at a given digestion duration of 1h. Our results showed that soil samples digested at 100, 110, and 120°C yielded comparable SOC contents (Fig. 2.11a). However, one data point (3 replications) with a high SOC content (~6.8%) was significantly lower at 120°C compared to those at 100 and 110°C. This difference may be caused by a short lifetime of the persulfate at a high digestion temperature (Doyle et al., 2004; Goulden and Anthony, 1978). On the other hand, soil digestion at 100°C resulted in a significantly lower TN content compared to those digested at 110°C and 120°C, which gave similar TN contents (Fig. 2.11b). These indicated that a digestion temperature of 110°C is sufficient to complete the oxidation of organic matter. Doyle et al. (2004) found that oxidation efficiency was not temperature sensitive as long as adequate time was allowed for persulfate radicals to be generated. As demonstrated in Fig. 2.10a and 10b that a 30-minute digestion duration at 110°C was insufficient to complete the oxidation, particularly for TN. Therefore, a digestion duration of approximately 1 hour at 110ºC is required to fully oxidize the organic material in our digestion system.

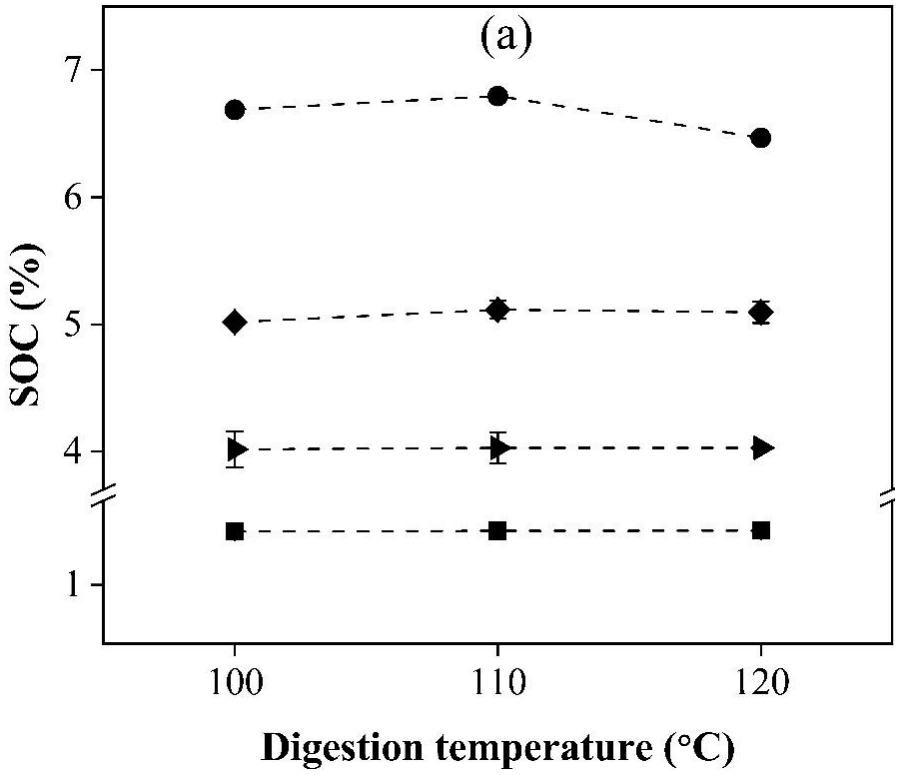


Fig. 2.11a Comparison of digestion temperatures for SOC analysis. Four soil samples with different SOC contents, collected from various land uses (volcanic, paddy, greenhouse, and orchard), were digested at 100, 110, and 120°C, respectively. Digestion conditions were maintained as described in Fig. 2.3. Each point represents the mean of three replications, with error bars representing standard errors (n = 4).

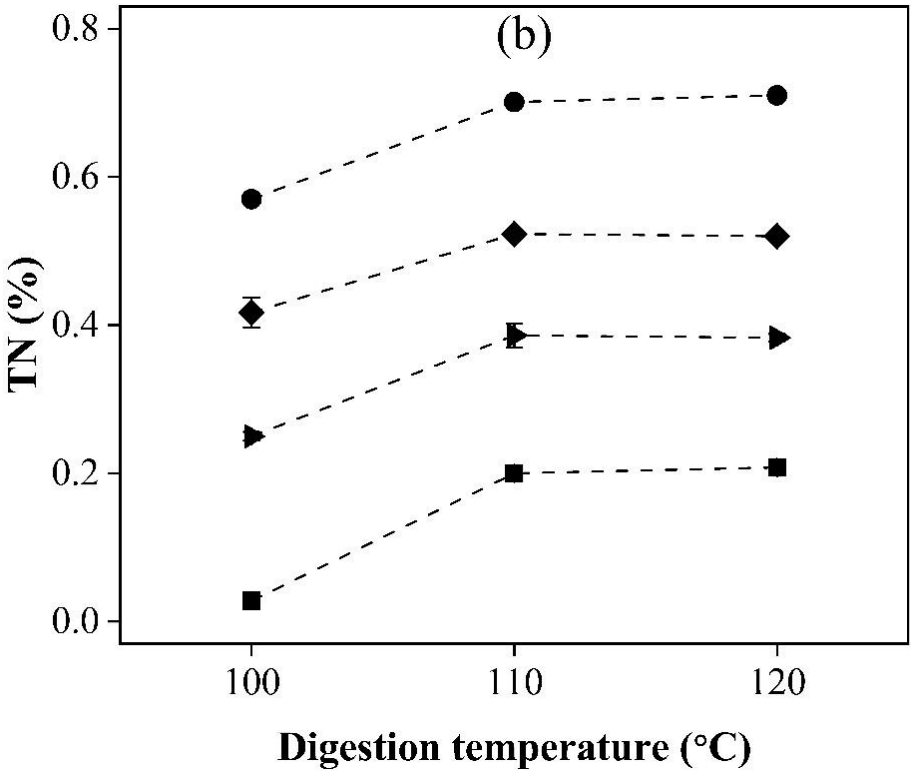


Fig. 2.11b Comparison of digestion temperatures for TN analysis. Four soil samples with different SOC contents, collected from various land uses (volcanic, paddy, greenhouse, and orchard), were digested at 100, 110, and 120°C, respectively. Digestion conditions were maintained as described in Fig. 2.3. Each point represents the mean of three replications, with error bars representing standard errors (n = 4).

2.4.10 Sample comparison with elemental analyzer

We compared samples analyzed by our alkaline persulfate digestion method to those analyzed by an elemental analyzer. The SOC and TN measured by our proposed method showed a good coincidence with those analyzed by the elemental analyzer (Fig. 2.12a and 12b). The paired t-test results revealed no statistically significant differences between the two methods for SOC ($t = 0.40$, $p \geq 0.69$, $df = 21$) and for TN ($t = 0.54$ and $p \geq 0.60$, $df = 21$). The SOC and TN of the selected soil samples fell within our standard ranges, with values ranging from 0.19% to 7.43% for C and 0.01% to 0.53% for N. The average SOC content of the topsoil was reported to be about 2% (min-max: 0.5-6.23%), whereas the average TN content was 0.12% (min-max: 0.08-0.19%) (Kim et al. 2021; Park et al. 2021). Generally, the SOC and TN contents of selected samples from different land uses, including paddy, upland, orchard, greenhouse, and volcanic ash, cover most of the soil in Korea.

The proposed method, in which CO_2 released from persulfate-oxidized soil is trapped in a basic pH indicator solution, offers a novel, cost-effective, and rapid way to determine both SOC and TN in the same soil digest. This method utilizes inexpensive standard equipment commonly found in most laboratories (i.e., autoclave, UV-VIS spectrophotometer, serum vial, white butyl rubber stopper, aluminum ring, and crimper) and can analyze over 100 samples per day, making it an efficient option for high-throughput analysis. Compared to known methods, our procedures appeared to be moderately simple and precise for both SOC and TN measurements.

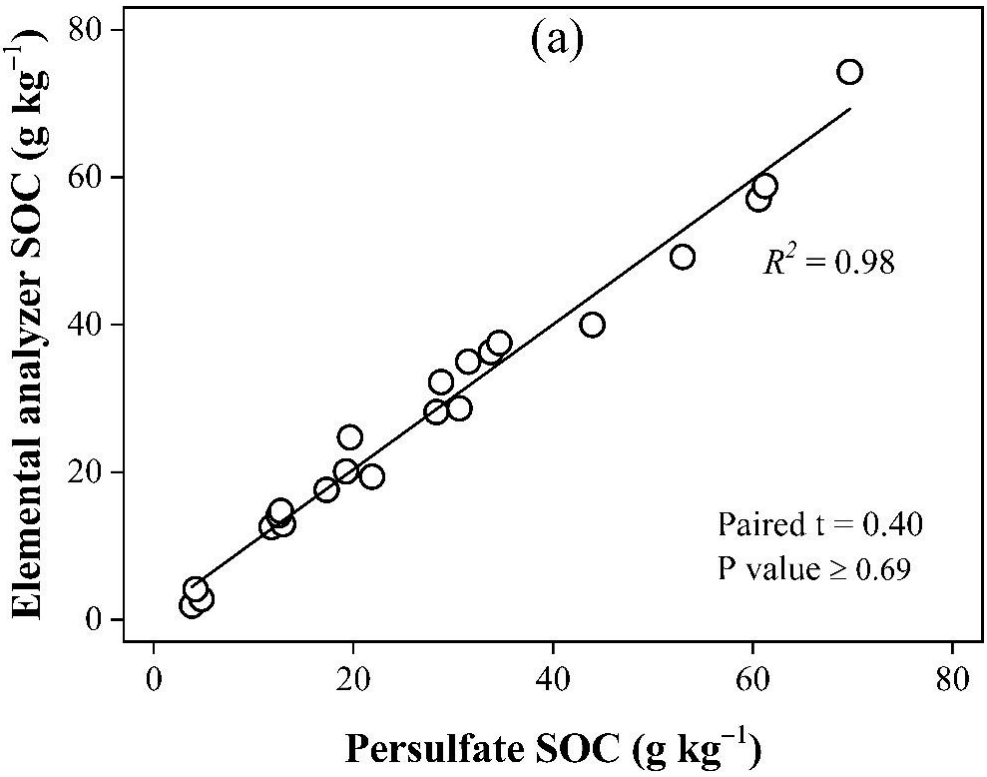


Fig. 2.12a Comparison of SOC contents analyzed by our proposed alkaline persulfate digestion method with those measured by an elemental analyzer. For our method, the digestion conditions were as described in Fig. 2.3. Twenty-two soil samples varying in SOC content, collected from various land uses (6 volcanic, 4 paddy, 4 greenhouse, and 4 orchard), were selected for analysis. Soil samples were analyzed in triplicate by our method, while the EA was performed with a single replicate. Paired t-test was performed to compare two means at $P \leq 0.05$.

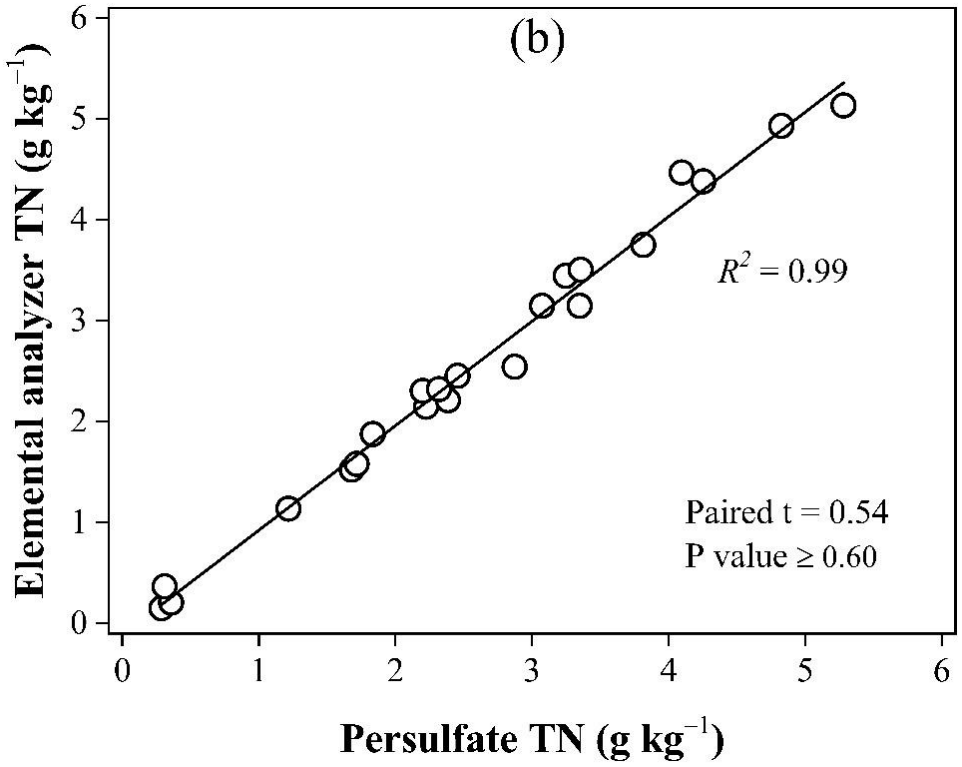


Fig. 2.12b Comparison of TN contents analyzed by our proposed alkaline persulfate digestion method with those measured by an elemental analyzer. For our method, the digestion conditions were as described in Fig. 2.3. Twenty-two soil samples varying in SOC content, collected from various land uses (6 volcanic, 4 paddy, 4 greenhouse, and 4 orchard), were selected for analysis. Soil samples were analyzed in triplicate by our method, while the EA was performed with a single replicate. Paired t-test was performed to compare two means at $P \leq 0.05$.

2.5 Conclusion

We found that AYR responds well to CO_2 absorption and remains stable at high digestion temperature. In the basic buffer solution, AYR exhibits a bathochromic shift at 490 nm and 375 nm, but the changes in absorbance at these two wavelengths are not linear in response to the amount of CO_2 absorbed. By applying the Henderson-Hasselbalch equation, we successfully established a new linear calibration curve (R^2 > 0.99) based on the absorbance ratio of 375 nm to 490 nm (A375/A490) for SOC determination with high precision (RSD < 3.53%) and low detection limit (0.012 mg C). In addition, the salicylic acid nitration method for TN determination also exhibited strong linear relationship (R^2 > 0.99), high precision (RSD < 2.23%), and low detection limit (1.27 ug N). These indicate that the proposed alkaline persulfate digestion and salicylic acid nitration methods are reliable for determining SOC and TN in soil digests. To ensure safe and accurate determination of SOC and TN in ground soil sample, all digestion conditions were optimized. For a 6 mL digestion volume, the optimal conditions for digesting 0.1 g of ground soil (with 8% C and 0.8% N) were found to be 5 mL of 0.4 M $Na_2S_2O_8$:0.6M NaOH (reaction concentration), 1 mL of 1.0 M K_2CO_3 + 15 mL of 0.1 (w/v%) AYR, and a digestion duration of 60 minutes at 110ºC. Under these conditions, our proposed method yielded statistically similar SOC (Paired t-test; t = 0.40, p ≥ 0.69) and TN (Paired t-test; t = 0.54 and p ≥ 0.60) values compared to those obtained from elemental analyzer. To measure SOC and TN in air-dried soil sample (passed through a 150µm screen) our proposed method offers a simple, rapid, and cost-effective approach, utilizing accessible laboratory equipment, and is capable of processing around a hundred samples per day, making it a valuable tool for high-throughput soil nutrient analysis.

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CHAPTER 3

Quantity and quality assessments of soil organic matter fractions in relation to the increment of soil organic carbon content across various land uses: Distinct shifts in carbon and nitrogen compositions along with humus formation

3.1 Abstract

Land uses and soil managements have profound effects on soil organic matter (SOM) status. Since total soil organic carbon (SOC) is formed through complex biological, chemical, and physical processes, determining its compositions may be more important than measuring its total concentration for assessing soil quality. The objectives of this study were (i) to investigate differences in organic matter fractions, such as non-humic substances (NHS), debris, and humic substances, including fulvic acid (FA), humic acid (HA), and humin (HM), in soils under different land uses; (ii) to examine the relative proportion of these fractions to the increment of the total SOC content; and (iii) to evaluate the impact of N on the formation and stability of humic substances by analyzing C and N contents in FA, HA, and HM fractions. To cover a wide range of SOC content, 362 soil samples (0-30 cm depth) were collected from six different land uses, including orchard, paddy, upland, greenhouse, volcanic ash, and reclaimed soils, across the Korean peninsula. All soil samples were analyzed for total SOC and only 30 samples covering low to high SOC were randomly selected from each land use. Thus, 180 soil samples were analyzed in this study. NHS and debris were extracted with 0.5 N HCl in 2.85 M MgSO4 and separted by filtration. Humic substances were chemically fractionated into FA, HA, and HM, and the C and N in these humus fractions were analyzed by the alkaline persulfate oxidation method. Regardless of land-use types, the C content in the SOC fractions followed the order:

NHS < debris < HA < FA < HM. On average, NHS and debris contributed about 0.7–1.4% and 6–14% of C to the total SOC, while FA, HA, and HM accounted for about 23–30%, 15–20%, and 35–43%, respectively. Regardless of land-use types, the relative proportion of NHS, debris, and FA decreased as SOC increased, whereas HA and HM increased with SOC. These findings indicated that HA and HM fractions are the major contributors to the increase in total SOC. However, we observed that in common land uses and reclaimed soils, the humification index (HI) increased and the degree of transformation (DT) decreased as SOC increased, whereas in volcanic ash soils, both the HI and DT increased with SOC. In most cases, the increase in HI and the decrease in DT were below and above the limit of 1.0, respectively. These findings indicated that a slow formation of stable humus in cultivated soils. Likewise, in common land uses and reclaimed soils, the C contents in FA, HA, and HM fractions increased, and the C/N ratio in FA and HA tended to decrease as SOC increased, while the ratio in HM increased with SOC. This pattern indicates that the formation of FA and HA requires N to stabilize the HM fraction. In contrast, in volcanic ash soils, the C contents in FA, HA, and HM increased, the C/N ratio in FA decreased, and those in HA and HM increased as SOC increased. These patterns suggest that young soils, like volcanic ash, need additional N to enhance the formation and stabilization of HA and HM fractions. In conclusion, our findings indicate that in assessing SOM quality in terms of soil quality, N appears to be critically important across all land use types.

Keywords: Fulvic acid, Humic acid, Humin fraction, Land-use types, Non-humic substances, Soil quality

3.2 Introduction

Soil organic matter (SOM) is a critical indicator of soil quality as it influencs key soil properties (Doran and Parkin, 1994; Wendling et al., 2010). It serves as both a primary source and a temporary reservoir for plant nutrients in agricultural systems, while also supporting biological diversity that reinforces soil health and ecosystem processes (Guimaraes et al., 2013). SOM contributes to maintaining soil structure, enhancing air and water infiltration, improving water retention, minimizing erosion, and influencing the effectiveness and behavior of applied pesticides (Gregorich et al., 1994). Preserving SOM is essential for sustaining the long-term productivity of agroecosystems. Assessment of SOM is, therefore, a crucial step in evaluating overall soil quality and may warrant its inclusion as a core component in global soil assessment frameworks.

One of the most common methods for quantifying SOM is to determine the total soil organic carbon (SOC) content. In general, the amount of SOC varies greatly across different land uses due to several factors, including parent materials, physio-chemical protections, altitude, climate conditions, as well as management practices (Lee et al., 2018; Zhong and Qiguo, 2001). For example, the volcanic ash soil (Andosols), which is developed from pyroclasic rocks, often have higher SOC conttent than soils under other land uses as it contains high amounts of allophance and imogolite minerals (Pizarro et al., 2003; Takahashi et al., 2020). These clay minerals possess highly reactive surface areas, characterized by abundant positive and negative charges and high water-holding capacities, enabling them to absorb SOC and chemically protect it through Al- and Fe-humus complexation (Brady and Weil, 2010; Yang et al., 2023). The effective sorption of SOC onto allopanic Andosols has been extensively studied (Torn et al. 1997; Parfitt et al. 2002; and Parfitt et al. 2009). Additionally, volcanic ash

soil provides significant protection of SOC from microbial mineralization due to Al toxicity at low pH levels (Illmer et al., 2003). Yang et al. (2023) found that the physico-chemical protection of SOC in forest Andosols was associated with a lower percentage of total SOC being respired, suggesting that microbes assimilate more C than they respire. On the other hand, common land uses, such as orchard, paddy, upland, and greenhouse may have intermediate SOC content as they typically formed from acidic rocks (Yang et al., 2023). Conversely, reclaimed soils often have the lowest SOC content because they were constructed in coastal areas with high salinity, elevated groundwater levels, and poor soil aggregation, all of which limit microbial activity (Park et al. 2022). Given that SOC is formed by the complex biological, chemical, and physical processes and vary significantly across land-use types, determining its compositions may be more important than measuring its total concentration for assessing soil quality (Asensio et al., 2014; Guimaraes et al.,2013; Ukalska-Jaruga et al., 2019).

SOM comprises a range of humified and biologically active compounds, including readily decomposable materials, plant litter and roots, and dead and living organisms. According to Brady and Weil (2010) and Stevenson (1994), SOM can be chemically classified into various operationally defined forms: debris, non-humic and humic substances. Debris, known as free or non-complexed SOM, consists of identifiable organic matter, including plant roots, root hairs, undecomposed plant residue, and partially decomposed products (ranging in size from < 3 mm to 0.3 mm) occluded in macroaggregates, as well as tiny organic particles (e.g., plant roots or particulate organic matter) with fragment sizes around 0.03 mm, coated in microaggregates (Brady and Weil, 2010; Golchin et al., 1994a, 1994b). Debris includes all fragments that can be dispersed in a high-density solution and collected by filtration. Golchin et

al. (1994a) applied a simple densimetric method to extract all remaining debris in soils and reported that partly decomposed root and plant fragments comprised about 0.6 – 4.34% of dry weight and accounted for 7 – 31% of total C and 6 – 22% of total N. They also observed that the contribution of C and N decreased as the fragment size decreased. On the other hand, non-humic substances (NHS) are also the chemically well-defined labile compounds that consist of low molecular weight aliphatic and aromatic acids, carbohydrates, amino acids, and their polymeric derivatives, such as polypeptides, proteins, polysaccharides, and waxes (Gregorich et al., 1994; Guimaraes et al., 2013). These compounds have a relatively rapid turnover in soil and are used readily as substrates by soil microorganisms (Tan, 2014). NHS accounts for about 20 to 30% of the soil humus (Brady and Weil, 2010) and the distribution of NHS gradually decreased as SOM undergoes further decomposition and stabilization into more recalcitrant humic substances (Tan, 2014). Khalafalla et al. (2019) found that NHS significantly decreased to below 5% compared to humic substances in soil after 60 days of incubation, regardless of fertilization. Likewise, Raiesi et al. (2021) reported that non-humified fractions accounted for about 3-5% of the total SOC in cultivated soils.

Humic substances generally are dark-colored amorphous substances found in soil, resulting from the decomposition of plant and animal residues (Stevenson, 1994). Humic substances are composed of huge molecules with variable structures characterized by aromatic rings, with molecular weight varying from 2000 to 300,000 g/mol (Brady and Weil, 2010). Because of their complexity, they are the organic materials most resistant to microbial attack and are very important in C sequestration and C cycling (Yang et al., 2004a and 2004b). Humic substances can improve soil buffering capacity, increase moisture retention, and supply plants with available

micronutrients (Guimaraes et al., 2013). Humic substances comprise about 60 to 80% of the SOM (Brady and Weil, 2010). Humic substances were chemically fractionated into fulvic acid (FA), humic acid (HA), and humin (HM) (Stevenson, 1994). FA is soluble in both acid and alkali solution, while HA is soluble in alkali and precipitate in acidic solution, and HM is insoluble in any solution (Khalafalla et al., 2019). These humus fractions differ in quantity and quality depending largely on soil type, land-use types, tillage practices, cultivation duration, parent materials, climate, altitude, vegetation type, and soil management (Khalafalla et al., 2019; Raiesi et al., 2021). Guimaraes et al. (2013) compared humus fractions in soil cultivated with different crops (conventional coconut, integrated coconut, citrus, and native forest) and found that native forest has significantly higher FA, but lower HA and HM than those of coconut and citrus crops. They also reported the distributions of FA, HA, and HM to the total SOC were about 19-32%, 15-25%, and 49-65%, respectively. Similarly, Ukalska-Jaruga et al. (2019) compared the humus fractions between grasslands and arable lands and found that the distribution of FA, HA, and HM to the total SOC was approximately 16.6-22%, 36.3-42%, and 35.6-42.6%, respectively. They also reported that arable lands had higher FA and HA but lower HM than grasslands. Generally, most previous studies have reported the distribution of humus fractions relative to the total SOC across different land-use types. However, no study has examined the relative proportion of these humus fractions in relation to the increment of SOC under various land uses. Understanding the relationship between these humus fractions to the increment of SOC is critical for elucidating SOM accumulation and stabilization process, which directly reflects overall SOM quality and its potential for C sequestration.

Previous studies have assessed SOM quality across various land-use types by evaluating humification index (HI) and degree of transformation (DT) (Asensio et al., 2014; Guimaraes et al., 2013; Ukalska-Jaruga et al. (2019; Yang et al., 2004a and 2004b). However, no study has examined the relationship between these indices and the increment of the total SOC content. Moreover, no study has reported the C/N ratio in FA, HA, and HM relative to the increment of the total SOC. N plays a key role in the formation of humic matter. Brady and Weil (2010) documented if the C/N ratio of organic matter added to soil exceeds about 25:1, the soil microbes will have to scavenge N sources (NH_4^- and NO_3^-) for the decomposition, thus immobilization and mineralization occur simultaneously in the soil. During organic matter decomposition, only one-third (two-third lost as CO_2) of C and considerable N is metabolized by microbes and incorporated into their cells. As decay proceeds, microbes polymerize some of the simpler new compounds with the complex residual products together into long and complex chains of high molecular weight compounds. These compounds interact with N-containing amino compounds, forming a significant component of stable humus, causing the C/N ratio to decrease. Tan (2014) also reported that the C/N ratio decreased with an increased rate of the degree of humification. The decrease of C/N ratio is due to microbes utilizing N to synthesize amino acids, proteins, DNA, and other nitrogenous compounds, which are essential precursors in the formation of stable humic substances (Beady and Weil, 2010; Tan, 2014). Moreover, previous studies have reported that long-term N fertilization leads to soil acidification, suppressing microbial activity, increasing lignin polymer concentration, which is a stable form of organic matter (Bai et al., 2018; Bonner et al., 2019; Hasegawa et al., 2021; Ye et al., 2018). In most soils, the C/N ratio falls within narrow limits to about 10 – 15, when the decomposition is completed, indicating organic matter decomposition is in equilibrium with the synthesis and accumulation of new organic materials (Brady and

Weil, 2010; Tan, 2014; Ukalska-Jaruga et al., 2018). Generally, the decrease of the C/N ratio in FA, HA, and HM characterizes well-developed humic matter (Tan, 2014). Therefore, understanding the dynamics of the C/N ratio in humus fractions in relation to the increase of SOC is critical for assessing SOM quality and improving soil management practices aimed at enhancing SOM stability and C sequestration.

The objectives of the study were (i) to investigate differences in organic matter fractions, including NHS, Debris, FA, HA, and HM in soils under six land-use types: orchard, paddy, upland, greenhouse, volcanic ash, and reclaimed soils; (ii) to examine the relative proportion of these fractions to the increment of the total SOC content; and (iii) to evaluate the impact of N on the formation and stability of humic substances by analyzing C and N contents in FA, HA, and HM fractions.

3.3 Materials and Methods

3.3.1 Study area and soil sample collection

To ensure a broad geographical distribution in the region, 362 soil samples, including orchard (65), upland (65), paddy (64), greenhouse (67), volcanic ash (58, and reclaimed (43) soils were collected randomly across South Korea peninsula. Orchards are mainly grown with apple, grape, peach, and pear fruits. Orchard farms were located extensively across the provinces, with a maximum separation distance of about 80 km. Within counties, for example, Chungju and Yesan, the average distance between farms was around 10 km. Similar field selection patterns were applied for upland, paddy, and greenhouse soils, considering their geographical separation (average 7-10 km apart). All reclaimed and volcanic ash soils, mainly cultivated with rice, were collected from Jeongbuk-do and Jeju-island, respectively, with a minimum

separation distance of about 2 km between fields. Triplicate soil samples (0-30 cm depth) were randomly collected from a field, regarding the overall slop and plant density in the field. For orchard farms, soil samples were collected from the points adjacent to canopies along the midpoint of the tree row. Three tree rows representing typical topographical distances, slopes, and plant densities within each orchard were carefully selected. One soil sample was taken from each tree row to reflect spatial heterogeneities within the farm. Soil samples were mixed in a pot, and about 500 g of soil samples were transferred into a plastic zipper bag. These fresh soil samples were returned to the laboratory within two days and air-dried under shadow for at least one week. The air-dried soil samples were crushed and sieved to pass a 2-mm screen, then stored in a 1 litter plastic bottle for later analysis.

3.3.2 Selection of soil samples

To select soil samples representing a wide range of organic carbon content in each land use, all 362 collected samples were analyzed for total SOC in a single replication. After analysis, 30 soil samples, covering low to high C content, were randomly selected from each land use. Thus, a total of 180 soil samples were analyzed in this study. These samples were used to determine SOC, TN, NHS, debris, FA, HA, and HM.

3.3.3 Extraction of non-humic and humic substances

The extraction of non-humic and humic substances is shown in Figure 3.1. Weigh 4 g of air-dried soil samples into a 20 mL PP tube (test tube cylin. PP tube 24 x 95, Kartell S. P. A, Italy), then add 12 mL of extraction reagent containing 0.5 N HCl in 2.85 M $MgSO_4$. The sample was stirred using a small stainless-steel spatula (HA.HSN009.18, L150, 2 Blade-2 x 40, Stem-ϕ1.2mm) for 30 seconds to float the

debris and the sample was stand at room temperature for about 10 minutes. Then, repeat the stirring for two more times. After the last stirring was finished, the PP tube was capped tightly using PP tube cap (Tappo polit. D 22, Kartell S. P. A, Italy), secured with O-ring (ϕ 18.9 mm x ϕ 23.3 mm) around the neck of the PP tube to ensure protection during high-speed centrifugation. The extract was then centrifuged at 8000 g for 5 minutes. After centrifugation was finished, transfer the supernatant (NHS + debris) to a 50 mL falcon tube. The extraction was repeated once with 0.5 N HCl in 2.85 M $MgSO_4$, following the same stirring procedure. A third extraction was then performed using distilled deionized water (DDW), adhering to the same stirring procedure as described. After three extractions, the total volume of the NHS and debris supernatant was 36 mL. The supernatant was kept aside for later separation of NHS and debris.

The precipitated soil particles remaining after NHS and debris extraction was used to extract fulvic acid (FA), humic acid (HA), and humin (HM). Twenty milliliters of fresh 0.5 M sodium hydroxide (NaOH) in 0.1 M sodium pyrophosphate ($Na_4P_2O_7$) and 2% of sodium hexametaphosphate ($Na_6P_6O_{18}$) was added to the precipitated soil particles, then the mixture was vortexed. The sample was then sonicated at 50% (200 W, 30°C) for 10 minutes, and shaken at 180 rpm for 6 hours. After shaking, the sample was centrifuged at 10,000 rpm for 5 minutes, and the supernatant was transferred into a 250 mL plastic bottle. The extraction was repeated once more for reclaimed soils and five more times for volcanic ash and common land uses, following the procedures described above. Thus, the total extraction volume was 40 mL for reclaimed soils and 120 mL for volcanic ash and common land uses. The total supernatant volume, containing FHA (FA + HA), was set aside for the subsequent separation of FA and

HA. The precipitated soil particles remaining after FHA extraction constituted HM. These soil particles were air-dried and ground to pass through a 150 µm sieve.

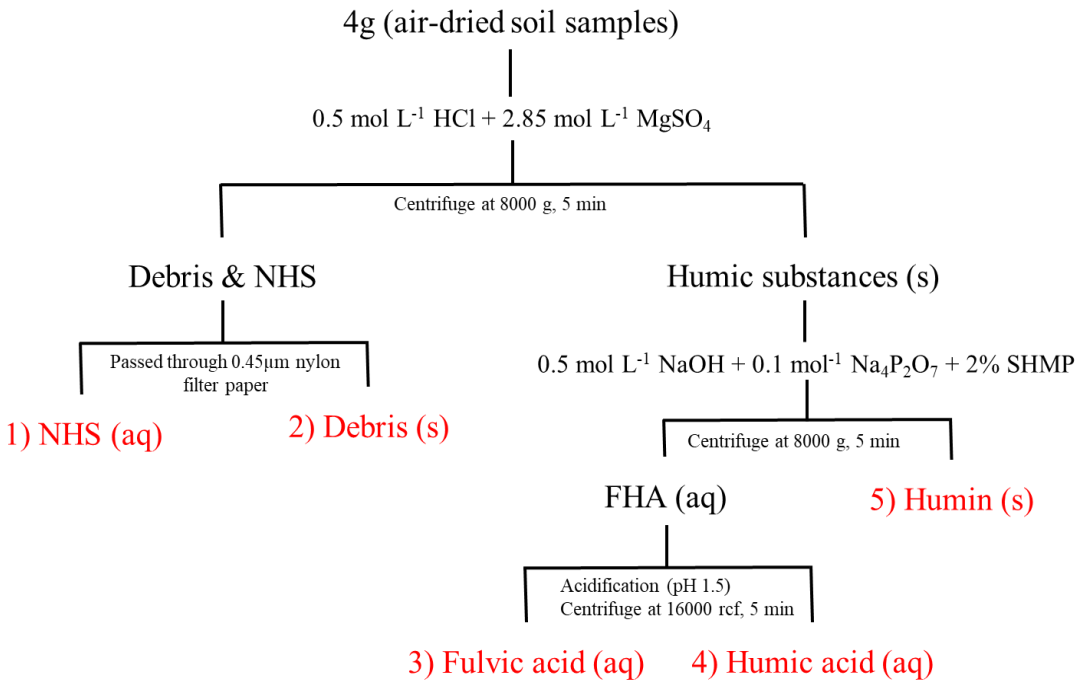

Fig. 3.1 Schematic diagram illustrating the separation of non-humic and humic substances, *adopted from Stevenson (1994) and Brady and Weil (2010)*.

3.3.4 Separation of NHS and debris

NHS and debris were separated by filtration. A 0.45 µm nylon filter was placed on the cap of a 50 mL Falcon tube assembled with a custom-designed filter unit, then tightly secured. Place a new falcon tube into the vacuum column, then position the custom-designed filter unit on top of the vacuum column (see the picture below). After operating the vacuum pump, the air valve was opened to initiate filtration. After the filtration was finished, the remaining debris on the filter and 3 new blank filters were

dried at 90°C until constant weight was achieved. The supernatant obtained after filtration represented the NHS sample.

Fig. 3.2 Procedures for the separation of debris and non-humic substances.

3.3.5 Separation of FA and HA fractions

FA and HA were separated by an acidification process. A 1.6 mL aliquot of FHA solution was transferred into a 2 mL Eppendorf tube, then 0.2 mL of 8.6 N (4.3 M) sulfuric acid (H_2SO_4) was added. The mixture was vortexed and allowed to stand at room temperature for about 3 hours. Generally, HA is soluble in alkaline or neutral pH and precipitates under acidic condition. After standing, the mixture was centrifuged at 16,000 rcf for 5 minutes. The supernatant was FA, and the precipitated particles left after centrifugation were HA.

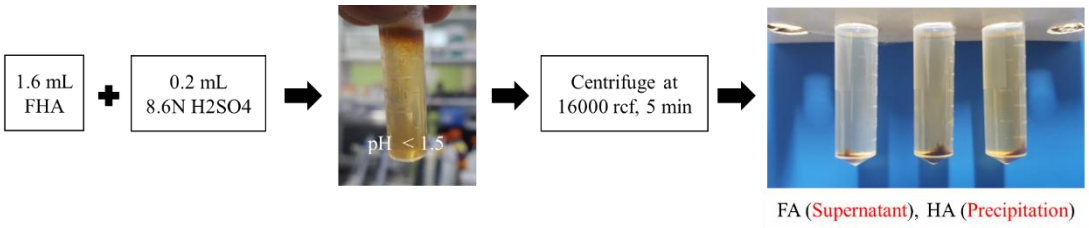

Fig. 3.3 Separation of fulvic acid and humic acid through the acidification process.

3.3.6 SOC and TN analysis

We followed the alkaline persulfate oxidation method described in Chapter 1 for the simultaneous determination of SOC and TN in the 180 selected air-dried soil samples in Section 2.3.2. About 5 g of soil per sample was crushed and sieve to pass a 150 µm screen. The analyses were performed in triplicate for each sample.

3.3.7 Analysis of non-humic substances

Non-humic substances (NHS) were analyzed by the TOC Analyzer (Sievers 5310 C). Briefly, 5 mL of NHS or standard solution (30, 60, 100, 140 ppm C, $C_8H_5KO_4$ stock, pH 2) was transferred into a 50 mL Falcon tube, and then 10 mL of acidification buffer (50 g of sodium hexametaphosphate and 12.5 mL of H_2PO_4) was added and swirled gently. After that, the NHS samples were centrifugated at 3,000 rpm for 5 minutes to remove the remaining Mg^{2+} residues, then the supernatant was transferred into new Falcon tube. The Falcon tube was then covered with aluminum foil and placed on the autosampler for analysis. TOC Analyzer (Sievers 5310 C) consists of 2 oxidizer cartridges, 15% Ammonium persulfate, and 6 M H_3PO_4. The oxidizer cartridges should be replaced every 2 to 3 months.

Fig. 3.4 Analysis of C in NHS using a TOC Analyzer (Sievers 5310C).

3.3.8 Preparations and digestion procedures for FHA and FA samples

We followed the alkaline persulfate digestion procedures described in Chapter 1, but the concentrations of C and N standards, the basic pH indicator solution, and oxidizing reagent were adjusted to match the concentration of FHA and FA samples.

3.3.8.1 Calibration curves for C and N

The calibration standard were potassium hydrogen phthalate (KHP; Kanto, Tokyo, Japan) and glycine (Junsei, Tokyo, Japan). A stock solution containing 10 mg C L^{-1} and 1 mg N L^{-1} (C/N ratio = 10) was prepared by dissolving 17.608 g of KHP and 5.360 g of Glycine in 500 mL of DDW, and diluting the volume to 1 L. The standard curve for FHA and FA ranged from 200 to 1600 ppm C (250 ppm interval).

3.3.8.2 The basic pH indicator

The basic pH indicator was 15 mL of 0.1% (w/v) AYR in 100 mM K_2CO_3 and 10 mM NaOH. 0.1 g of AYR was dissolved in 80 mL of DDW, then diluted to a final

volume of 100 mL. The basic pH indicator solution was prepared immediately before use by dissolving 13.89 g of K_2CO_3 in 500 mL of distilled-deionized water (DDW), then mixing with 15 mL of 0.1% (w/v) AYR, and adjusting the volume to 1 L. The solution was stored in an amber bottle and purged with N_2 to minimize the absorption of atmospheric CO_2.

3.3.8.3 Oxidizing reagent

The reaction concentration of oxidizing reagent was 0.4 M $Na_2S_2O_8$ in 0.6 M NaOH. For FHA, the total digestion volume is 2 mL, with 1 mL of oxidizing reagent. Therefore, the final concentration of the oxidizing reagent was 0.8 M $Na_2S_2O_8$ in 1.2 M NaOH. Dissolve 48.59 g of $Na_2S_2O_8$ and 12.25 g of NaOH in 150 mL of DDW, then mess up to a final volume of 250 mL.

3.3.8.4 Digestion procedures

FHA digestion procedures: Transfer 1.0 mL of FHA into a 2 mL Eppendorf tube and centrifuge at 10,000 rcf for 3 minutes. Subsequently, transfer 0.8 mL of the supernatant into a 10 mL vial, and add 0.2 mL of 2.0 N H_2SO_4 to neutralize the pH of FHA to approximately 7.0. Then, transfer 0.5 mL of the standard solution (C/N = 10) into the vial, then add 0.5 mL of DDW. Next, 1 mL of oxidizing reagent was added and swirled gently. The treated samples were set aside for digestion.

FA digestion procedures: After the separation of FA from HA by centrifugation (see Section 3.3.5), the FA sample was strongly acidic (pH < 1.5). Transfer 1.0 mL of FA into a vial, then add 0.2 mL of 2.25 M NaOH to adjust the pH to around 7.0. Next, transfer 0.5 mL of the standard solution (C/N = 10) into the vial and add 0.7 mL of DDW. The treated samples were set aside for digestion.

A glass tube containing 1 mL of a basic pH indicator solution was carefully inserted vertically into the FHA or FA vials. Then, the vial containing both the mixture solution in the outer space and the basic pH indicator solution in the inner tube was immediately sealed with silicone rubber stopper and aluminum rings, then crimp tightly using electronic crimper (CSR 6A20C0). Then, the vials were autoclaved at 110°C for 1 h, which is analogous to the conditions used in previous studies (Zhou et al., 2003 and Suzumura et al., 2008). The CO_2 released during soil oxidation was trapped by the basic pH indicator solution inside the digestion flask.

3.3.8.5 Determination of C and N in FHA and FA samples

After cooling to room temperature for 10 min, the digestion vials were refrigerated at 4°C for 1 h to facilitate CO_2 absorption. Prior to measurement, the caps and stoppers were carefully removed. An aliquot of the basic pH indicator solution was measured for C at 375 nm and 490 nm using a spectrophotometer (U3900, Hitachi, Tokyo, Japan). The color of the basic pH indicator solution gradually changed from red to yellow (pH ~12.0 to 10.0) as CO_2 absorption increased, which in turn caused the absorbance at 490 nm to decrease and the absorbance at 375 nm to increase. The changes in absorbance at 490 nm and 375 nm did not follow a linear relationship with increasing C contents. Based on the Henderson-Hasselbach equation (pH dependence of the conjugate acid and base ratio) (Harris, 2000), the absorbance ratio between 375 nm and 490 nm (A375/A490) was utilized for the determination of SOC content.

An aliquot of the digestion mixture was analyzed for NO_3^- by the salicylic acid-nitration method (Cataldo et al., 1975). Briefly, 200 μL of the aliquot was mixed with 0.6 mL of 5% (w/v) salicylic acid dissolved in 10 mL of concentrated H_2SO_4. After standing for 10 min, it was alkalized with 6 mL of 4.05 M NaOH, and the absorbance

at 414 nm was measured. Soil particles in the aliquot were removed beforehand by centrifugation at 16000 g for 3 min.

The HA fraction is calculated by substacting FA from FHA (HA = FHA – FA).

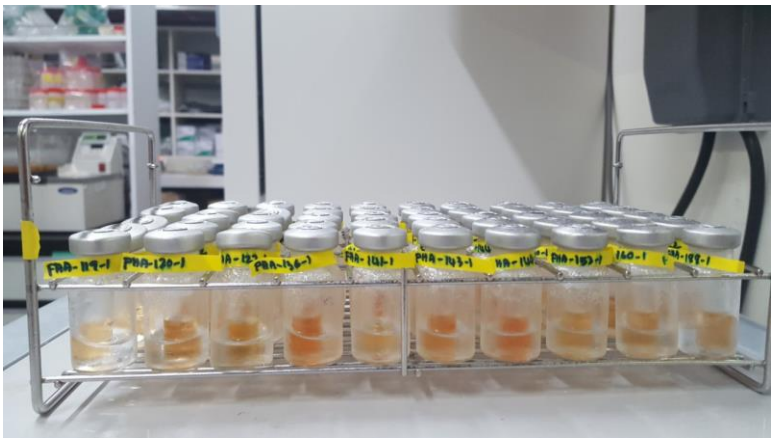

Fig. 3.5 FHA and FA samples after digestion and cooling processes.

3.3.9 HM digestion procedure

The precipitated soil particles remaining after FHA extraction constituted HM (see Section 2.3.3). After the soil particles were air-dried and ground to pass through a 150 µm sieve, 0.2 g of ground soil sample was weighed into 30 mL vial, then add 2 mL of DDW and kept aside for digestion. 2 mL of standard solution (C/N =10; 500 – 3000 ppm C, 500 ppm C interval) was transferred to new vials. 5 mL of oxidizing reagent (0.56 M Na_2S_2O8 in 0.84 M NaOH) was added, and the mixture was swirled gently. A glass tube (10 mm OD, 33 mm height, Wheaton, USA, assembled with teflon tube on the top 17 mm height) containing 1 mL of the basic pH indicator solution (15 mL of 0.1% (w/v) AYR in 1.0 M K_2CO_3) was carefully inserted vertically into the vial.

Then, the vial containing both the mixture solution in the outer space and the basic pH indicator solution in the inner tube was immediately sealed with a silicone rubber stopper (13 mm ID x 20 mm OD, white color) and aluminum rings (WH.224191, 20 mm, solid-top type, Wheaton, USA), then crimp tightly using electronic crimper (CSR 6A20C0). The vials were autoclaved at 110°C for 1 h. The CO_2 released during soil oxidation was trapped by the basic pH indicator solution inside the digestion vial.

3.3.10 Determination of C and N in HM samples

For the determination of C and N in the HM sample, we followed the procedures described in Chapter 2, Section 2.3.6.

3.3.11 Assessement of soil humus quality

The humification index (HI) was calculated as the ratio of HA to FA (HA/FA). An HI ratio lower than the limit of 1.0 indicates the predominance of FA over HA, while an HI ratio higher than 1.0 signifies a high degree of conversion of FA into more stable HA. The degree of transformation (DT) was calculated as the sum of FA and HA over HM (FA+HA)/HM. Lower DT (DT < 1) indicates greater SOM stability. On the other hand, the C/N ratio in the FA, HA, and HM fractions were computed, respectively, to examine the impact of land use and management practives on the formation and stability of SOM.

3.3.12 Statistical analysis

Analysis of variance (one-way ANOVA) was conducted to test for significant differences in the relative proportions of NHS-C, debris-C, FA-C, HA-C, and HM-C within each land use and among these fractions across different land uses at $P \leq 0.05$.

Post hoc analysis using the least significant difference (LSD) was applied for multiple mean comparisons. Statistical analysis was performed using SAS 9.4.

3.4 Results and Discussion

3.4.1 SOC contents of the selected soil samples

To cover a wide range of SOC, all 362 soil samples were analyzed, and only 30 soil samples covering low to high SOC content were randomly sellected from each land-use type. SOC content differed significantly amoung land uses and followed the order: volcanic ash > common land uses (orchard, paddy, upland, and greenhouse) > reclaimed soils (Fig 3. 6). The SOC content in volcanic ash soils varried from 15 to 83 g kg^{-1}, while common land uses ragned from 2 to 60 g kg^{-1} and reclaimed soils varied from 2-8 g kg^{-1}. On average, volcanic ash soils had more than 2 times the total SOC compared to the common land uses and more than 10 times compared to the reclaimed soils. The higher SOC in volcanic ash soils compared to the common land uses may result from thier parent materials (Yang et al., 2023), physico-chemial protections through allophane and imogolite (Torn et al., 1997; Parfitt et al., 2009), and through complexation of humus with aluminum (Al) and iron (Fe) (Pizzaro et al., 2003; Takahashi et al., 2020). The volcanic ash soil developed from pyroclastic rocks, whereas soils under common land uses formed from acidic rocks (Yang et al. 2023). The lowest SOC in reclaimed soils compared to the other land uses mainly attributed to the fact that the reclaimed soils have been constructed in coastal areas (to increase food security) with high salinity and high ground water levels (Jeong et al., 2020; Lim et al., 2020). In salt-affected soil like reclaimed soils, soduim (Na) replaces with polyvalent cations, such as Ca^{2+}, forming Na-organic or Na-humate linkages that are highly soluble and prone to leaching (Sumner et al., 1998). As a result, SOC contents

are frequently lower than in other land uses (Wong et al., 2010). The SOC content of the selected reclaimed soils reported here was lower than those reported recently by Park et al. (2022) who analyzed 72 rice paddies and found SOC levels ranging from 6.6 to 12.8 g C kg^{-1}). The differences can be attributed to variations in sampling design and timing, as well as the frequent imput of organic matter, such as compost or rice root residue left after harvesting (Lim et al. 2020). However, both our SOC levels and theirs were lower than the optimum SOC levels recommended by the Korean government (11.6-17.4 g C kg^{-1}) (RDA, 2017).

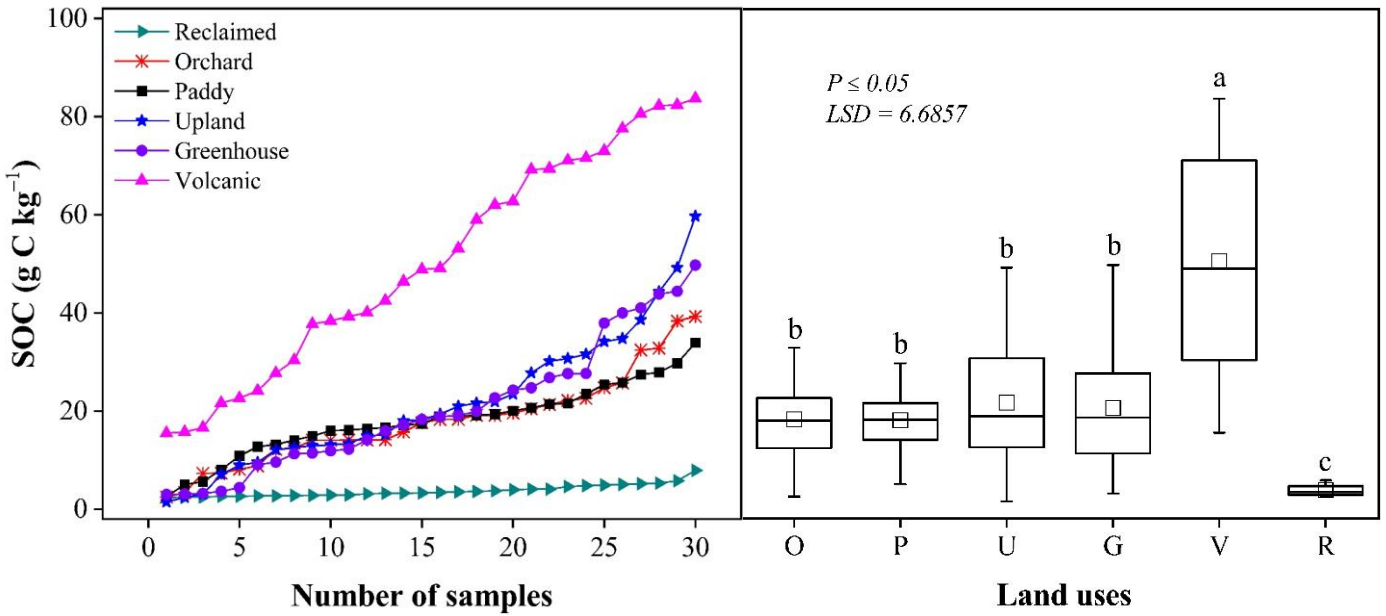

Fig. 3.6 SOC contents of the selected soil samples. To cover a wide range of SOC, several soil samples were collected from orchard (O), paddy (P), upland (U), greenhouse (G), volcanic ash (V), and reclaimed (R) soils across the Korean peninsula. All samples were analyzed for SOC using alkaline persulfate digestion method. 30 soil samples, with low to high SOC contents were selected from each land use. Squares and lines in the box plots represent mean and median values, respectively. One-way ANOVA test followed by Post-hoc LSD were applied for multiple mean comparison, with different lower-case letters indicating significant differences ($P \leq 0.05$). Statistical analysis was performed using SAS 9.4.

3.4.2 C content in SOC fractions

The fractions of SOC in selected soil samples collected from different land uses (orchard, paddy, upland, greenhouse, volcanic ash, and reclaimed soils) are shown in Fig. 3.7. Regardless of land use types, the non-humic and humus fractions of SOC increase in a following order: NHS-C < debris-C < HA-C < FA-C < HM-C. HM-C is approximately 40, 5, and 2 times greater than NHS-C, debris-C, FA-C and HA-C, respectively. HA-C in common land uses and reclaimed soils was lower than FA-C but the differences were not statistically significant. In contrast, HA-C in volcanic ash soil was significantly lower than FA-C. Volcanic ash soils are known for their high content of amorphous minerals like allophane and imogolite, as well as Al-humus complex, which bind to organic acids such as FA more effectively than HA, thereby promoting FA stability (Takahashi and Dahlgren, 2016). In contrast, regular cultivation and liming in common land uses and reclamation soils, along with organic and inorganic fertilization, can increase microbial activity and C mineralization. This process may reduce Al-humus complex formation, leading to more dynamic cycling of FA and HA, ultimately balancing these fractions within the soil (Takahashi et al., 2006a; Stevenson, 1994).

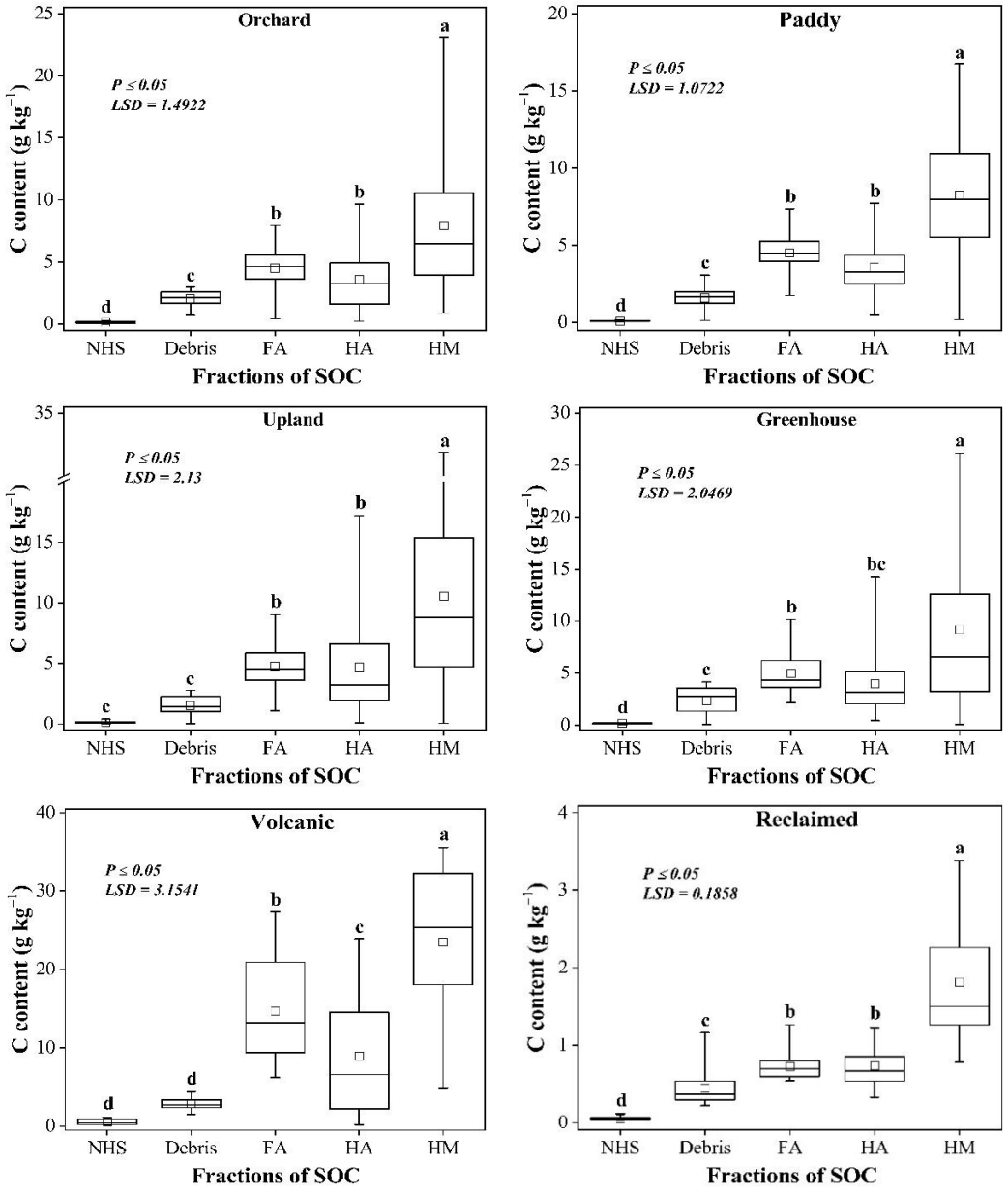

Fig. 3.7 The contribution of NHS-C, debris-C, FA-C, HA-C, and HM-C to the total SOC in soil samples collected from paddy, upland, orchard, greenhouse, volcanic ash, and reclaimed soils. Squares and lines in the box plots represent mean and median

values, respectively. One-way ANOVA and Post hoc LSD were performed for multiple mean comparison. Different lower-case letters indicate significant difference at $P \leq 0.05$. Statistical analysis was performed using SAS 9.4.

3.4.3 Relative proportion of NHS-C to the increment of SOC content

On average, the relative proportion of NHS-C to total SOC ranged from 0.7 to 1.5% (Fig. 3.8a). The NHS-C in reclaimed soils was significantly higher than in other land uses, followed by orchard, greenhouse, and volcanic ash soils, while the lowest was observed in paddy soils. Although the NHS-C was significantly different among selected land uses, we found that their contribution to SOC was very low and tended to decrease as SOC increased, regardless of land use types (Fig. 3.8b). Our findings aligned with those of Khalafalla et al. (2019), who reported that NHS decreased as incubation duration increased, with its proportion falling below 5% after 60 days of incubation, regardless of fertilizer applications. The low level of NHS in these regular cultivated soils would indicate a high rate of SOM decomposition, resulting in strong depletion of most labile C forms (Raiesi et al., 2021). NHS, which serve as food and energy sources for microorganisms, is defined to include all substances released by the decomposition of plants and other organisms residues in the forms of identifiable chemical and physical properties, such as carbohydrates, amino acids, protein, lipids, waxes, nucleic acids, lignin, and many other organic compounds that make this NHS fraction different from the humus fraction (Brady and Weil, 2014; Tan, 2014). The accumulation of SOC enhances the process of humification, where all NHS forms are progressively transformed and eventually contribute to the formation of more stable humic substances, thereby reducing the concentration of NHS released into the soil (Kogel-Knabner, 2000; Stevenson, 1994; Tan, 2014). Although the NHS contributes only a small portion of C, it is essential for the formation of humic matter. Therefore,

enhancing NHS through the application of organic and inorganic fertilizers are recommended (Tan, 2014).

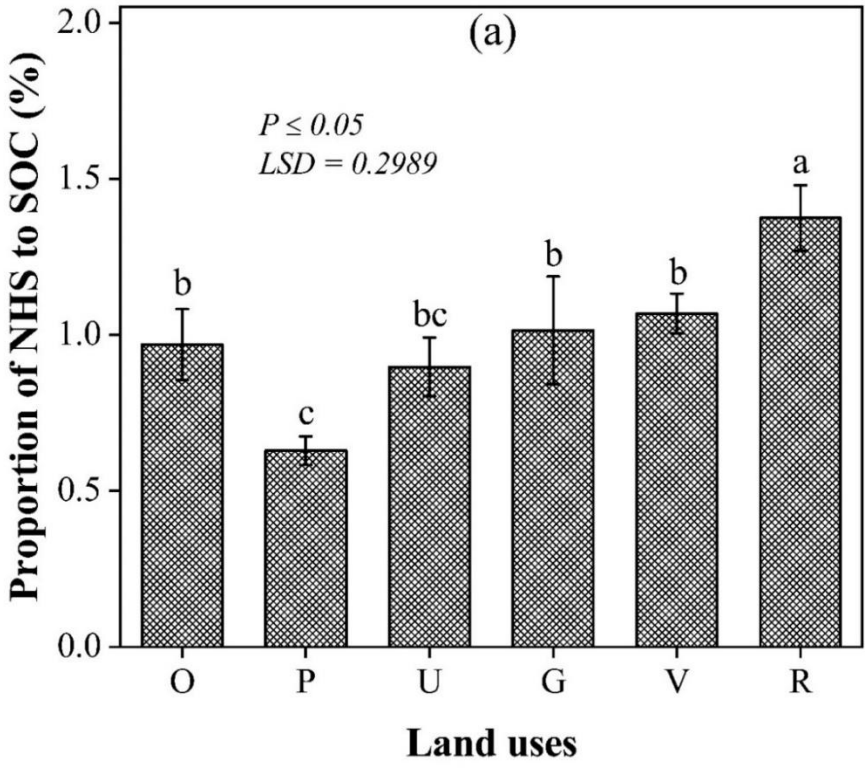

Fig. 3.8a The proportion of NHS-C to the total SOC in selected soil samples collected from orchard (O), Paddy (O), upland (U), greenhouse (G), volcanic ash (V), and reclaimed (R) soils. Means followed by different lower-case letters indicate significant difference at $P \leq 0.05$ based on one-way ANOVA followed by Post-hoc LSD test. Vertical bars represent standard errors (n=3). Statistical analysis was performed using SAS 9.4.

(b)

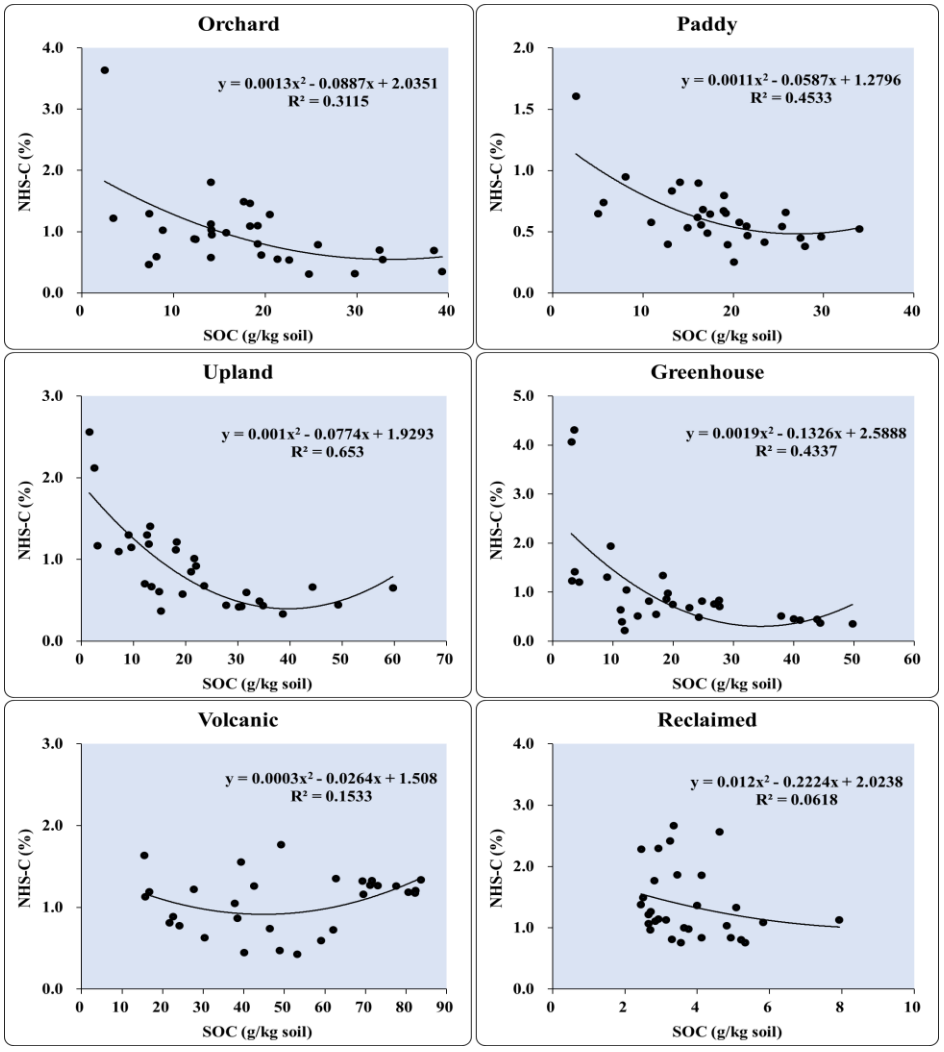

Fig. 3.8b Relative proportions of NHS-C to the increment of SOC in selected soil samples collected from orchard (O), Paddy (O), upland (U), greenhouse (G), volcanic ash (V), and reclaimed (R) soils. Thirty soil samples with varying SOC contents, ranging from low to high, were selected from each land use. Soil samples were analyzed in triplicate.

3.4.4 Relative proportion of Debris-C to the increment of SOC content

On average, debris-C comprised about 6 to 14% of total SOC (Fig. 3.9a). Our results aligned with those of Golchin et al. (1994a) who reported the debris-C contributions ranging from 6.9-31.3% of the total C. Orchard soils had significantly higher debris-C ($P \leq 0.05$) than other land uses, followed by greenhouse, and reclaimed soils, while the lowest was observed in paddy and volcanic ash soils. The lower debris-C in the paddy and volcanic soils may be due to less input of organic matters, as aboveground residues (i.e., rice straw) are often removed from the fields for use in livestock farming (RDA, 2019). The higher debris-C in orchard than in other land use types may be attributed to the formation of soil aggregates in a cultivated soil with minimal disturbance (Six et al., 1998). The above and below ground plant residues are the primary source of SOM. As they decompose, these residues function as binding agents that help hold soil particles together, forming aggregates and promoting soil C storage (Osborne et al., (2014). The aggregates are mainly stabilized by carbohydrate-rich root or plant debris, which occluded within them (Golchin et al., 1995). However, we observed that debris-C decreased as SOC increased, regardless of land use types (Fig. 3.9b). The decrease in debris-C can be attributed to the reduction in particle sizes of debris fractions (Rovira and Vallejo, 2002). Golchin et al. (1994a) also observed a decrease in debris-C as the fragment size decreased.

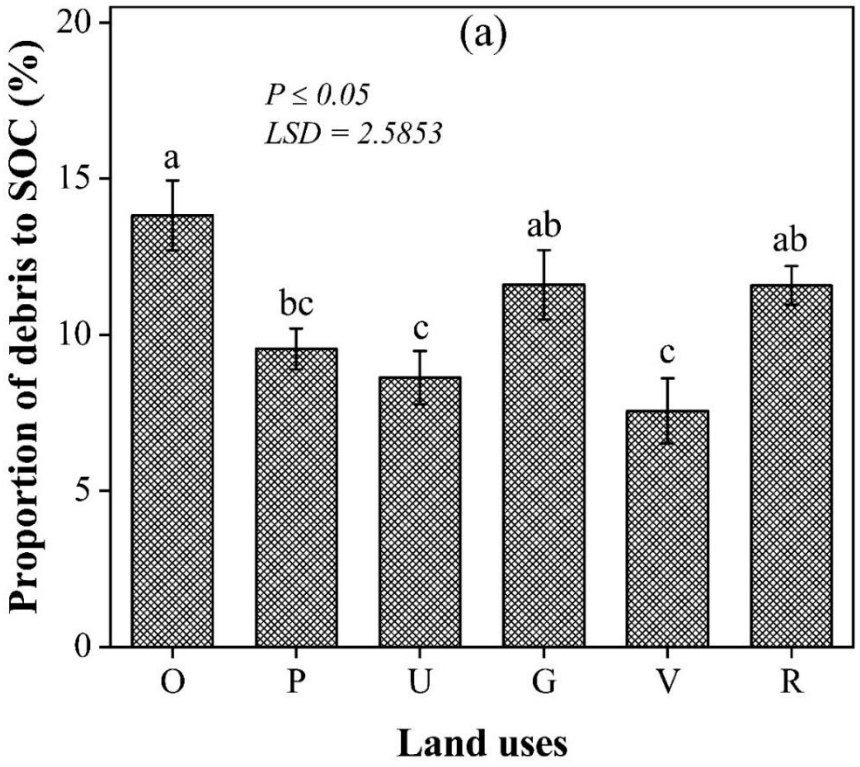

Fig. 3.9a The proportion of debris-C to the total SOC in selected soil samples collected from orchard (O), Paddy (O), upland (U), greenhouse (G), volcanic ash (V), and reclaimed (R) soils. Means followed by different lower-case letters indicate significant difference at $P \leq 0.05$ based on one-way ANOVA followed by Post-hoc LSD test. Vertical bars represent standard errors (n=3). Statistical analysis was performed using SAS 9.4.

(b)

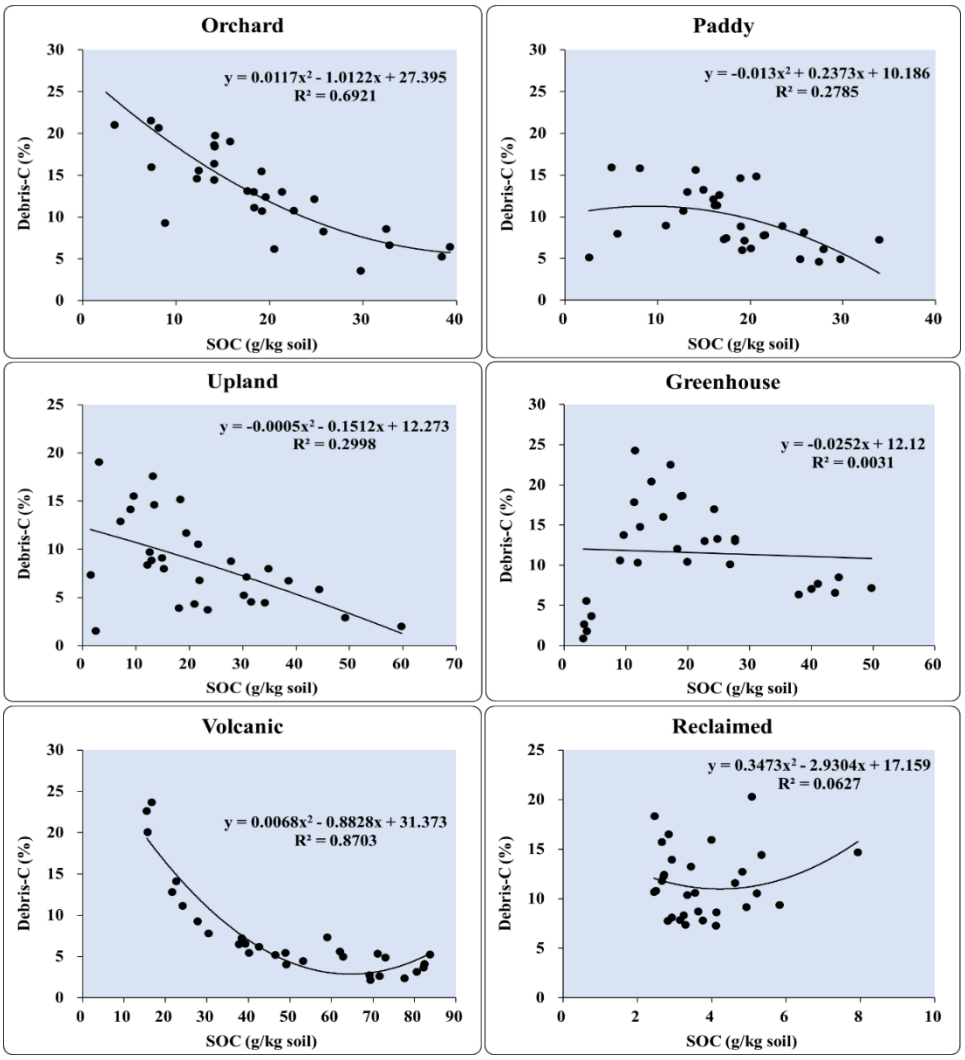

Fig. 3.9b Relative proportions of debris-C to the increment of SOC in selected soil samples collected from orchard (O), Paddy (O), upland (U), greenhouse (G), volcanic ash (V), and reclaimed (R) soils. Thirty soil samples with varying SOC contents, ranging from low to high, were selected from each land use. Soil samples were analyzed in triplicate.

3.4.5 Relative proportion of FA-C to the increment of SOC content

Fig. 3.10a and 10b demonstrated the relative proportion of FA-C to SOC of selected soil samples collected from orchard, paddy, upland, greenhouse, volcanic ash, and reclaimed soils across the Korean peninsula. On average, FA-C accounted for about 20 to 32% of the total SOC (Fig. 3.10a). Our results were similar to those reported by Reddy et al. (2012), Yang et al. (2004b), and Yang et al. (2006) who reported the relative proportion of FA to SOC ranged from about 23 to 30% in most arable land use systems. The FA-C in greenhouse, upland, paddy, and volcanic ash soils were statistically similar ($P \leq 0.05$; LSD =5.5751). However, the FA-C in greenhouse soils was significantly higher than in orchard soils, while reclaimed soils had the lowest FA-C among all land uses. The lower proportion of FA-C in the reclaimed soils may be caused by high salinity that inhibits microbial activity, which is essential for the decomposition and humification process (Lim et al., 2020). Park et al. (2022) found that SOC concentration decreased with increasing EC. Generally, in salt-affected soils, Na^+ replaces with polyvalent cations, such as Ca^{2+}, forming highly soluble Na-humate linkages, which are prone to be carried away in runoff and leaching faster than other land uses (Summer et al., 1998). Wong et al., (2010) reported that salt-affected soils are usually subjected to increased losses due to dispersion, erosion, and leaching, as a result, SOC contents are often lower than in other land uses. However, we observed that the relative proportion of FA-C decreased as SOC content increased for all studied land uses and when SOC reached about 20 to 30 g kg^{-1}, the decrease of FA-C remained stable, even as SOC continued to increase (Fig. 3.10b). This highlight supports the results of Sun et al. (2012) and Seddaiu et al. (2013), who stated that cultivation destabilizes humus formation and intensifies mineralization processes by increasing soluble forms of FA. We found that the reduction pattern of FA-C in relation to SOC increases differed among land uses. This result reflects the

shifts in organic matter composition and humification dynamics (conversion of FA into more stable form) within the SOM pools due to inherent soil characteristics (allophanic or non-allophanic soil, OM content, aggregations, soil water content, soil compaction) and management practices (Reddy et al., 2012).

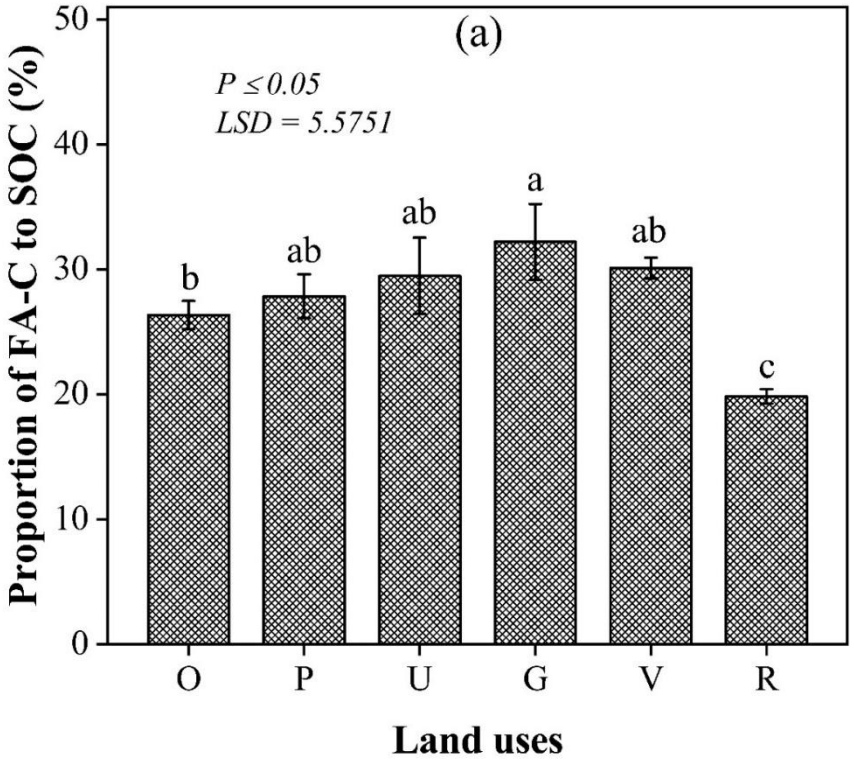

Fig. 3.10a The proportion of FA-C to the total SOC in selected soil samples collected from orchard (O), Paddy (O), upland (U), greenhouse (G), volcanic ash (V), and reclaimed (R) soils. Means followed by different lower-case letters indicate significant difference at $P \leq 0.05$ based on one-way ANOVA followed by Post-hoc LSD test. Vertical bars represent standard errors (n=3). Statistical analysis was performed using SAS 9.4

(b)

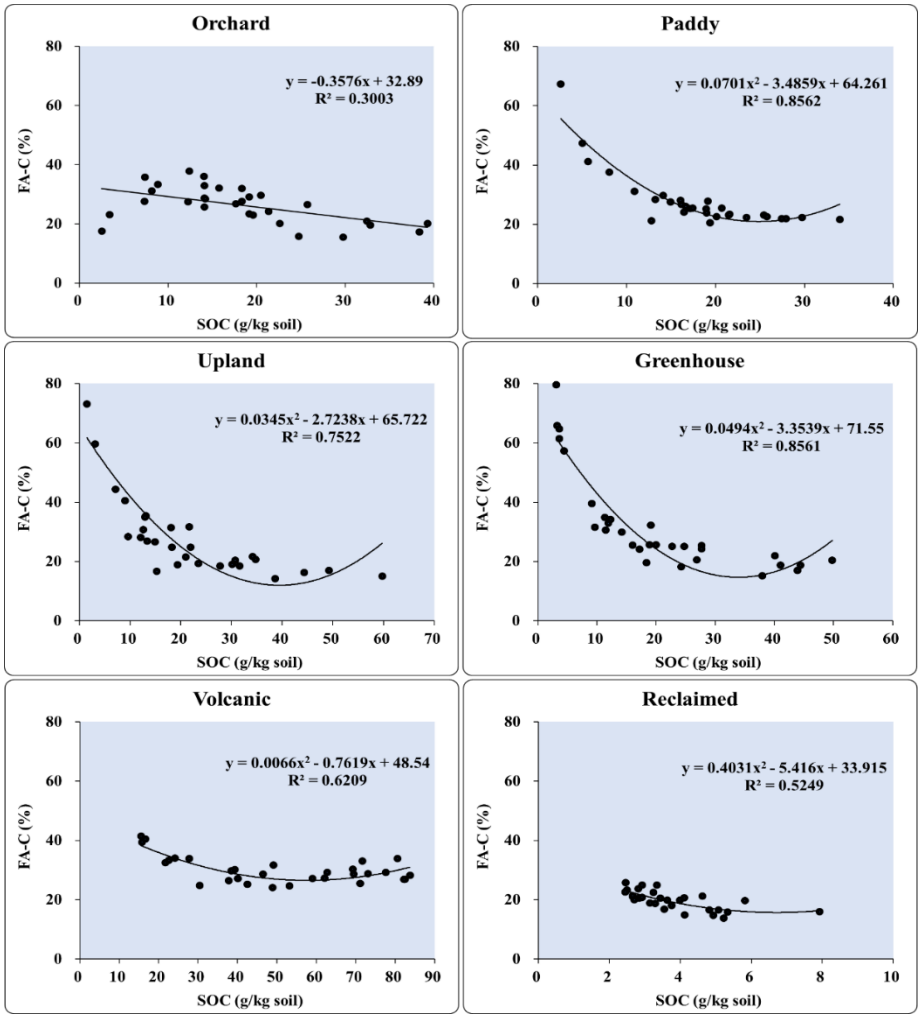

Fig. 3.10b Relative proportions of FA-C to the increment of SOC in selected soil samples collected from orchard (O), Paddy (O), upland (U), greenhouse (G), volcanic ash (V), and reclaimed (R) soils. Thirty soil samples with varying SOC contents, ranging from low to high, were selected from each land use. Soil samples were analyzed in triplicate.

3.4.6 Relative proportion of HA-C to the increment of SOC content

On average, HA-C accounted for about 15 to 20% of SOC (Fig. 3.11a). The contribution of HA-C to SOC in common land uses and in reclaimed soils was similar (~19%; $P \leq 0.05$; LSD = 2.8381). Our results were similar to those reported by Raiesi et al. (2021), Reddy et al. (2012), and Yang et al. (2004b) who reported the relative proportion of HA to SOC ranged from about 19 to 26% in most arable land use systems. Among all land uses, only volcanic ash soils exhibited a significantly lower relative proportion of HA-C to SOC. This lower proportion may be due to less input of organic materials to soil and difference in mineral composition (Park et al. 2022; Yang et al., 2023). Yang et al. (2023) found that the allophane content was greater in Andosols (volcanic ash soils) compared to Inceptisols, which can strongly adsorb organic carbon and enhance overall SOC retention, but often result in lower levels of HA. In addition, a study by Maie et al. (2002) found that the humification process of HA (the formation of middle size molecules) increased but it was lower in Andosoils (Andisols or volcanic ash) compared to paddy soils. However, we found that the relative proportion of HA-C increased as SOC increased, except for greenhouse and reclaimed soils, and the increase of the relative proportion differed among land uses (Fig. 3.11b). Our finding is consistent with results of Maie at al. (2002) and Kunlanit et al., 2019) who reported a positive correlation between SOC and HA. This result suggests that the increase of SOC is ultimately linked to the HA stock, as HA serves a critical role as a cementing agent for aggregate formation, particularly microaggregates, which contribute to stabilization and accumulation of SOC (Zhang et al. 2019).

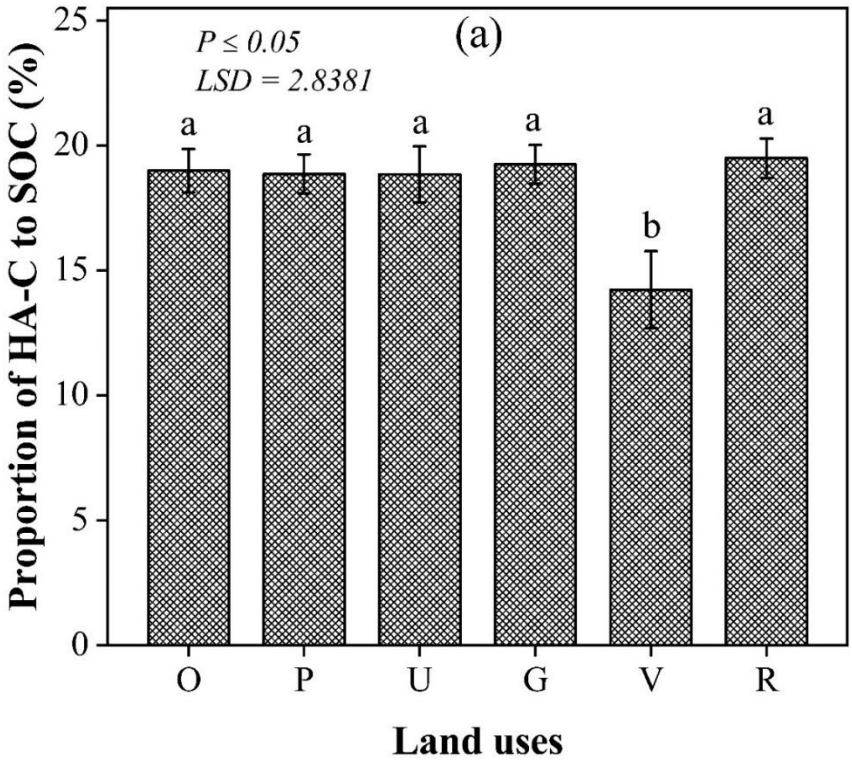

Fig. 3.11a The proportion of HA-C to the total SOC in selected soil samples collected from orchard (O), Paddy (O), upland (U), greenhouse (G), volcanic ash (V), and reclaimed (R) soils. Means followed by different lower-case letters indicate significant difference at $P \leq 0.05$ based on one-way ANOVA followed by Post-hoc LSD test. Vertical bars represent standard errors (n=3). Statistical analysis was performed using SAS 9.4.

(b)

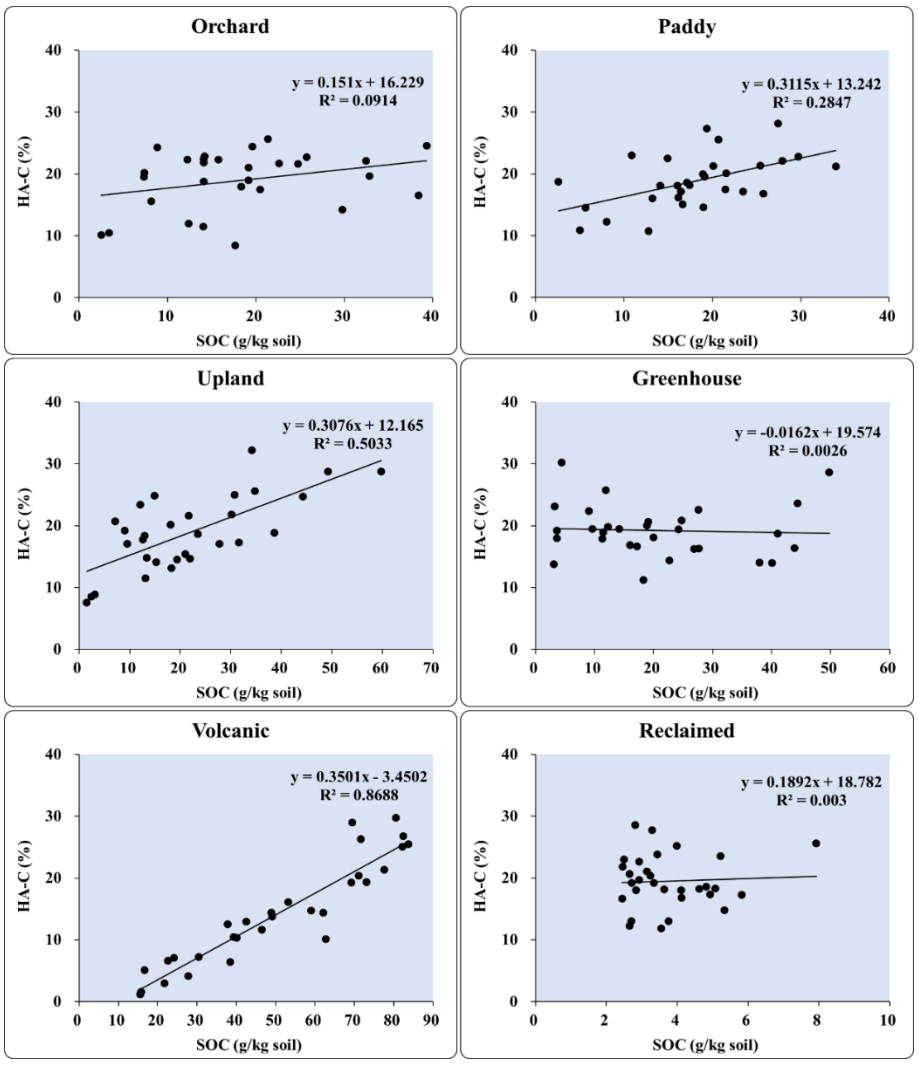

Fig. 3.11b Relative proportions of HA-C to the increment of SOC in selected soil samples collected from orchard (O), Paddy (O), upland (U), greenhouse (G), volcanic ash (V), and reclaimed (R) soils. Thirty soil samples with varying SOC contents, ranging from low to high, were selected from each land use. Soil samples were analyzed in triplicate.

3.4.7 Relative proportion of HM-C to the increment of SOC content

The relative proportion of HM-C to total SOC varied significantly among studied land uses ($P \leq 0.05$; LSD = 5.9688) (Fig. 3.12a). The volcanic ash and reclaimed soils exhibited the highest proportions of HM-C to SOC, both exceeding 47%, and were significantly different from greenhouse soils, which had the lowest proportion (~36%). Orchard, paddy, and upland soils showed intermediate HM-C proportions, falling between the values for greenhouse and volcanic soils. The higher relative proportion of HM-C in volcanic and reclaimed soils suggests enhanced stabilization of SOC in these systems, likely due to soil mineral interactions that protect HM from microbial degradation. Volcanic soils are known to contain high allophane and imogolite content, which are clay minerals with high surface areas and reactive sites that facilitate the adsorption and stabilization of organic matter, especially HM fraction. This is consistent with findings in other studies that highlight the strong organic matter retention capabilities of Andosols, primarily due to their unique mineralogy (Torn et al., 1997; Parfitt et al., 1997). The lower HM-C proportion in greenhouse soils may be attributed to intensive cultivation practices that accelerate organic matter decomposition and destabilize HM structures. Continuous crop production, frequent fertilization, and intensive soil disturbance in greenhouse systems can enhance microbial activity and promote mineralization, thus reducing the stable HM fraction relative to the total SOC (Seddaiu et al., 2013; Sun et al., 2012). Intermediate HM-C proportions observed in orchard, paddy, and upland soils suggest a moderate level of SOC stabilization, possibly influenced by balanced organic matter inputs and relatively lower disturbance levels compared to greenhouse soils. Paddy soils, for example, often have anaerobic conditions that slow down decomposition and favor the accumulation of more stable organic carbon fractions like HM, despite frequent organic matter inputs from crop residues (Mi et al., 2019).

However, we found that the relative proportions of HM-C in paddy, upland, greenhouse, and volcanic ash soils increased as SOC increased, but tended to decrease when total SOC exceeded about 30 g kg^{-1} (Fig. 3.12b). These trends may be influenced by N availability in the soil. In general, as organic matter begins to decompose, microbes polymerize some of the simpler new compounds with more complex residual products, forming long, complex chains that resist further decomposition. These high molecular weight compounds (e.g., lignin) interact with N-containing amino compounds, leading to the formation of resistant humus (Berg and Matzner, 1997; Brady and Weil, 2010). However, when nitrogen availability exceeds microbial requirements, it can promote the breakdown of more recalcitrant SOC, reducing the efficiency of carbon stabilization in HM, but this phenomenon is less pronounced in regularly cultivated soils due to management practices that mitigate excessive N availability (Berg and Matzner, 1997; Li et al., 2023; Li et al., 2024). Conversely, in low-N soils, HM may form more slowly and with reduced chemical complexity due to limited microbial activity and a shortage of N-containing compounds (Li et al., 2023). On the other hand, less soil disturbance may enhance the soil C/N ratio balance and aggregate stability, both of which are critical for HM formation (Kaushik et al., 2018; Li et al., 2024). As highlighted in orchard soils shown in Fig. 3.12b, the relative proportion of HM-C gradually increased as the total SOC increases. These finding suggests that management practices such as reduced tillage, N fertilization or the incorporation of N-rich organic materials (e.g., compost, legume residues) can enhance HM stabilization and promote a balanced humification process (Li et al., 2023; Li et al., 2024).

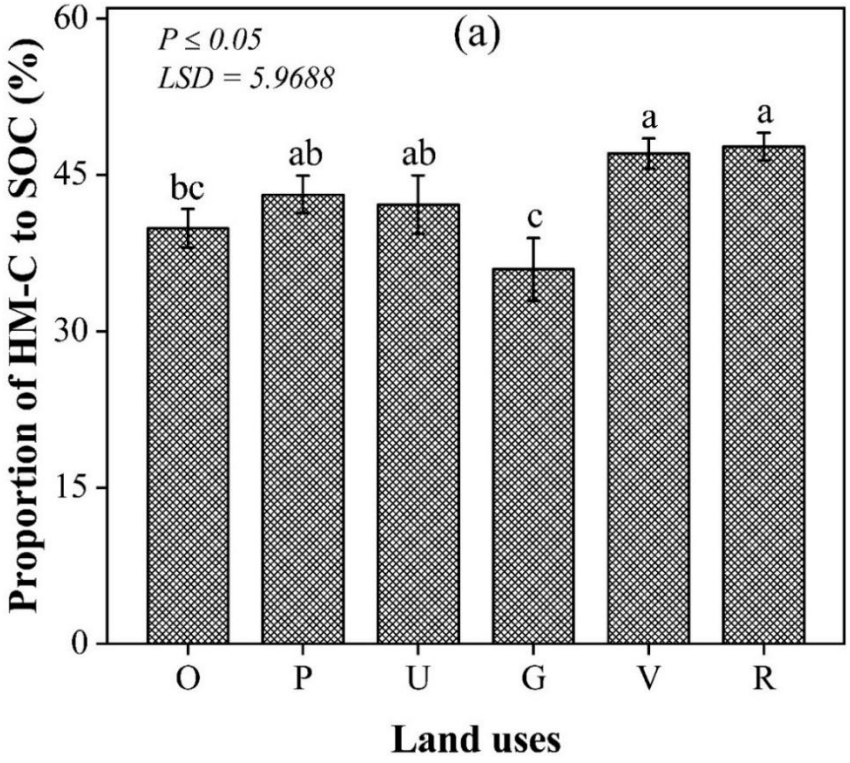

Fig. 3.12a The proportion of HM-C to the total SOC in selected soil samples collected from orchard (O), Paddy (O), upland (U), greenhouse (G), volcanic ash (V), and reclaimed (R) soils. Means followed by different lower-case letters indicate significant difference at $P \leq 0.05$ based on one-way ANOVA followed by Post-hoc LSD test. Vertical bars represent standard errors (n=3). Statistical analysis was performed using SAS 9.4.

(b)

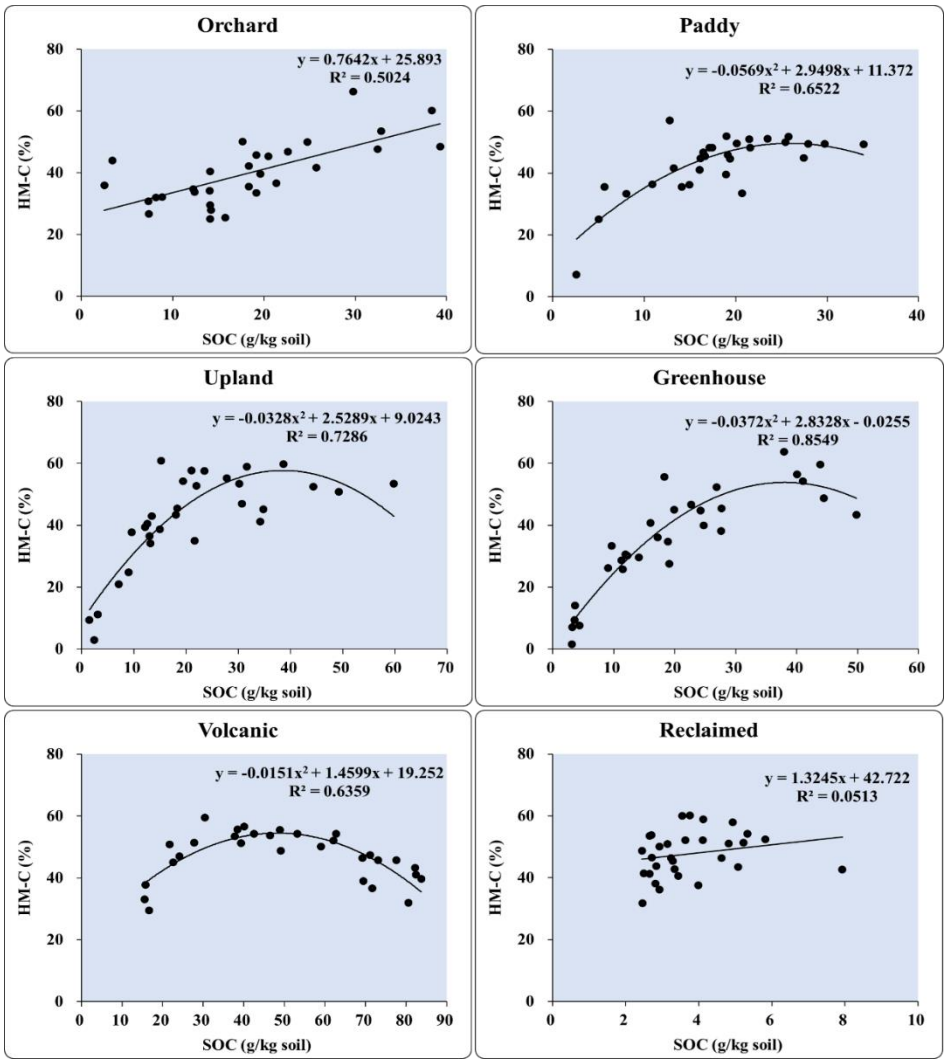

Fig. 3.12b Relative proportions of HM-C to the increment of SOC in selected soil samples collected from orchard (O), Paddy (O), upland (U), greenhouse (G), volcanic ash (V), and reclaimed (R) soils. Thirty soil samples with varying SOC contents, ranging from low to high, were selected from each land use. Soil samples were analyzed in triplicate.

3.4.8 HI and DT indies in relation to the increment of SOC content

The relationship between HA-C and FA-C (HA/FA) expressed by the humification index (HI) indicates the quality of organic material that could enhance soil physical properties and improve plant growth (Ukalska-Juagar et al., 2019). The higher HI than 1.0 (HI > 1.0) is indicative higher humification rates (conversion of FA to HA) or HA persisted longer in the soil (Moraes et al., 2011; Watanabe et al., 2001). On the other hand, a low degree of transformation (DT), (FA+HA)/HM, can be related to a strong interaction between the SOC and the soil mineral phase, resulting in high SOC stability in the more recalcitrant HM fraction (Guimaraes et al., 2013; Ukalska-Juagar et al., 2019). The main factors affecting HI and DT are the amount of organic C inputs and time of active plant residue transformation in soil affected by microbial activity, climate and soil texture (Feng-bo et al., 2015). In our study, we found that the HI increased as SOC increased in all land uses, but the HI values of the individual soil sample were lower than 1.0 (Fig. 3.13a), indicating a predominant of FA than HA, particularly in the volcanic ash soils. In contrast, the DT in common land uses and reclaimed soils decreased as SOC increased, while the DT in volcanic ash soils tended to increase as SOC increased (Fig. 3.13b). From our results, 2 distinct patterns emerged: (1) in common land uses and reclaimed soils, the HI increased, and DT decreased as SOC increased; and (2) in the volcanic ash soil, both the HI and DT increased with SOC. According to Liu et al. (2016), the increase in HI and decrease in DT indices indicate relatively fertile soil with good water infiltration capacity, aggregate stability, microbial activity and nutrient supply. The increase in both HI and DT parameters implies that humic substances occur mainly in high-polymerized organic forms (Liu, 2016; Moraes et al., 2011). However, the increase in HI was below the limit of 1.0, while the decrease in DT was above the limit of 1 for most individual soils, regardless of land-use types. These results suggest that cultivated soils slowly

enhance both the humification and the degree of transformation processes of SOM. The slow process of HI and DT can be attributed to a slow rate of SOM decomposition, or frequent inputs of fresh organic residues. HI and DT are the major parameters for evaluating changes in the soil quality as a function of soil management, but it is insufficient for a detailed assessment of SOM mobility or resistance in soils.

(a)

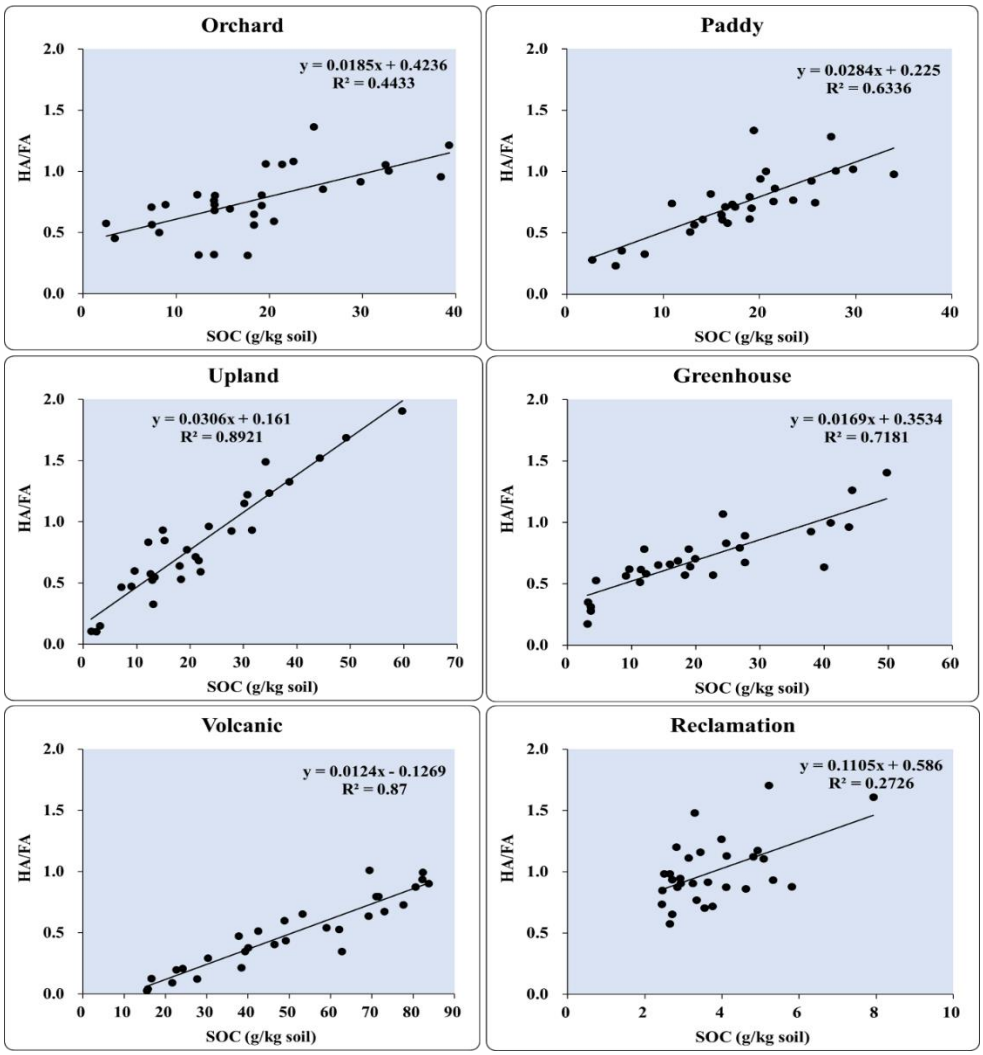

Fig. 3.13a The humification index (HI) in relation to the increment of SOC in soils collected from 6 land uses: orchard (O), Paddy (O), upland (U), greenhouse (G), volcanic ash (V), and reclaimed (R) soils. Thirty soil samples varying in SOC contents (ranging from low to high) were selected from each land use. The individual soil sample was analyzed in triplicate. The HI increased as SOC increased.

(b)

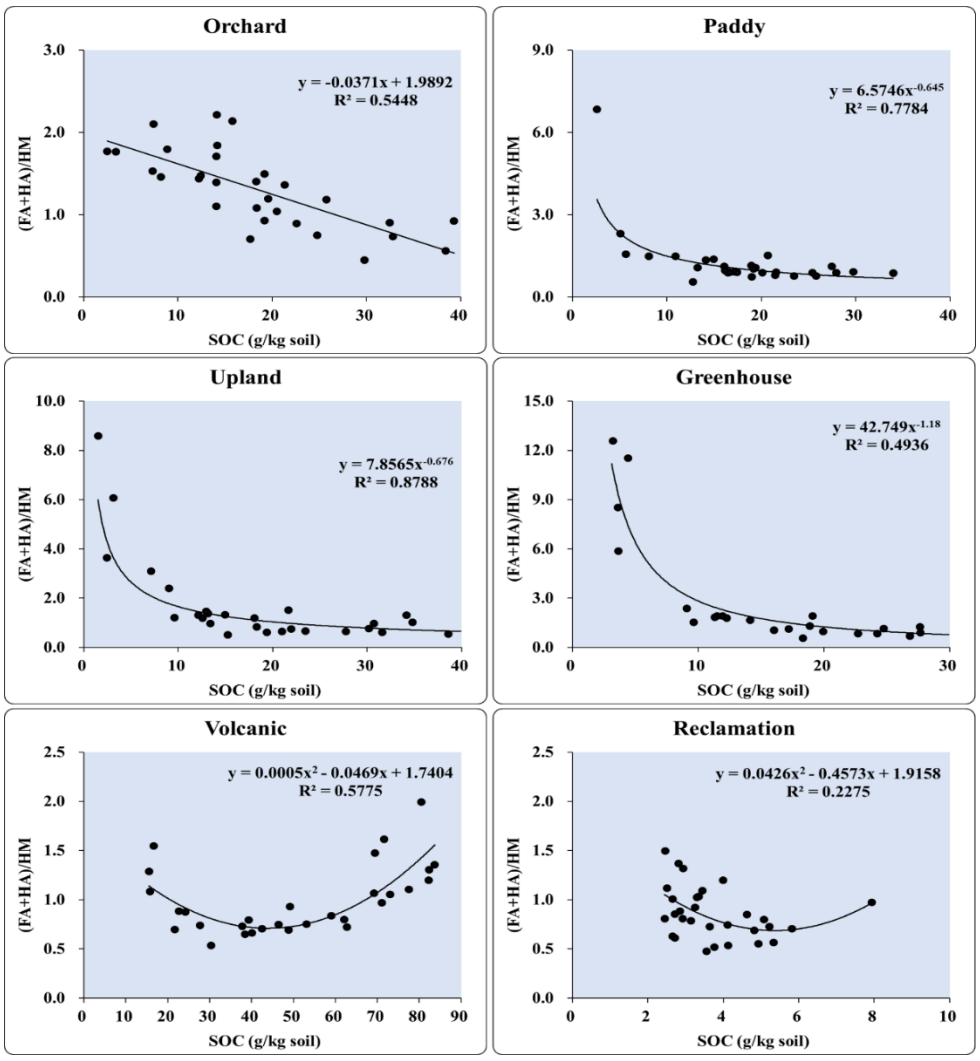

Fig. 3.13b The degree of transformation (DT) in relation to the increment of SOC in soils collected from 6 land uses: orchard (O), Paddy (O), upland (U), greenhouse (G), volcanic ash (V), and reclaimed (R) soils. Thirty soil samples varying in SOC contents (ranging from low to high) were selected from each land use. The individual soil sample was analyzed in triplicate. DT tended to decrease as SOC increased.

3.4.9 Assessment of SOM quality under different land uses

The C/N ratios of FA, HA, and HM reflect the formation and stabilization of SOC, which is the major indicator of SOM quality. In our study, the C/N ratio of FA was high (>10) when FA content was low, however, the C/N ratio tended to decrease as FA content increased in all land uses (Fig. 3.14a to19a). The C/N ratio of FA reached a steady level of around 5 to 6 as the FA-C content continued to increase. These findings suggested that the formation of FA was closely associated with the progressive stabilization of both organic C and N during the humification process.

Unlike FA, the C/N ratio of HA was lower than that of FA when HA content was low, but it tended to increase as HA content increased in most land use types (Fig. 3.14b to 18b). In reclaimed soils, the C/N ratio of HA was generally higher than that of FA (Fig. 3. 19b). On average, the C/N ratio of HA ranged from about 8 to 14, exceeding the C/N ratio of FA. The higher C/N ratio of HA compared with FA indicates the depletion of N during the formation of the HA fraction, which is consistent with the observation that N in HA was generally lower than that in FA and HM fractions (Fig. 3. 14d to 19d).

As in the case of HA, the C/N ratio of HM also exhibited different patterns across land use types, reflecting differences in organic matter dynamics influenced by land use and management practices. In orchard, upland, and greenhouse soils, the C/N ratio was lower than that of HA when HM content was low, but it tended to increase as HM content increased (Fig. 3. 14c, 16c, and 17c). In contrast, the C/N ratio of HM was consistently higher than that of HA in paddy, volcanic ash, and reclaimed soils (Fig. 3. 15c, 18c, and 19c). In most cases, the C/N ratio of HM in orchard, upland, and greenhouse soils was lower than that of HA, varying from about 7 to 8 on average,

even as HM content continued to increase. In contrast, the C/N ratio of HM in paddy, volcanic ash, and reclaimed soils was generally higher than that of HA, ranging from about 11 to 34 on average. The lower C/N ratio of HM compared to that of HA as HM content was low suggests a higher degree of N enrichment during initial humus formation, while the observed increase in the C/N ratio as HM content increased could indicate the gradual accumulation of more carbon-rich organic materials over time or the preferential depletion of N through microbial activity.

The C/N ratio of FA, HA, and HM showed that SOM quality in orchard, upland, and greenhouse soils was higher than that in paddy, volcanic ash, and reclaimed soils. According to Tan (2014), during the decomposition of plant residues, some of the C and a considerable amount of N liberated are incorporated into microbial cells or fixed in substances used for the formation of humic matter. In addition, long-term application of N fertilizers can lead to soil acidification, which suppresses microbial activity, including the lignin-degrading fungi. This suppression reduces SOM decomposition, increasing lignin polymer concentration, and forming stable organic matter (Bai et al., 2018; Bonner et al., 2019; Hasegawa et al., 2021; Ye et al., 2018). This process also leads to decreasing the C/N ratios, eventually reaching relative constant values in the soils. In most soils, the C/N ratio falls within narrow limits to about 10–15, when the decomposition is completed, meaning organic matter decomposition is in equilibrium with the synthesis and accumulation of new organic materials (Brady and Weil, 2010; Tan, 2014). Generally, the decrease of the C/N ratio of FA, HA, and HM characterizes well-developed humic matter. In contrast, when the C/N ratio increases, humic substances start to decompose, and microorganisms scavenge N source for stabilization process (Tan, 2014). In conclusion, in assessing SOM quality, N appears to be very important in all land use types.

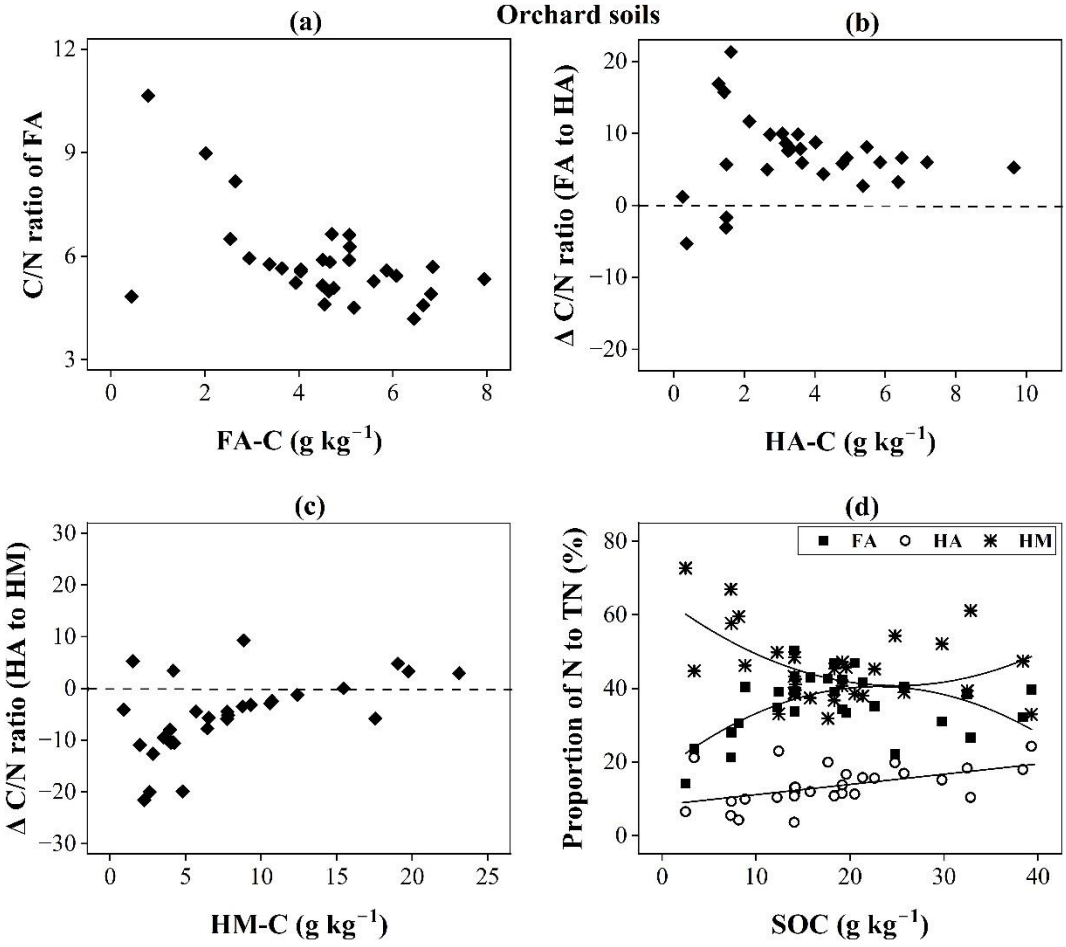

Fig. 3.14 Changes in the C/N ratio and N content in response to increasing C content in orchard soils: **(a)** the C/N ratio of FA in relation to the increase in FA-C content; **(b)** the C/N ratio of HA relative to the C/N ratio of FA in relation to the increase in HA-C content; **(c)** the C/N ratio of HM relative to the C/N ratio of HA in relation to the increase in HM-C content; and **(d)** the relative proportions of FA-N, HA-N, and HM-N in relation to the increase in SOC content.

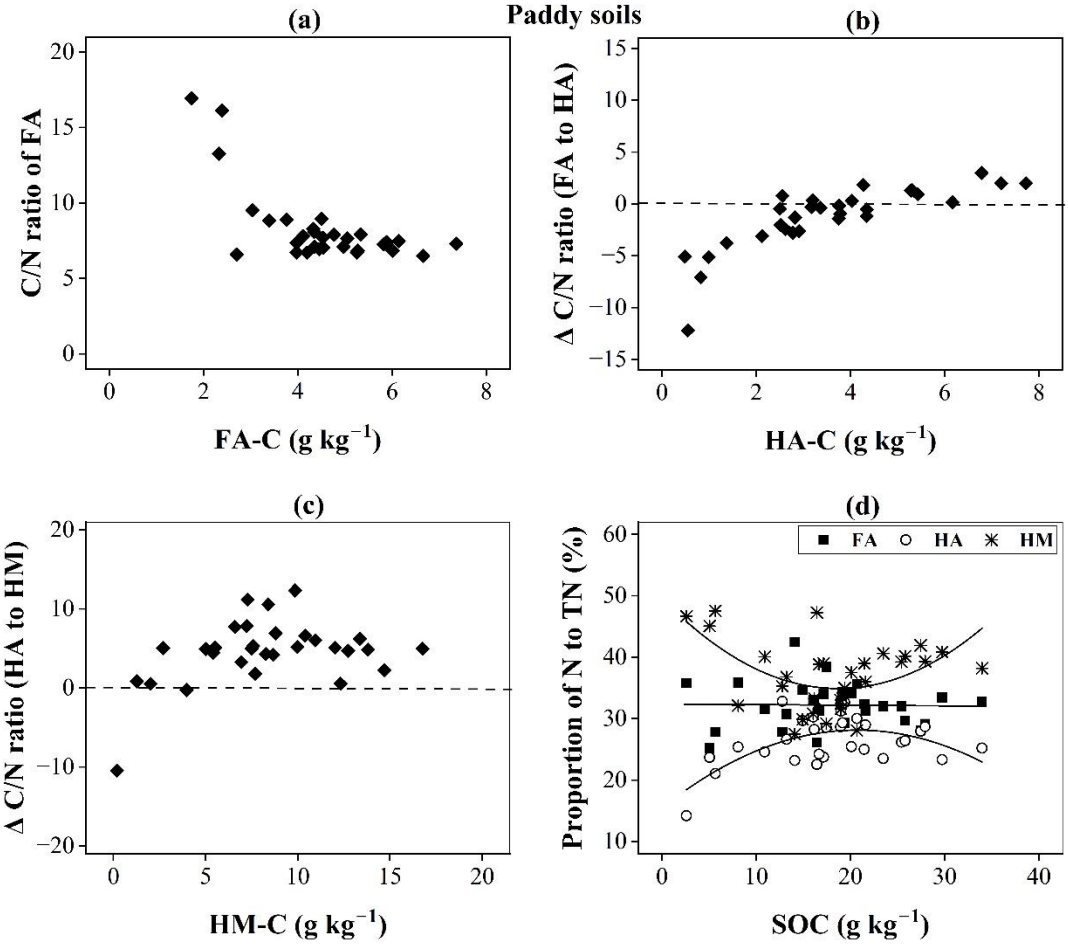

Fig. 3.15 Changes in the C/N ratio and N content in response to increasing C content in paddy soils: **(a)** the C/N ratio of FA in relation to the increase in FA-C content; **(b)** the C/N ratio of HA relative to the C/N ratio of FA in relation to the increase in HA-C content; **(c)** the C/N ratio of HM relative to the C/N ratio of HA in relation to the increase in HM-C content; and **(d)** the relative proportions of FA-N, HA-N, and HM-N in relation to the increase in SOC content.

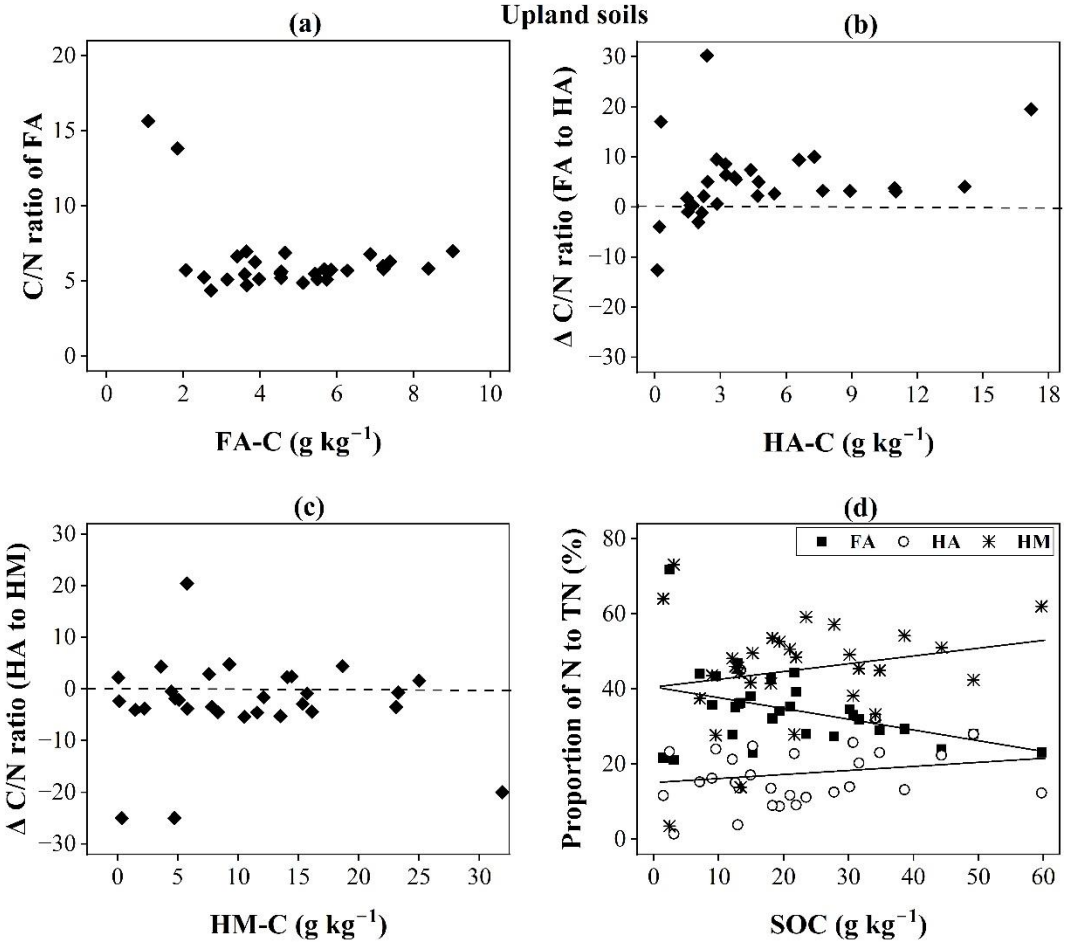

Fig. 3.16 Changes in the C/N ratio and N content in response to increasing C content in upland soils: **(a)** the C/N ratio of FA in relation to the increase in FA-C content; **(b)** the C/N ratio of HA relative to the C/N ratio of FA in relation to the increase in HA-C content; **(c)** the C/N ratio of HM relative to the C/N ratio of HA in relation to the increase in HM-C content; and **(d)** the relative proportions of FA-N, HA-N, and HM-N in relation to the increase in SOC content.

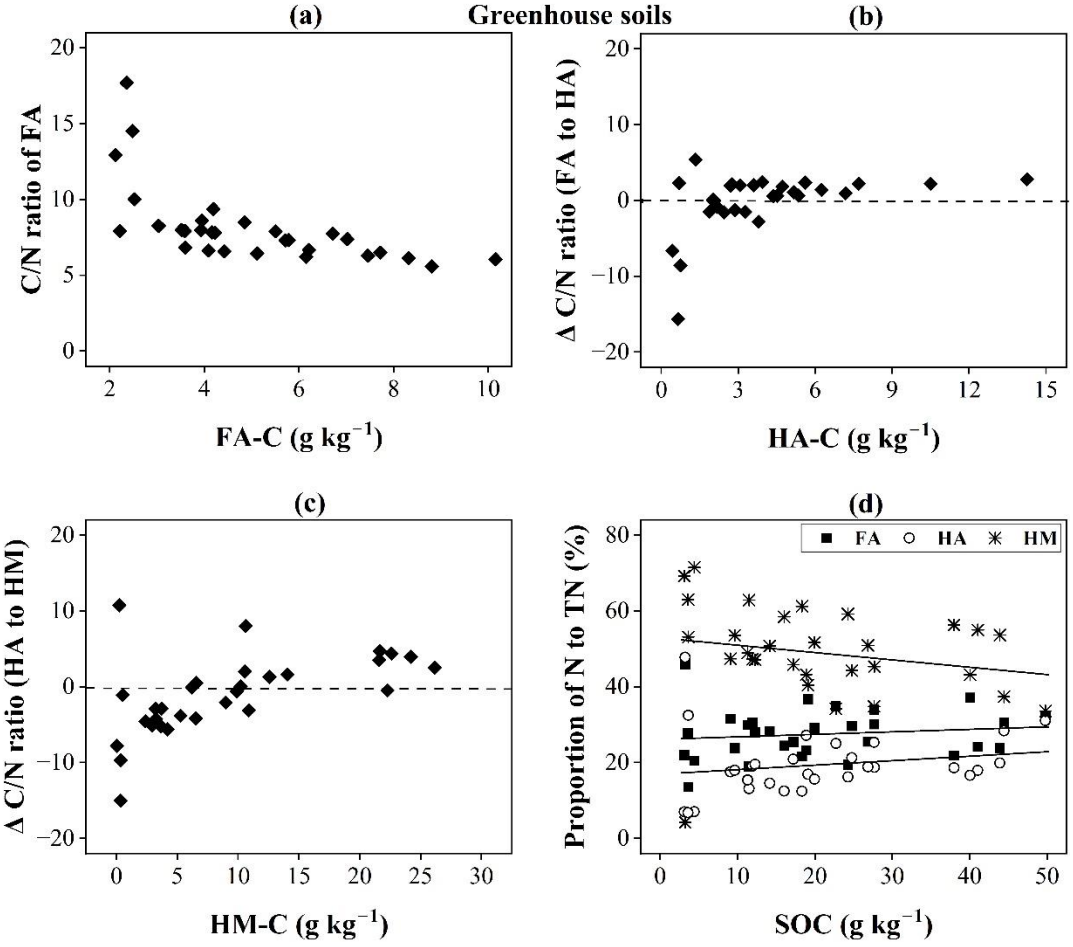

Fig. 3.17 Changes in the C/N ratio and N content in response to increasing C content in Greenhouse soils: **(a)** the C/N ratio of FA in relation to the increase in FA-C content; **(b)** the C/N ratio of HA relative to the C/N ratio of FA in relation to the increase in HA-C content; **(c)** the C/N ratio of HM relative to the C/N ratio of HA in relation to the increase in HM-C content; and **(d)** the relative proportions of FA-N, HA-N, and HM-N in relation to the increase in SOC content.

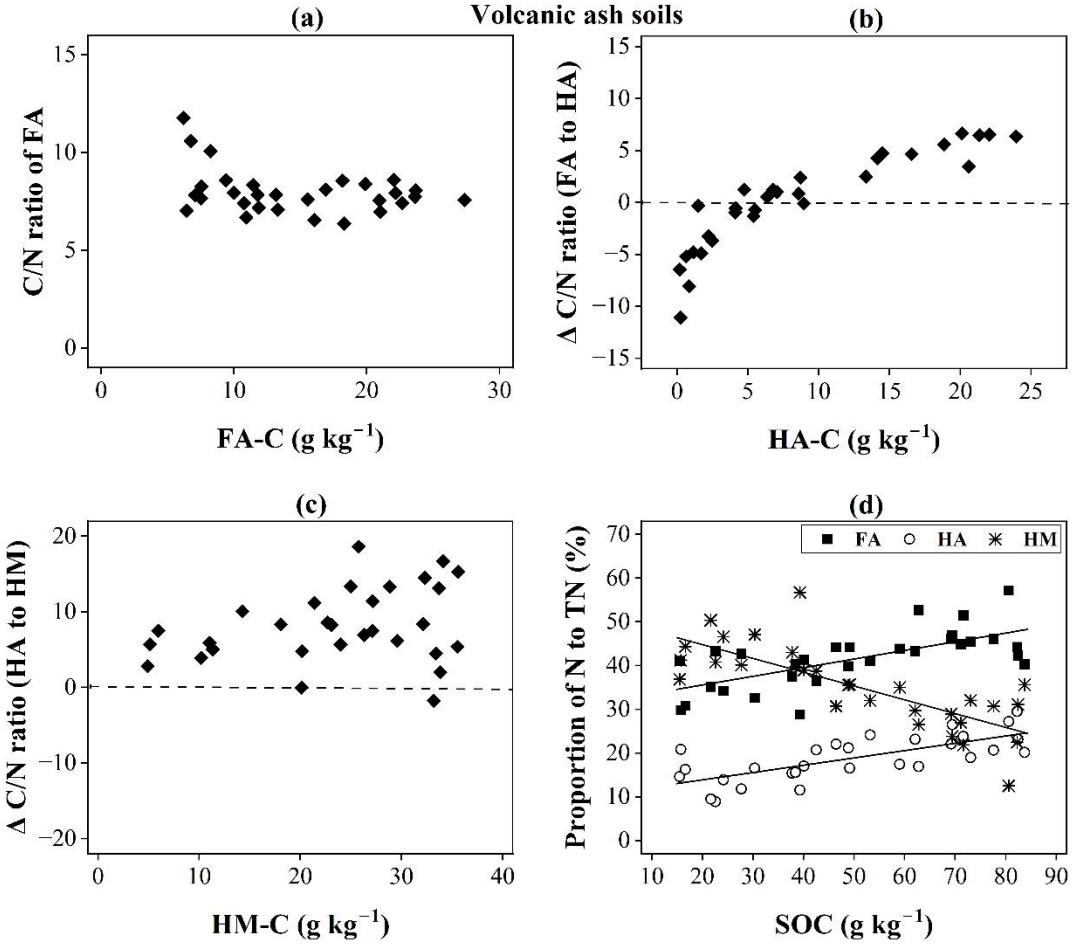

Fig. 3.18 Changes in the C/N ratio and N content in response to increasing C content in Volcanic ash soils: **(a)** the C/N ratio of FA in relation to the increase in FA-C content; **(b)** the C/N ratio of HA relative to the C/N ratio of FA in relation to the increase in HA-C content; **(c)** the C/N ratio of HM relative to the C/N ratio of HA in relation to the increase in HM-C content; and **(d)** the relative proportions of FA-N, HA-N, and HM-N in relation to the increase in SOC content.

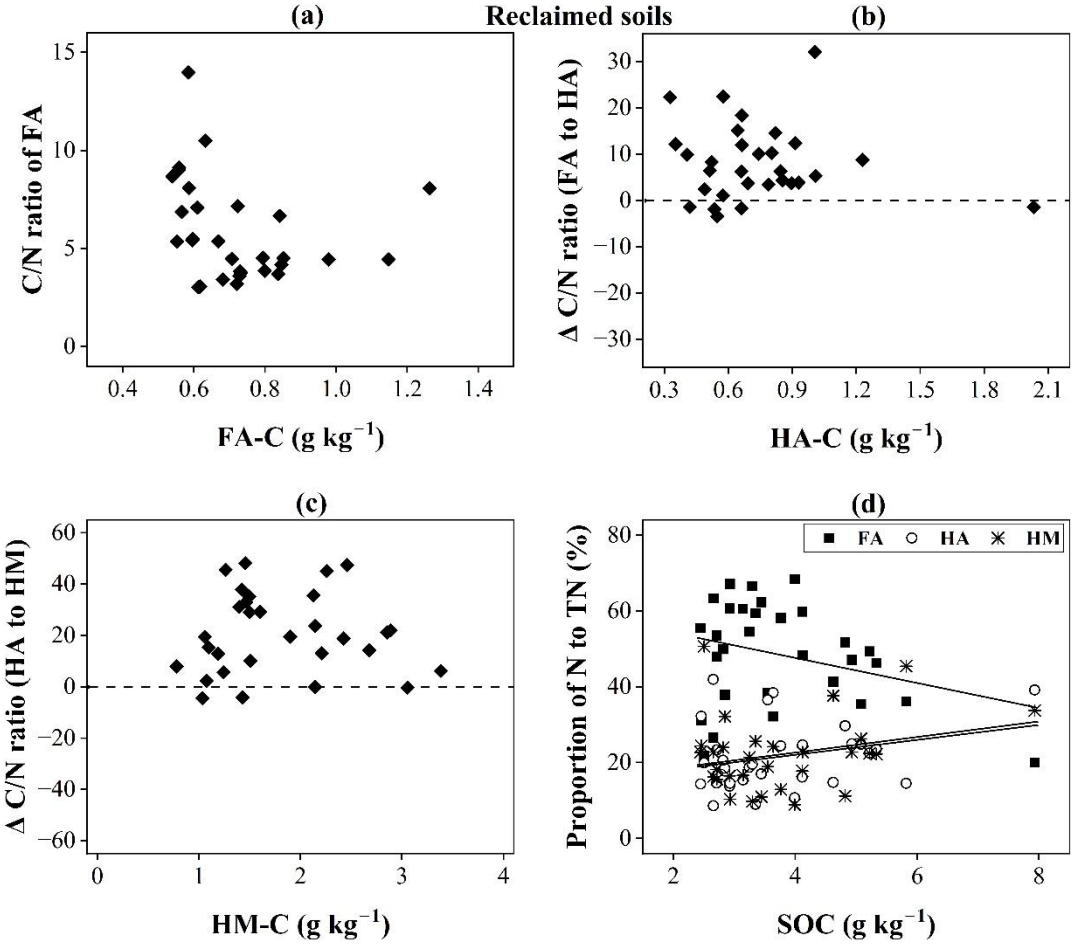

Fig. 3.19 Changes in the C/N ratio and N content in response to increasing C content in Reclaimed soils: **(a)** the C/N ratio of FA in relation to the increase in FA-C content; **(b)** the C/N ratio of HA relative to the C/N ratio of FA in relation to the increase in HA-C content; **(c)** the C/N ratio of HM relative to the C/N ratio of HA in relation to the increase in HM-C content; and **(d)** the relative proportions of FA-N, HA-N, and HM-N in relation to the increase in SOC content.

3.5 Conclusion

In summary, the total SOC content varied significantly from volcanic ash soils (15 to 83 g kg^{-1}) to common land uses (orchard, paddy, upland, and greenhouse) (2 to 60 g kg^{-1}) and reclaimed soils (2-8 g kg^{-1}). On average, volcanic ash soils had more than 2 times the total SOC compared to the common land uses and more than 10 times compared to the reclaimed soils. Regardless of land-use types, the C content in the SOC fractions followed the order: NHS-C < debris-C < HA-C < FA-C < HM-C. On average, NHS and debris contributed about 0.7–1.4% and 6–14% of C to the total SOC, while FA, HA, and HM accounted for about 23–30%, 15–20%, and 35–43%, respectively. Regardless of land-use types, the relative proportion of NHS, debris, and FA decreased as SOC increased, whereas HA and HM increased with SOC, demonstrating that HA and HM fractions are the major contributors to the increase in total SOC. In common land uses and reclaimed soils, the HI increased, and DT decreased as SOC increased, whereas in volcanic ash soils, both the HI and DT increased with SOC. In most cases, the increase in HI and the decrease in DT were below the limit of 1.0, suggesting that the formation of stable humus progresses relatively slowly in all cultivated soils. The C/N ratio of FA was high as FA content was low and it decreased as FA content increased, suggesting the formation of FA was closely associated with the progressive stabilization of both organic C and N during the humification process. In contrast, the C/N ratio of HA and HM tended to increase as HA or HM continued to increase, indicating the depletion of N during the formation of HA and HM fractions, particularly in paddy, volcanic ash, and reclaimed soils. The results of the C/N ratio of FA, HA, and HM showed that SOM quality in orchard, upland, and greenhouse soils was higher than that in paddy, volcanic ash, and reclaimed soils. In conclusion, our findings indicate that in assessing SOM quality in terms of soil quality, N appears to be critically important across all land-use types.

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CHAPTER 4

Changes in microbial populations and humic fractions in soil amended with organic and inorganic fertilizers in a long-term incubation system

4.1 Abstract

The application of organic, inorganic, and compost fertilizers, either alone or in combination, influences not only carbon dioxide (CO_2) emissions and microbial populations but also substantially impacts the composition and quality of SOM. Given that SOM consists of various pools ranging from labile to stable fractions, assessing the effect of soil amendments on SOM quality through chemical fractionation is a more sensitive method than measurement of total SOC. Therefore, the objectives of this study were to evaluate the effects of amendments on CO_2 emission and microbial populations, including bacteria, fungi, and actinomycetes; to analyzer C and N dynamics in SOM fractions, including debris, FA, HA, and HM; and to assess changes in humification index (HI), degree of transformation (DT), and C/N ratio in FA, HA, and HM fractions. To assess the impact of amendments, we incubated soils amended with different fertilizers, including hairy vetch (HV), corn leave (CL), corn stover (CS), rice straw (RS), compost-pig manure (CP), CMS, oil cake (OC), CMS+RS, NPK, and withiout fertilization (Nil) for about 5 months. The fertilizer application rate was 250 kg N ha^{-1}. The incubation was conducted with three replications, and soil samples were collected at four intervals: weeks 2, 6, 12, and 21. Results showed that organic and inorganic amendments significantly increased CO_2 emissions and altered microbial populations, with crop residues and CMS+RS producing higher emissions. Bacterial populations increased initially and then stabilized, while fungi and actinomycetes decreased during the incubaiton period. Soils amended with RS and CMS+RS had higher SOC, while CS increased TN. Combined fertilization (CMS+RS)

promoted more stable SOM than organic amendments alone, as evidenced by the changes in HI, DT, and C/N ratios in FA, HA, and HM fraction. To enhance SOM quality and stability, which is a key indicator of soil quality, regular addition of organic N or a combination of orgnic and inorganic N is necessary.

Keywords: CO_2 emissions, Humic substances, Microbial populations, Soil amendements, Soil quality indicators, SOM fractions

4.2 Introduction

Soil organic matter (SOM) is a critical component of soil health by directly influencing its physical structure, chemical properties, and biological activity (Doran and Parkin, 1994; Lehtinen et al., 2014; Mi et al., 2019). SOM provides energy and substrates and promotes biological diversity that helps to maintain soil quality and ecosystem functionality (Guimaraes et al., 2013). Enhancing SOM stability is essential not only for improving soil fertility, but also for C sequestration, and sustainable land management (Follett, 2001). While SOM accumulation is governed by several factors, including the quality and quantity of organic and inorganic inputs (Manzoni et al., 2008), the mechanisms driving SOM transformations and stabilization require further investigation, particularly in systems that integrate diverse amendments.

Total SOC represents the largest active C reservoir that interacts with atmospheric carbon dioxide (CO_2), thus changes in the SOC pools highly influential on global climate dynamics (Kunlanit et al., 2019). Maintaining increasing SOC stocks and reducing CO_2 emissions are essential for the long-term sustainability of agriculture (Li et al., 2018). Emissions of CO_2 indicate heterotrophic microbial activity and mineralization (Lehtinen et al., 2014). CO_2 emission from farmland soil is controlled by the decomposition rate and types of soil amendments (Li et al., 2018). Organic amendments, particularly those rich in labile carbon compounds, often result in elevated CO_2 emissions during the initial stages of incubation. Yan et al. (2019) investigated the decomposition characteristics of rice straw and found that the loss of cellulose and hemicellulose as CO_2 was significantly greater than that of lignin, with a rapid release occurring in the first and second years after straw return. In addition, Zhou et al. (2019) incubated soils amended with rice straw, hairy vetch, and their mixtures at different C/N ratios and found that residue mixtures (rice straw + hairy

vetch) induced non-additive effects on CO_2 emissions, with synergistic (the combined effect is greater than the sum of the individual effects) increases in emissions at lower C/N ratios and interactions driven by the quality of the amendments. Similarly, Li et al. (2013) observed that pig compost, maize straw, and their combination with NPK significantly increased CO_2 emissions due to organic matter decomposition, while NPK alone had minimal impact on cumulative CO_2 emissions as it lacks organic C. Although several studies have addressed the impact of various soil amendments on CO_2 emissions, materials such as CMS, oil cake (fermented food wastes), hairy vetch, corn leaves, corn stover, rice straw, compost, and NPK have been less frequently compared under conditions without plant growth in a system with less disturbance.

In addition to CO_2 emission, types of amendment applied significantly influence the microbial populations, including bacteria, fungi, and actinomycetes, which are key drivers of organic matter decomposition and nutrient cycling. Previous studies have reported that organic amendments, such as crop residues, often promote rapid microbial proliferation during the early stages of decomposition due to the availability of labile C and nutrients. Singh and Dhar (2011) demonstrated that organic farming in the rice-wheat-greengram cropping system, through the application of bio-inoculants, vermicompost, blue-green algae, farmyard manure, and Azolla, whether applied alone or in combination, significantly increased bacterial, fungal, and actinomycetes populations over the years. Similarly, Meshram et al. (2016) under the continuous use of organic manure along with balance fertilization significantly increased microbial populations (bacteria, fungi, and actinomycetes) in the Vertisol. They also found that organic addition coupled with NPK fertilizer exerted a stimulating influence on the growth and activities of these microorganisms. The enhancement of microbial population growth and activity under organic or combined organic systems were also

reported by many previous studies (Das and Dkhar, 2011, Meena et al., 2013, and Selvi et al., 2004). While several studies have explored the impact of organic amendments or combined fertilization on microbial populations in fields under crop growth, there is limited research comparing the effects of diverse amendments in plant-free conditions. This comparison is important for evaluating which amendments most effectively enhance microbial populations without the interaction or support from plant root exudates.

The application of organic, inorganic, and compost fertilizers, either alone or in combination, influences not only CO_2 emissions and microbial populations but also substantially impacts the composition and stability of SOM. These impacts are largely determined by the quality and quantity of organic and inorganic inputs (Manzoni et al., 2008), as these factors dictate the availability of nutrients and carbon for soil processes. Specifically, plant species and the C/N ratio of organic residues, which are major indicators of organic matter quality, play a critical role in shaping SOM composition and stability. Given that SOM consists of various pools ranging from labile to stable fractions, assessing the effect of soil amendments on SOM quality through chemical fractionation is a more sensitive method than measurement of total SOC. The debris fraction which can be identified or occluded in soil aggregate represents visually more decomposed organic material with stronger association to soil mineral particles (You et al., 2014). The humic substances, which represents 60-80% of SOC, consists of more processed materials, has a slower turnover rate, and is generally a more stable and high-density organomineral fraction with a higher degree of physical protection then the light fraction (Brady and Weil, 2010).

Humic substances can be divided into 3 major fractions according to their mobility and reactivity in soils: fulvic acid (FA), humic acid (HA), and humin (HM) (Ukalska-Jaruga et al., 2019). The stability of SOM is commonly evaluated by measuring the humification index (HI) and the degree of transformation (DT) (Moraes et al., 2011, Masmoudi et al., 2024, Khalafalla et al., 2019, Ukalska-Jaruga et al., 2019). However, N plays a vital role in the formation of SOM stability. Trinsoutrot et al. (2000) found that residues with a C/N ratio below 24 induced net N mineralization, whereas those with a C/N above 24 caused net immobilization of soil mineral N. They concluded that the mineralization and immobilization of residue N are controlled by soluble polyphenol, which form complexes with proteins, making them inaccessible to microorganisms. In addition, See et al. (2005) observed the C/N ratio in the low molecular weight size fraction of the humics decreased greatly during the formation of stable humus due to the binding of NH_4^+ to humic substances. While previous studies have explored the role of the C/N ratio, polyphenols, and NH_4^+ binding in influencing SOM stability and the formation of humic substances, limited research has systematically examined how different soil amendments affect the dynamics of C and N within FA, HA, and HM fractions, along with changes in humification indices under varying C/N ratios.

Therefore, the objectives of the study were (i) to investigate the effects of soil amendments, including hairy vetch, corn leave, corn stover, rice straw, compost, CMS, oil cake, and CMS + rice straw, on soil respiration (CO_2 emissions) and microbial populations, including bacteria, fungi, and actinomycetes as indicator of micobial activity and SOM decomposition; (ii) to analyze C and N dynamics in SOM fractions, including debris, FA, HA, and HM in soils amended with organic, inorganic, and

combined fertilizers; (iii) to assess changes in humification, degree of transformation, and C/N ratio in FA, HA, and HM fractions as measures of SOM quality.

4.3 Materials and Methods

4.3.1 Experimental description

To investigate the effects of organic and inorganic amendments on the SOM quality or its fractions (debris, FA, HA, and HM), ten treatments were decided: Nil, Hairy vetch (HV), Corn leave (CL), Corn stem (CS), Rice straw (RS), Compost (CP), Oil cake (OC), CMS-granuala (CMS), CMS + Rice straw (CMS + RS), and NPK (24-10-15). The incubation experiment was initiated in 2016, starting from 22 May to 29 October, lasting about 5 months.

Organic fertilizers, including corn leave, corn stem, hairy vetch, and rice straw, were collected from upland and paddy fields located at the CBNU research station in October 2015. These crop residues were dried in an oven at 75ºC for one week, then chopped and ground into fine particles using a common fruit grinder machine. These crop residuers, as well as CMS, OC, and CP, were analyzed for total nitrogen (TN) content by the Kjeldahl method. The TN contents of these amendments were 4.08%, 1.90%, 1.25%, 1.16%, 2.50%, 3.80%, 11.00%, and 24.00% for HV, CL, CS, RS, CP, OC, CMS, and NPK, respectively.

The fertilizer was appled based on an N rate of 25 kg 0.1 ha^{-1}. To calculate fertilizer amount per column, first we calculated soil weight per 1000 m^2. Assuming that in a 1000 m^2 (0.1 ha) field with a depth of 0.15 m, the dry soil weight for this field size was calculated as 1000 m^2 x 0.15 m = 150 m^3. Assuming that the soil density is

1000 kg m^3, the total soil weight for 0.1 ha is 150 m^3 x 1000 kg m^3 = 150,000 kg. According to the fertilizer recommendation rate (25 kg N 0.1 ha^{-1}), the amount of N in a gram dry soil was calculated as 25 kg N / 150,000 kg soil x 1000 = 0.167 g.

Since this incubation experiment was conducted using soil columns, fertilizer application rates were calculated based on the dry soil weight per column. The radius of the column used in this experiment was 3.75 cm, and the soil thickness in the column was 7 cm (see Fig. 4.1). This thickness was chosen to facilitate easier control of soil water content. To calculate the size of the column, we used the formula for the volume of a cylinder: $V=\pi r^2 h$, where π equals 3.1416. As a result, the volume of the column was calcuated to be approximately 309.25 cm^3. Therefore, the amount of N (based on the recommended rate) per column was calculated as 0.167 g N / kg soil x 309.25 cm^3 / 1000 = 0.052 g.

To calculate the amount of fertilizer per column, the N content in individual fertilizers and the recommended N per column (0.052 g) were taken into account. For example, the N content in HV was 4.08%. Thus, the amount of HV per column was calculated as 0.052 g N / (4.08 / 100) = 1.263 g HV. Fertilizers, soil columns, and other apparatus were prepared prior to go for soil sampling.

4.3.2 Soil sample collection

To minimize the influence of soil variability, one well-defined location was used for sampling. Soil samples were collected from the upland filed of the CBNU research station (36º37’27.3”N and 12º727”20.1”E). After removing plants and non-soil materials from the topsoil surface, around 100 kg of soil samples from a 0–30 cm depth was collected and immediately returned to the laboratory. Wet soil samples were

sieved to pass a 2-mm screen. The sieved soil sample was then kept in a big plastic container for soil incubation treatment. Small amounts of soil samples were immediately used for colony counting.

4.3.3 Laboratory incubation

Prior to preparing soil columns, the sieved soil sample was tested for soil water conten in order to maintain the dry soil weight of 309.25g per column. Briefly, 2-3g of soil sample (3 replications) was weighed onto a glass plate and then dried in the microvawe for 3 minites. The drying process was repeated until a constant weight was achieved. The soil water content was calculated by subtracting the dry weight from the initial weight, then dividing by the dry weight and multiplying by 100. The soil water content of our soil was around 17%. Thus, the mass of wet soil sample per column was calculated as 309.25 g x (1+17/100) = 362 g.

A 362 g of soil sample was weighed in a container, then the calculated amount of fertilizer was added, and soils and fertilizer were mixed using laboratory spatula stainless steel to ensure homogeneity. The soil-fertilizer mixture was then transferred into a column and leveled to a thickness of 7 cm. The soil column was closed with a silicon stopper and immediately connected to the incubation system.

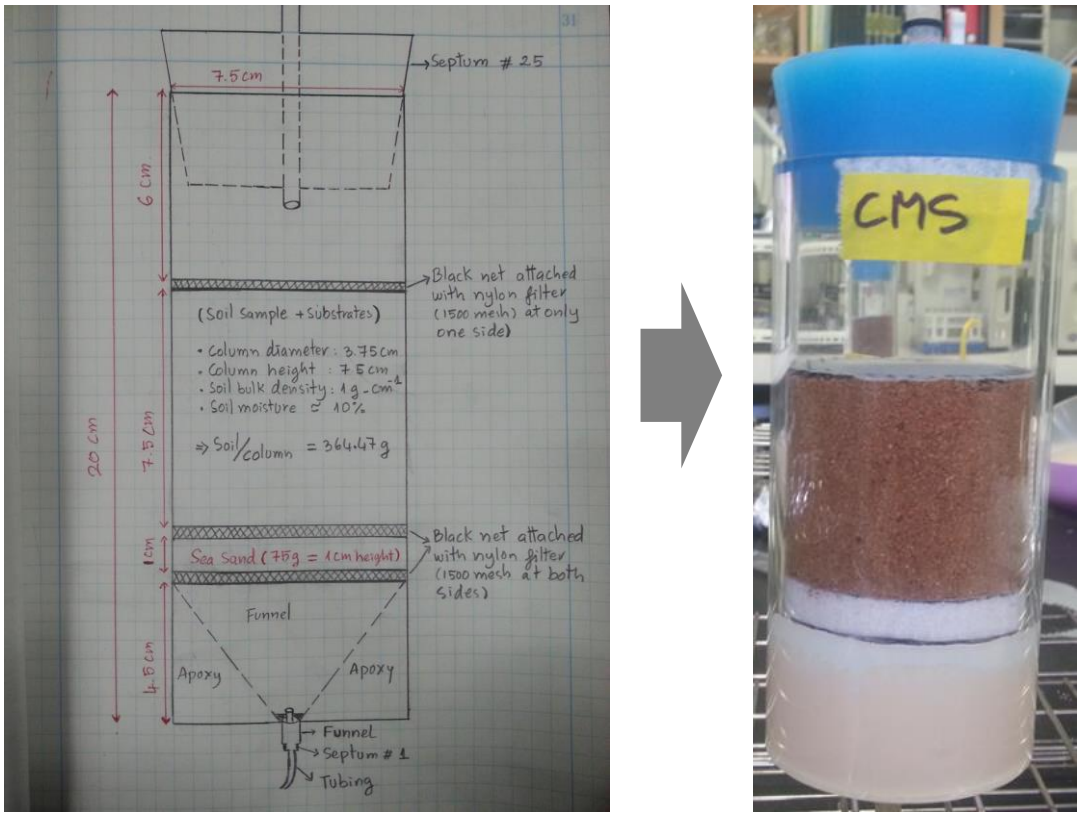

Fig. 4.1 Preparation of a soil column: (left) schematic diagram of the manually designed soil column and (right) the column filled with soil samples.

The incubation unit, called the Enforced Aeration Respirometer (EAR), uses a pump to introduce ambient air into the system, circulating it through each column at an airflow rate of 2 L per minute. The incubation temperature was set at 29ºC. During the incubation period, CO_2 emissions from each soil column were automatically measured by the CO_2 Gas Analyzer LI-840. Soil water content was maintained at about 50–60% by adding water twice a week. Leachates from each soil column were also collected throughout the incubation period.

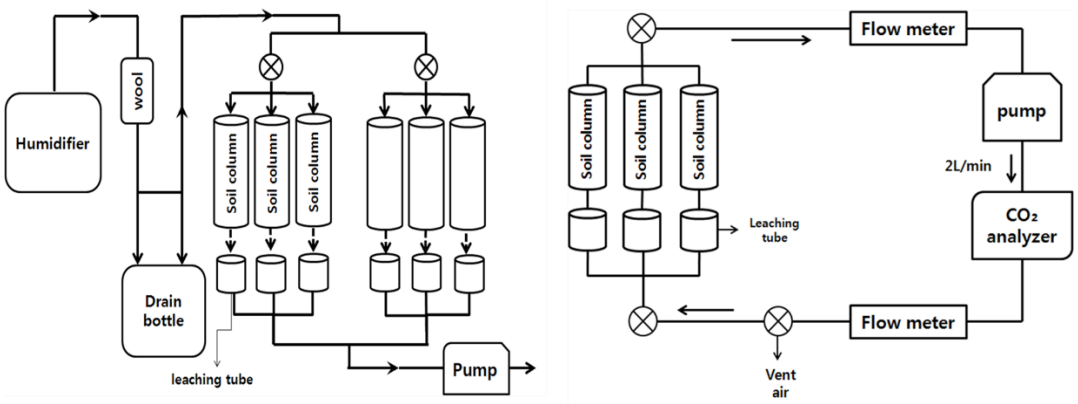

Fig. 4.2 The schematic diagrams of EAR system: (left) Aeration circulation system; (right) CO_2 measurement system.

Fig. 4. 3 Preparation of soil columns and soil incubation.

4.3.4 Soil extraction and analysis

During the incubation period, soil samples were collected four times: at 2 weeks, 6 weeks, 12 weeks, and 21 weeks. Each soil sample from a column was divided into three subsamples. Two-thirds of the samples were air-dried and used to measure SOC, TN, and C and N in debris, FA, HA, and HM fractions. The extraction and analysis procedures for these SOM pools were described in Chapters 1 and 2. Approximately 15 g of fresh soil samples were used to measure soil microbial populations, while the remaining samples were freeze-dried and stored at -80°C.

4.3.5 Counting of microbial populations

The serial dilution plate technique was employed to count soil bacteria, fungi, and actinomycetes. R2A agar medium was used for bacteria count (Reasoner and Geldreinch, 1985). Actinomycetes were estimated by using Starch-Casein agar (Williams and Davies, 1965). Martin's Streptomycin-rose Bengal agar medium was used for fungi count (Martin, 1950). The inoculated Petri plates were incubated at 28ºC ± 1ºC for 3 days. After the incubation period, the colony forming units were counted manually and expressed as CFU g^{-1} soil.

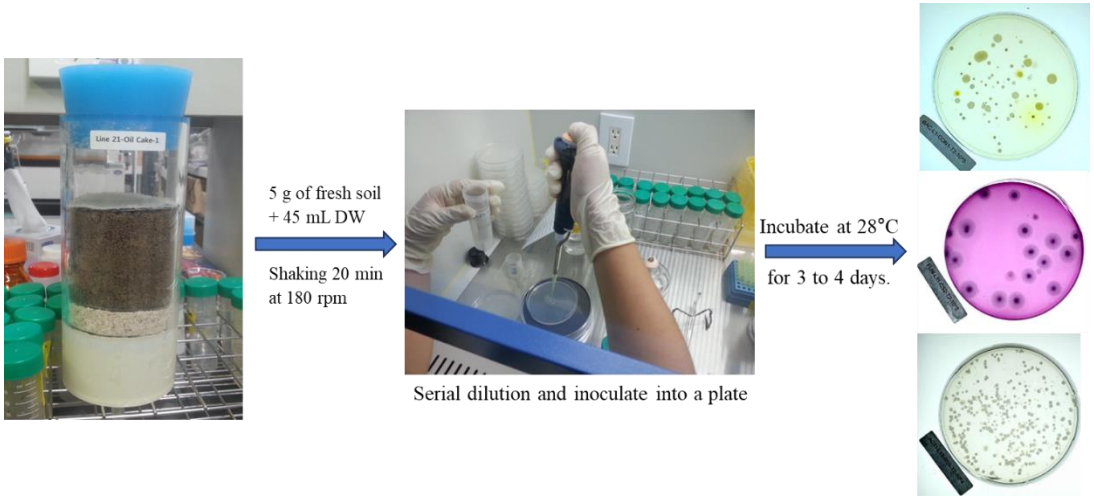

Fig. 4.4 Serial dilution plate technique for colony counting.

4.4 Results and Discussion

4.4.1 CO_2 emissions from soils amended with different fertilizers

Soil microbial respiration, quantified through CO_2 production, serves as a direct indicator of microbial activity and an indirect measure of organic material availability. We observed that the incorporation of organic and inoragnic fertilizers had immediate effects on the CO_2 emissions (Fig. 4.5). The CO_2 emission was high in soils amended with crop residures alone (i.e., RS, CL, CS, HV) and combined fertilization (CMS+RS) compared to those amended with CP, CMS, OC, and NPK. RS application increased CO_2 emissions during the first four weeks of incubation, then declined dramatically and remained steady after three months of incubation. This result aligns with the findings of Li et al. (2018), who reported that rice straw application increased CO_2 emissions during the early incubation period and decreased as incubation progressed. In contrast, in soils amended with other organic and inorganic fertilizers, including Nil, CO_2 emissions decreased dramatically from the first day to the 60th day

and then remained steady over the rest of the incubation period. The different CO_2 emissions patterns between RS treatment and other treatments may be attributed to substrate availability for microbial mineralization and changes in soil microbial communities during the incubation period (Tian et al., 2014). RS contains significant amounts of cellulose, hemicellulose, and easily decomposable compounds such as soluble sugars and starches. These labile carbon sources are rapidly metabolized by microbes during initial decomposition, resulting in increased CO_2 emissions (Islam et al., 2023; Sanoja-Lopez et al., 2024). In addition, substrate with high C/N ratio (> 57:1) like rice straw can initially stimulate microbial activity but later limits nitrogen availability, slowing the decomposition (Li et al., 2018; Zhou et al., 2019).

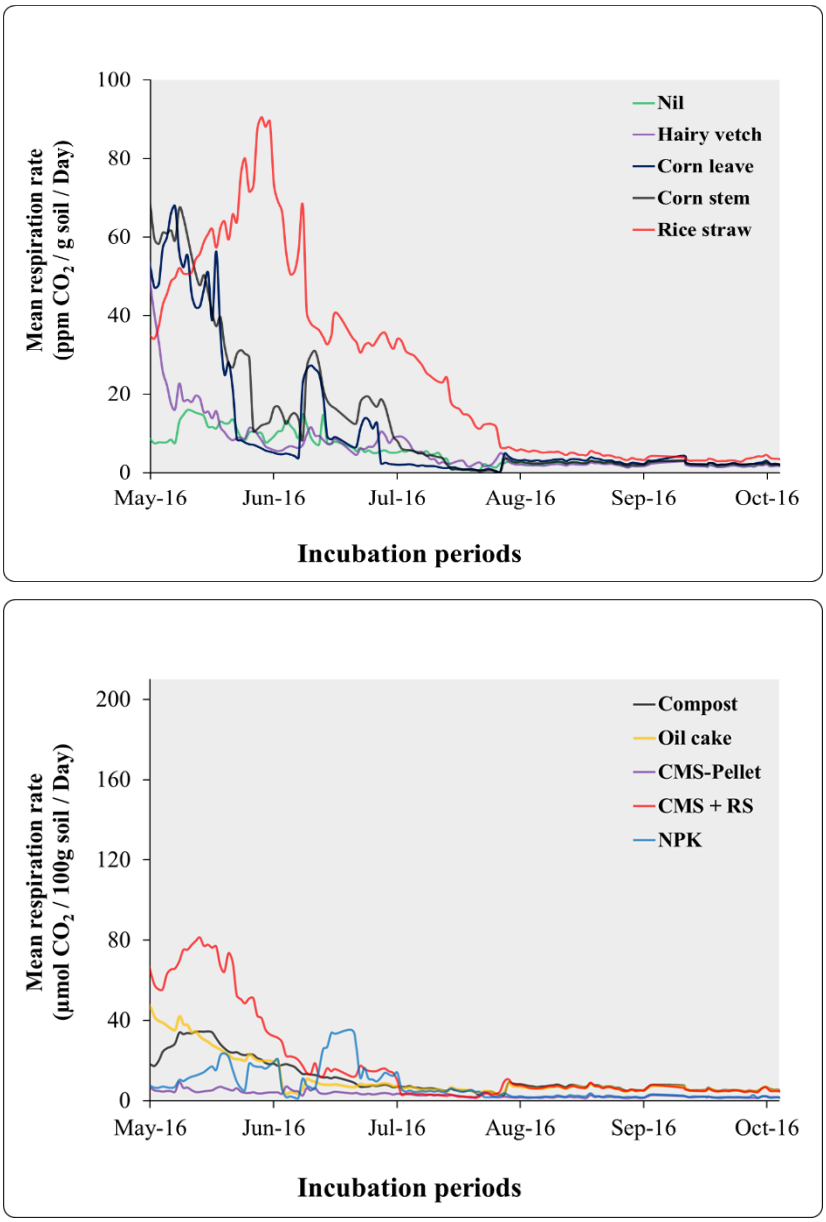

Fig. 4.5 The CO_2 emission rate in soils amended with hairy vetch, corn leave, corn stem, rice straw, compost, oil cake, CMS, CMS+RS, and NPK fertilizers and without fertilization (Nil).

4.4.2 Microbial populations: bacteria, fungi, and actinomycetes

The microbial dynamics in soils amended with various fertilizers revealed distinct patterns in bacterial (Fig. 4.6a and 6b), fungal (Fig. 4.7a and 7b), and actinomycetes populations (Fig. 4.8a and 8b). The bacterial populations in the per-incubation soil (week 0) were 460 x 10^6 CFU g^{-1} soil (Fig. 4.6a). Soils incubated with or without amendments increased bacterial populations about twofold in Nil, NPK and compost treatments; about threefold in HV, RS, CMS, and CMS+RS treatments; about fivefold in OC treatment; and seven-to eightfold in CL and CS treatment compared to the bacterial population in the pre-incubation soil. After 6 weeks, bacterial population decreased and reached a steady state after 12 weeks of the incubation period. The observed maximum increase in bacterial populations during the first 6 weeks of incubation in amended soils aligns with previous studies that reported rapid microbial colonization in response to both organic and inorganic amendments (Cleveland et al., 2006; Meshram et al., 2016). The subsequent decrease to a steady state by 12 weeks suggests a stabilizing effect, likely due to the depletion of readily available nutrients and the establishment of a more equilibrium microbial community (Cleveland et al., 2007). This pattern of early abundance followed by stabilization is typical in soil microbial dynamics following nutrient input (Cleveland et al., 2006; Cleveland et al., 2007). In contrast, treatments like CMS + RS and NPK showed lower bacterial populations, which could be attributed to either the lower nutrient content or the more recalcitrant nature of rice straw, which is harder for bacteria to degrade compared to other organic matter (Zhou et al., 2019).

The fungal and actinomycetes populations in the pre-incubation soil were about 600 x 10^4 CFU g^{-1} soil (Fig. 4. 7a and 7b), and 400 x 10^6 CFU g^{-1} soil (Fig. 4.8a and 8b), respectively. Unlike the bacterial population, fungal and actinomycetes counts in

all treatments decreased about fivefold and threefold, respectively, compared to those in the pre-incubation soil, and reached a steady state after 6 weeks of the incubation. Our findings are contrasted with those of Meshram et al. (2016) and Singh and Dhar (2011), who observed that organic fertilizers or organic + NPK enhanced the growth of not only bacteria but also fungi and actinomycetes. The inconsistency between our results and theirs may stem from differences in experimental conditions and sampling design. In our study, fertilizers were applied only once at the beginning of the incubation period, and the experiment was conducted without plant growth. Conversely, Singh and Dhar (2011) applied farmyard manure (FYM), vermicompost, and bioinoculants during the rice phase, with applications timed to coincide with plant growth stages. This periodic addition of organic inputs and the presence of plants likely sustained a continuous supply of carbon substrates through root exudates and decomposing organic matter, which supported fungal and actinomycete populations. Similarly, Selvi et al. (2004) reported that the integration of FYM (10 t/ha annually) with NPK fertilizers in a continuous cropping system stimulated microbial activity, including fungi and actinomycetes. The absence of plants in our study could explain the decline in not only fungi but also actinomycetes, as these groups rely more heavily on readily available organic substrates and root-derived carbon sources compared to bacteria, which can exploit a wider range of organic and inorganic nutrients. Thus, our finding highlighted the importance of sustained organic input and plant-microbe interactions in maintaining diverse soil microbial communities during crop production.

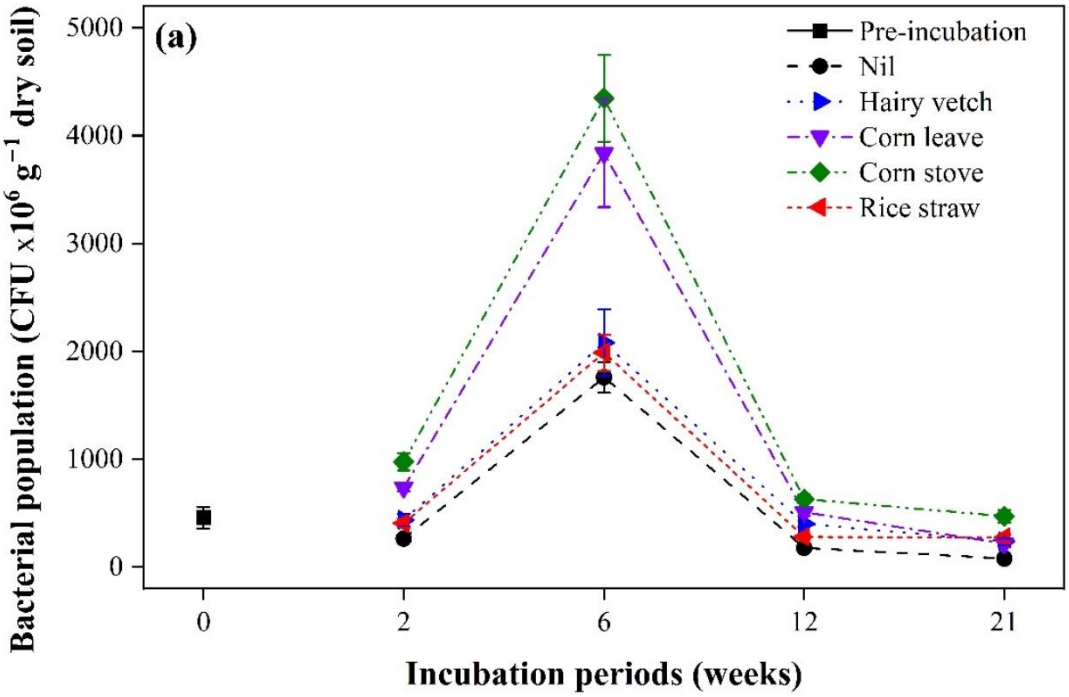

Fig. 4.6a Bacterial population in soils amended with hairy vetch, corn leave, corn stover, rice straw, and without fertilization (Nil). Soil samples were collected 4 times during the incubation period: 2, 6, 12, and 21 weeks.

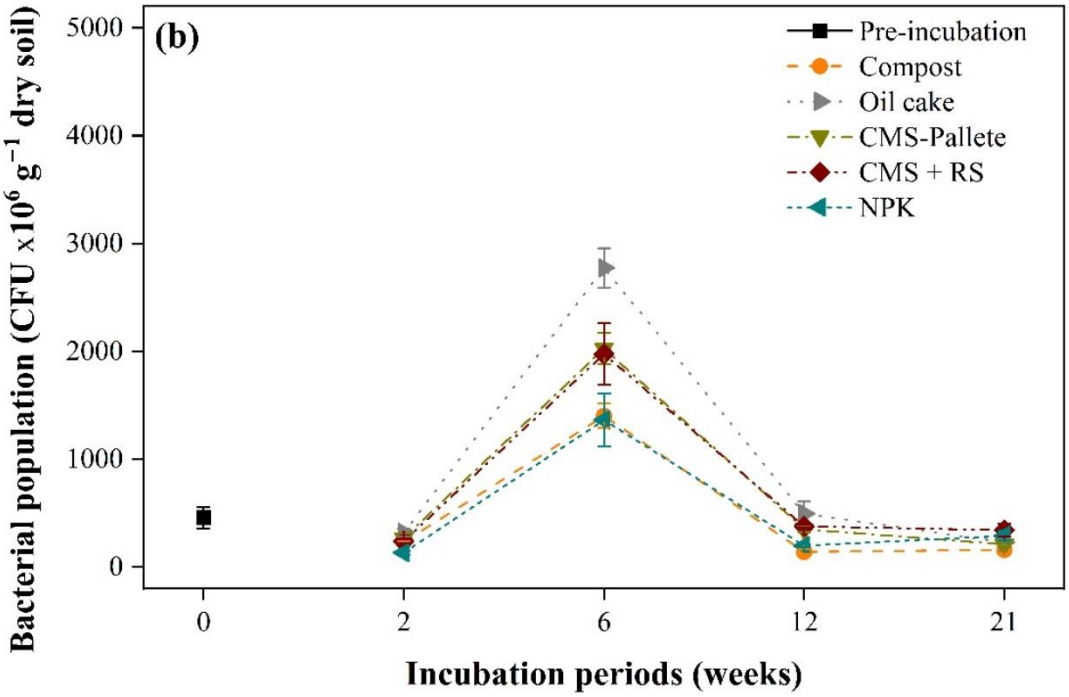

Fig. 4.6b Bacterial population in soils amended with organic and inorganic fertilizers and without fertilization (Nil). Soil samples were collected 4 times during the incubation period: 2, 6, 12, and 21 weeks.

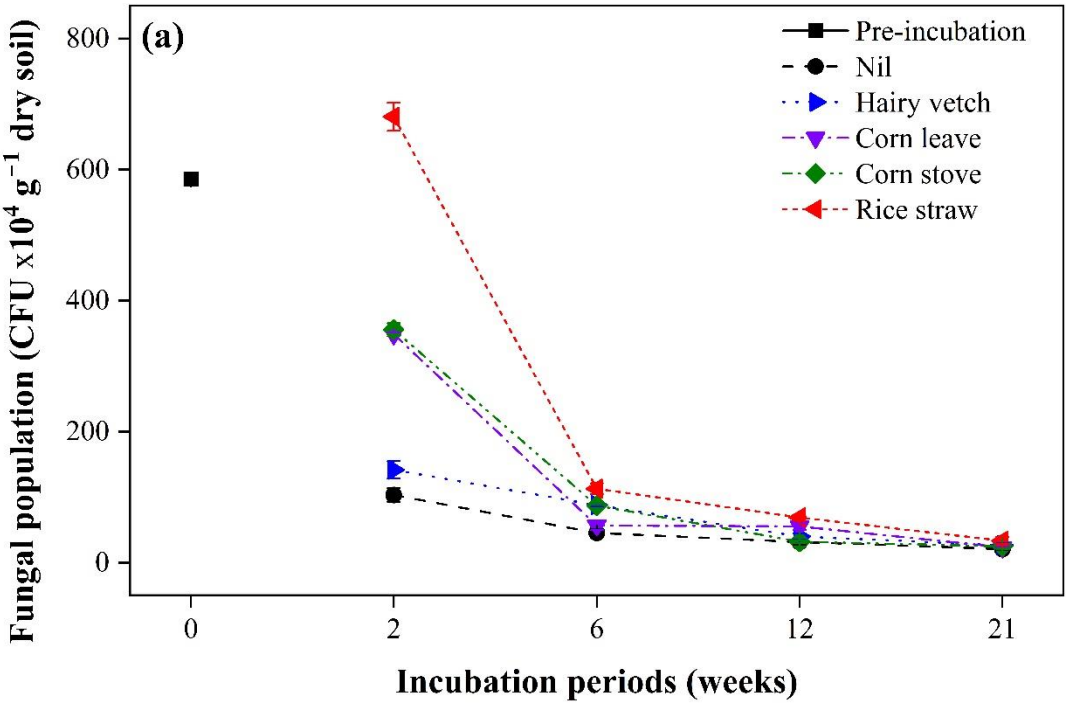

Fig. 4.7a Fungal population in soils amended with organic and inorganic fertilizers and without fertilization (Nil). Soil samples were collected 4 times during the incubation period: 2, 6, 12, and 21 weeks.

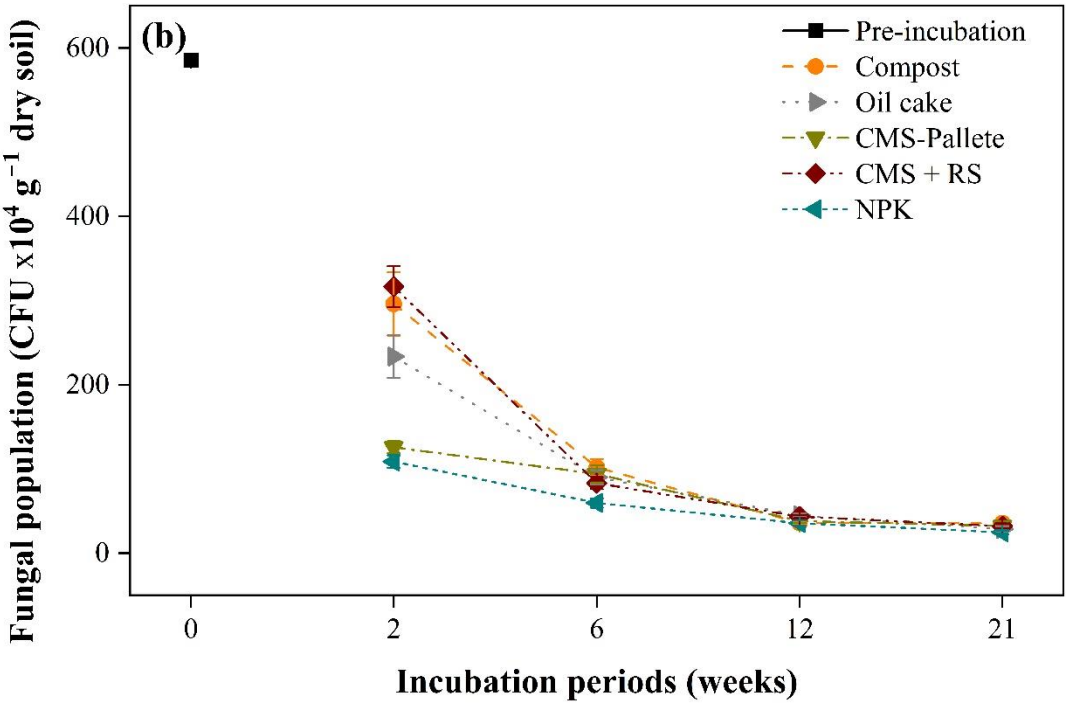

Fig. 4.7b Fungal population in soils amended with organic and inorganic fertilizers and without fertilization (Nil). Soil samples were collected 4 times during the incubation period: 2, 6, 12, and 21 weeks.

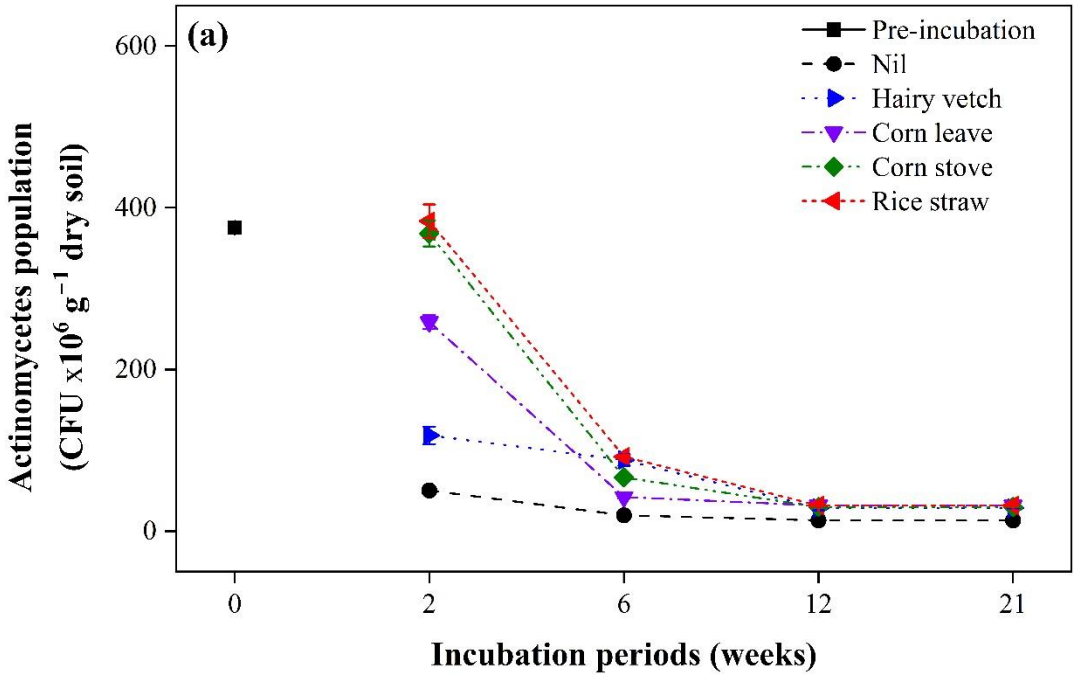

Fig. 4.8a Actinomycetes population in soils amended with organic and inorganic fertilizers and without fertilization (Nil). Soil samples were collected 4 times during the incubation period: 2, 6, 12, and 21 weeks.

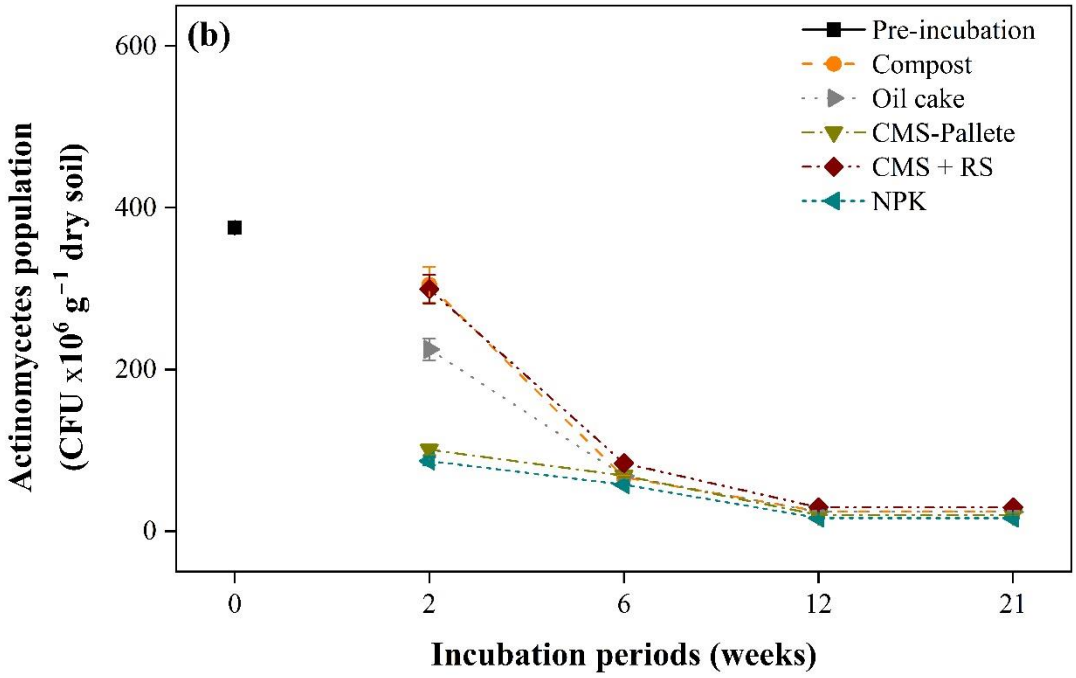

Fig. 4.8b Actinomycetes population in soils amended with organic and inorganic fertilizers and without fertilization (Nil). Soil samples were collected 4 times during the incubation period: 2, 6, 12, and 21 weeks.

4.4.3 Total SOC and TN

Total SOC and TN in soil amended with organic and inorganic fertilizers varied from 9-15 g kg^{-1} and 1-1.7 g kg^{-1}, respectively (Fig. 4.9a and 9b). Total SOC and TN were high in soil amended with crop residues and combined fertilization (CMS+RS), followed by CP, OC, and CMS. The lowest SOC and TN was in Nil treatment. However, we found that SOC and TN in soils incubated with or without fertilizers decreased throughout the incubation period. The observed decline in SOC and TN during the incubation period can be attributed to the decomposition of organic matter (Li et al., 2018; Tian et al., 2014; Zhou et al., 2019). In soils amended with organic fertilizers, readily decomposable carbon fractions are rapidly utilized by soil microorganisms, leading to CO_2 emissions and the conversion of organic N into inorganic forms via mineralization processes (Graaff et al., 2011; Zhou et al., 2019). This aligns with findings by Six et al. (2002), who reported that soil microbial activity intensifies in the presence of labile organic substrates, accelerating C and N losses during incubation. In addition, the rate of SOC and TN decline likely depended on the quality of the organic amendments. Amendments such as corn leaves, hairy vetch, and compost, rich in easily decomposable compounds (e.g., polysaccharides and proteins), promoted faster initial microbial activity compared to more resistant materials like rice straw and corn stover. This pattern corroborates findings by Trinsoutrot et al. (2000), who observed that substrate quality significantly influences mineralization rates during soil incubation experiments. The continuous decline in SOC and TN over time emphasizes the importance of balancing organic inputs with microbial demand (Fottett, 2001). Without periodic replenishment of organic material, soils may experience nutrient depletion and reduced fertility (Guimaraes et al., 2013).

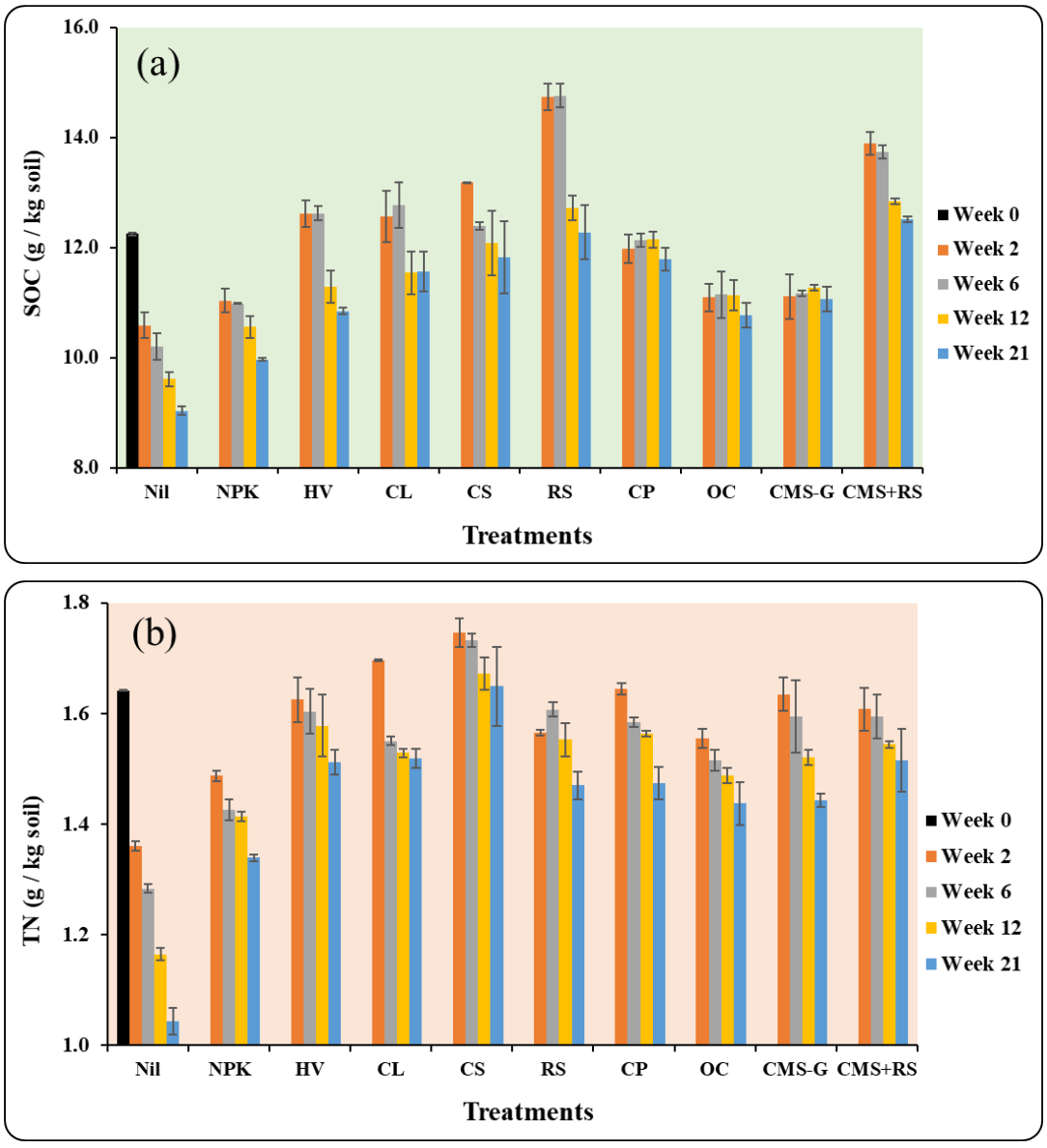

Fig. 4.9 Total SOC **(a)** and TN **(b)** contents in soils amended with organic and inorganic fertilizers. HV, CL, CS, RS, CP, and OC represent hairy vetch, corn leave, corn stove, rice straw, compost, and oil cake, respectively. Error bars represent standard error (SE; n=3).

4.4.4 C and N content in debris fraction

The C and N contents in the debris fraction varied from about 2.5 to 5.5 g kg^{-1} and 0.3-0.6 g kg^{-1}, respectively (Fig. 4.10a and 10b). High C and N was observed in CMS+RS, followed by crop residues, CP, CMS, OC, NPK, and Nil treatments. As in the case of SOC and TN, C and N contents in the debris fraction (coarse particulate organic matter) in soils amended with organic and inorganic fertilizers decreased throughout the incubation period. The initial C and N contents were high in soils treated with CMS + RS, followed by organic amendments (i., e. rice straw, hairy vetch, corn leave, and corn stem, compost, and oil cake, while Nil was the lowest. The decrease of C and N in the debris fraction reflects the progressive decomposition and mineralization of organic residues. Debris fraction consists of coarser plant-derived materials that are readily accessible to microbial activity in early stages of decomposition (Brady and Weil, 2010; Golchin et al., 1994a, 1994b). The decline in C and N over time may also indicate the conversion of the active decomposition phase, characterized by the breakdown of easily degradable compounds, to the stable phase, where recalcitrant organic matters become dominant and can be protected through macro-or-microaggregates (Osborne et al. 2014; Rovira and Vallejo, 2002). Therefore, our findings emphasize that combined fertilization like CMS+RS, which exhibited a decreasing trend yet remained higher than other treatments, may offer a balance between rapid nutrient release and longer-term carbon storage.

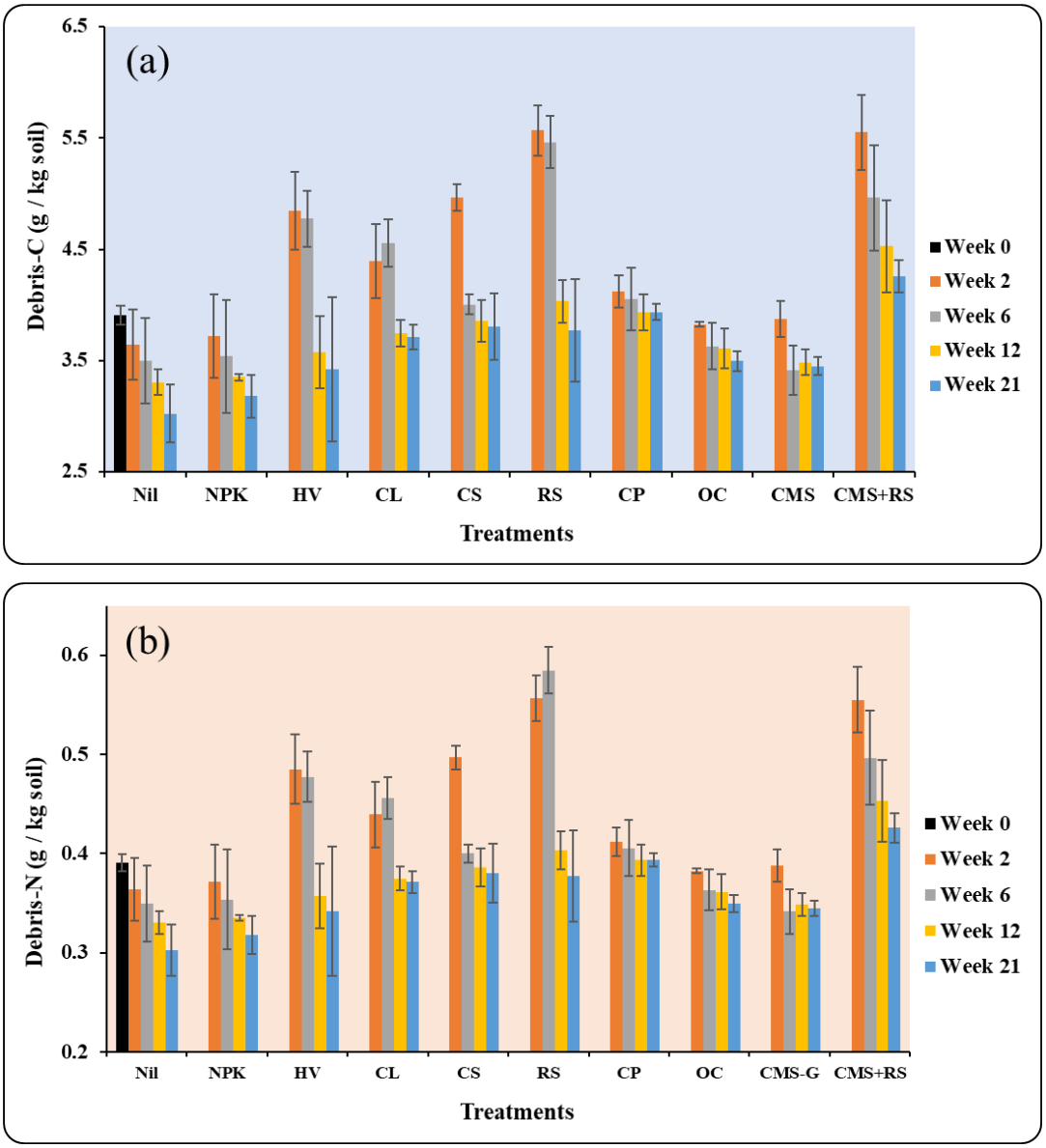

Fig. 4.10 The C **(a)** and N **(b)** contents in the debris fraction in soils amended with organic and inorganic fertilizers. HV, CL, CS, RS, CP, and OC represent hairy vetch, corn leave, corn stove, rice straw, compost, and oil cake, respectively. Error bars represent standard error (SE; n=3).

4.4.5 C and N content in FA fraction

The C and N in the FA fraction ranged from 1-3g kg^{-1} and 0.2-0.6 g kg^{-1} (Fig. 4. 11a and 11b). High C content was observed in HV, CL, CS, RS, CP, and CMS+RS. The highest N content was found in the CS treatment, while the other treatments showed similar N levels, with the lowest observed in the Nil treatment. As in the cases of SOC and debris, soils incubated with or without fertilizers also consistently decreased in both C and N contents in the FA fraction during the incubation period. The decrease in both C and N in the FA fraction over time is indicative of ongoing microbial degradation and humification processes. Treatments with higher initial organic matter content, such as HV, CL, CS, RS, and CP, showed the most pronounced reductions in FA-C, and FA-N indicating that these amendments may provide a larger pool of labile organic substrates for microbial activity early in the incubation. Brady and Weil (2010) and Tan, (2014) documented that during the initial stages of decomposition, labile organic compounds are converted into more soluble organic acids, including FA. As the incubation period extended, microbial populations may have used up readily available C and N, leading to a reduction in the production of soluble organic acids like FA. Thus, in our study, the decline in FA fractions may reflect the shift from more soluble organic compounds towards more stable humic substances. This suggests that after an initial phase of intense microbial activity, the decomposition slows down and stabilizes, which is consistent with findings from Bhatnagar et al. (2018) and Lehtinen et al. (2014), who emphasized the role of microbial communities in driving the mineralization and transformation of organic matter into more stable forms. Therefore, our finding that C and N in the FA fraction decrease during the incubation period highlights the role of microbial activities in stabilizing SOM over time, emphasizing the need for regular organic inputs and the

careful management of nutrient cycling to maintain soil health (Follett, 2001; Guimaraes et al., 2013).

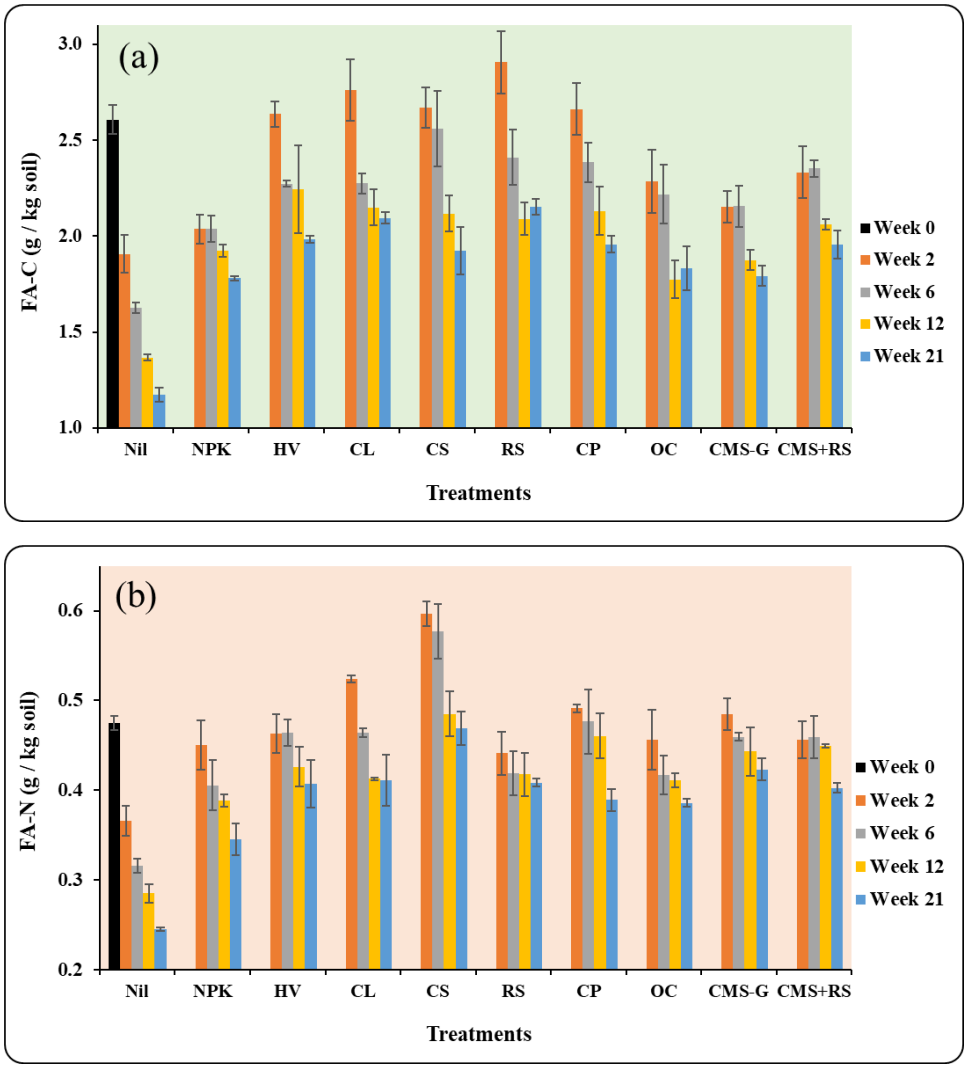

Fig. 4.11 The C **(a)** and N **(b)** contents in the FA fraction in soils amended with organic and inorganic fertilizers. HV, CL, CS, RS, CP, and OC represent hairy vetch, corn leave, corn stove, rice straw, compost, and oil cake, respectively. Error bars represent standard error (SE; n=3).

4.4.6 C and N content in HA fraction

The C and N contents in the HA fraction varied from about 1-2.2 g kg^{-1} and 0.15-0.27 g kg^{-1}, respectively (Fig. 4.12a and 12b). Unlike FA fractions, C and N in the HA fraction in soils amended with organic and inorganic fertilizers tended to increase during the first 12 weeks of incubation before they decreased as the incubation goes longer. The observed increase in C and N in the HA fraction during the early stages of incubation aligns with the mineralization and immobilization of labile organic matter, followed by humification processes, in which soluble fractions are converted into more stable humic matter (Tan, 2014). In contrast, HA-C and HA-N in NPK and Nil treatments declined consistently from the beginning of the incubation. This highlights the importance of organic amendments in sustaining humic substance formation. These results emphasize the synergistic role of organic inputs in enhancing humification and sustaining soil fertility over time. Substrates such as RS, CL, CS, CMS, CMS+RS, and OC resulted in high HA-C and HA-N levels. However, we observed that soil amended with CP, CS, OC, CMS, and CMS+RS gradually increased N in the HA fraction. The gradual increase in HA-N observed in these treatments likely reflects the slower mineralization and immobilization of nitrogen-rich compounds, which allow these compounds to be integrated into HA structures over time (Ming et al., 2024). Brady and Weil (2010) and Tan (2014) reported that during organic matter decomposition, one-third of C and considerable N is metabolized by microbes and incorporated into their cells. As decay proceeds, microbes polymerize some of the simpler new compounds with the complex residual products together into long and complex chains of high molecular weight compounds. These compounds interact with N-containing amino compounds, forming a significant component of stable humus. Therefore, high HA-N in these amendments may highlight their potential for improving soil fertility and N retention compared to other amendments.

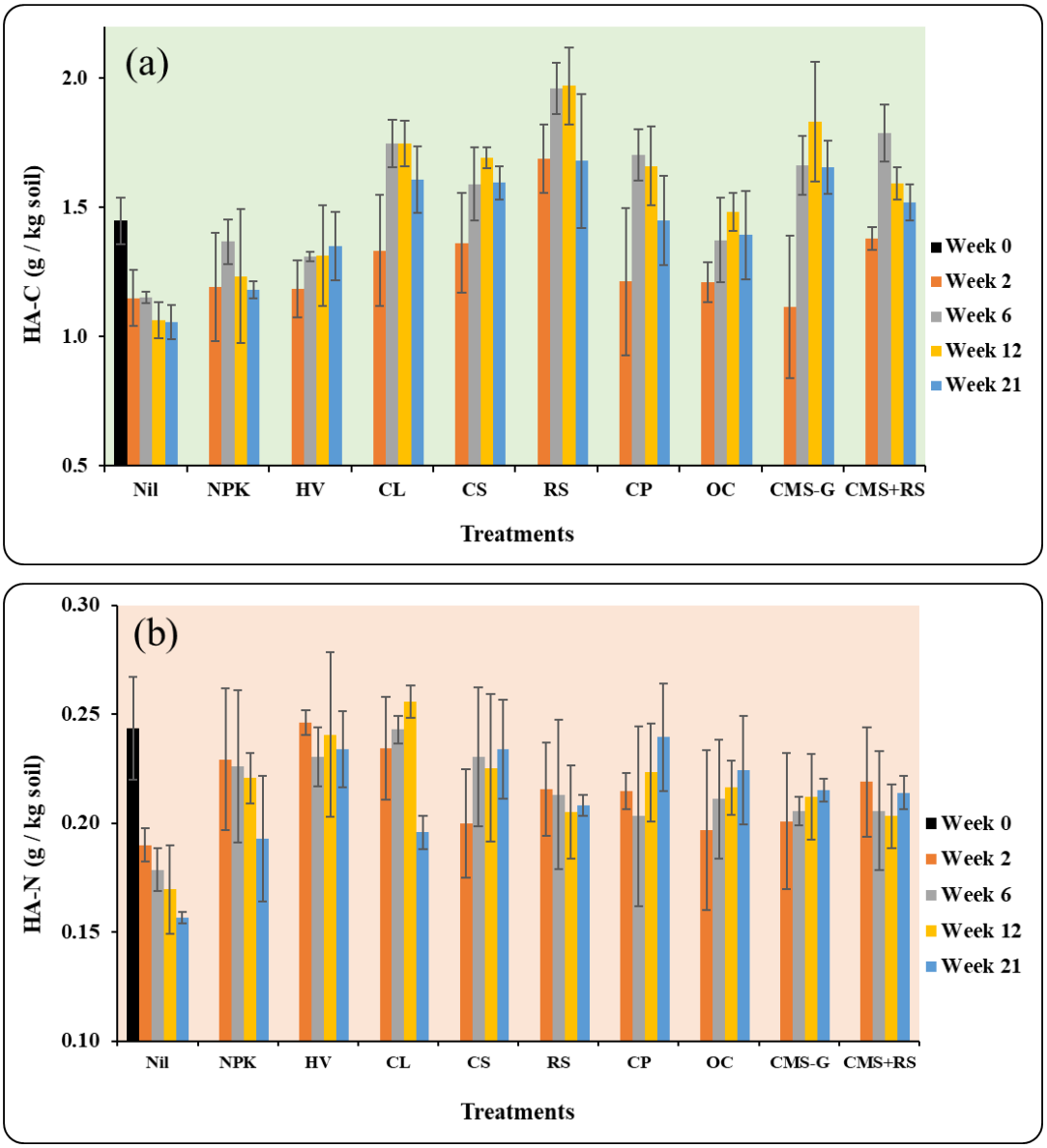

Fig. 4.12 The C **(a)** and N **(b)** contents in the HA fraction in soils amended with organic and inorganic fertilizers. HV, CL, CS, RS, CP, and OC represent hairy vetch, corn leave, corn stove, rice straw, compost, and oil cake, respectively. Error bars represent standard error (SE; n=3).

4.4.7 C and N content in HM fraction

The C and N contents in the HM fraction demonstrated four distinct patterns (Fig. 4.13a and 13b). First, soils incubated without fertilizer application (Nil) decreased in both C and N. This result clearly highlights the importance of organic amendments in enhancing the formation of HM fraction. Second, soils incubated with NPK fertilizer decreased C but increased N. This can be attributed to the process of immobilization, consistent with findings by Okorkov et al. (2016). As microbes decompose organic materials remained in soils, N may be incorporated into the HM fraction through microbial byproducts, microbial biomass residues, or microbial transformation of organic matter into more stable N-containing compounds, leading to an increased storage of N in HM (Berg and Matzner, 1997; Brady and Weil, 2010). Third, soils amended with CP, CMS, and OC increased C but decreased N. Compost, a humus-like compound, typically contains both labile and recalcitrant components, such as lignin and polyphenols. During the early stages of decomposition, large amounts of N was released as gas forms, while the recalcitrant C fractions play a key role in stabilizing SOM (Masmoudi et al., 2024). CMS and OC (wastes remaining after fermentation) may provide much N in more labile forms, which are susceptible to mineralization. Im et al. (2015) reported that the half-life of organic N in soil treated with OC fertilizer was 7 to 21 days, indicating that it was mineralized within 3 weeks of application. Lastly, soils amended with plant residues alone or combined fertilizers increased both C and N. Particularly, soils amended with CMS+RS contributed to a higher increase in C than in soils treated with crop residues alone. This result showed that organic residues amended into the soils provided sustained N availability, which is critical for the formation of N-enriched humic substances, while a combined fertilization increased further the stable SOM (Berg and Matzner, 1997; Brady and Weil, 2010).

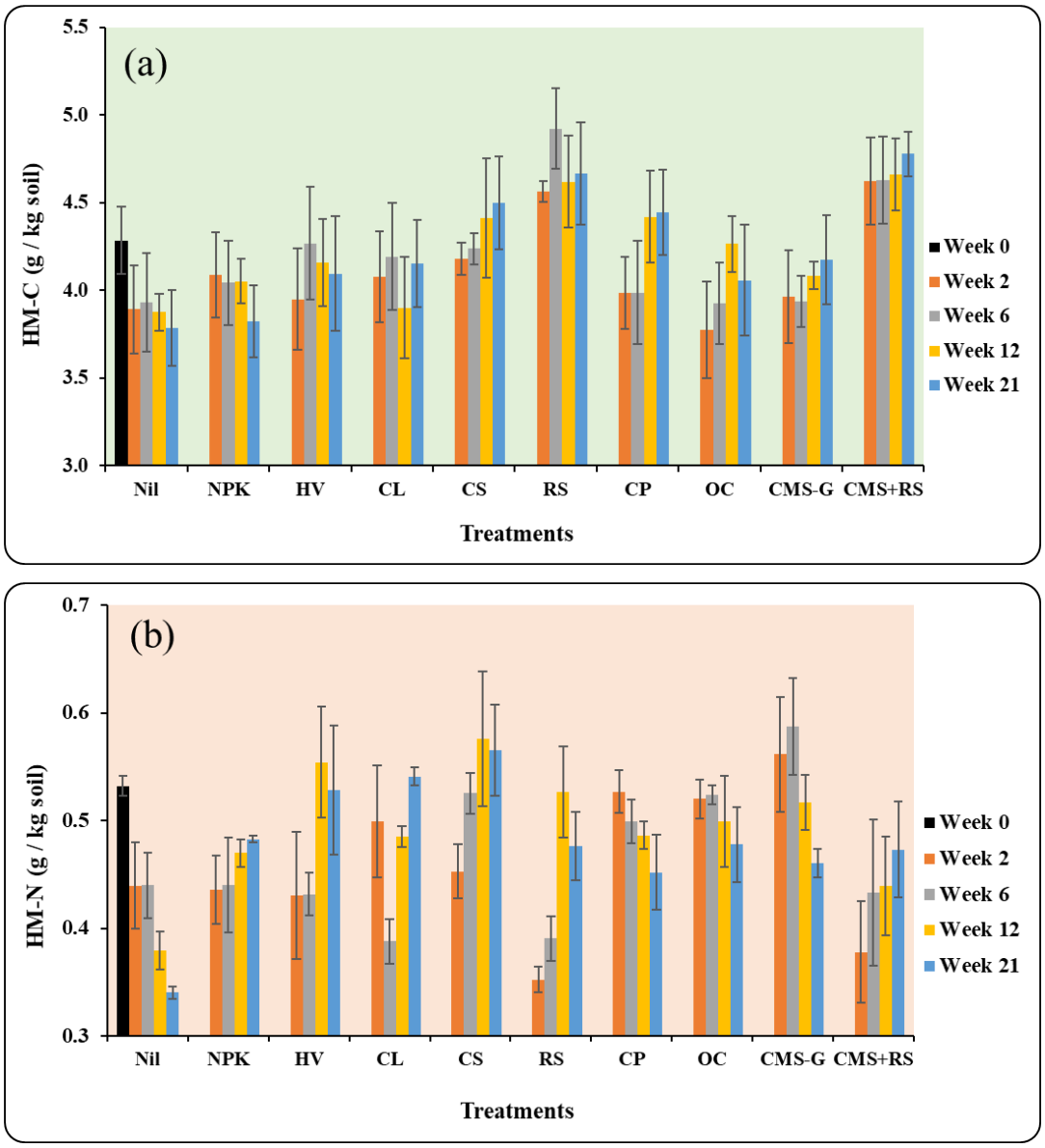

Fig. 4.13 The C **(a)** and N **(b)** contents in the HM fraction in soils amended with organic, inorganic, and combined fertilizers. HV, CL, CS, RS, CP, and OC represent hairy vetch, corn leave, corn stove, rice straw, compost, and oil cake, respectively. Error bars represent standard error (SE; n=3).

4.4.8 Degree of polymerization

The humification index (HI), HA/FA, indicates the intensity of OC humification processes and mobility of C in the soils. (Masmoudi et al., 2024; Khalafalla et al., 2019). In our study, the HA/FA ratio in all treatments gradually increased during the incubation period, but the magnitudes of the increase differed among treatments (Fig. 4.14a). RS, CP, and CMS+RS showed the highest increase in HI (0.55 – 1.0), followed by CMS, CS, CL, HV, and OC (0.55 – 0.9), while NPK and Nil treatments exhibited a plateauing increase (0.55 – 0.7). This result indicated that RS, CP, and CMS+RS contributed to a higher humification rate than other soil amendments. However, HI value showed a decreasing trend after 12 weeks of incubation period in most treatments. Only CMS+RS treatment maintained a liner increasing trend throughout the incubation period. This result suggested that combined fertilization enhanced humification rate more effectively than fertilizing soil with crop residues or chemical alone. On the other hand, the degree of transformation (DT), expressed as (FA + HA) / HM, consistently declined across all treatments, reflecting the gradual stabilization of organic matter as labile fractions (FA and HA) transformed into the more recalcitrant HM fraction (Fig. 4.14b). Among the treatments, CMS+RS exhibited the most pronounced linear decrease in DT, suggesting that combined fertilization practice enhanced the stability of SOM more effectively than the use of an individual amendment. According to Moraes et al. (2011) and Ukalska-Jaruga et al. (2019), the higher HI and lower DT imply that humic substances occur mainly in high-polymerized organic forms. Therefore, results of HI and DT highlighted the potential of integrated organic amendments in improving soil C stability and long-term fertility.

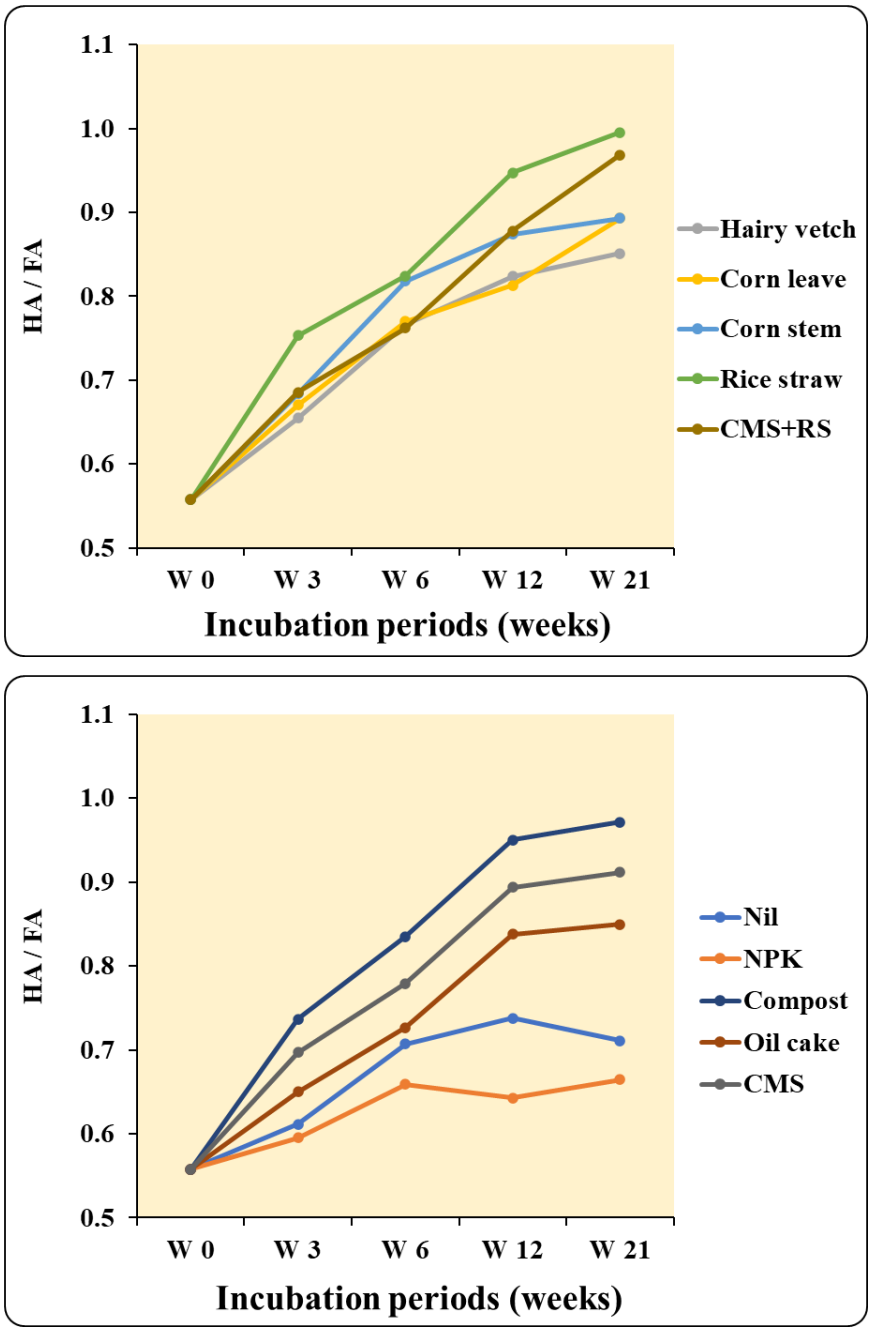

Fig. 4.14a Humification index (HI) in soils amended with organic, inorganic, and combined fertilizers.

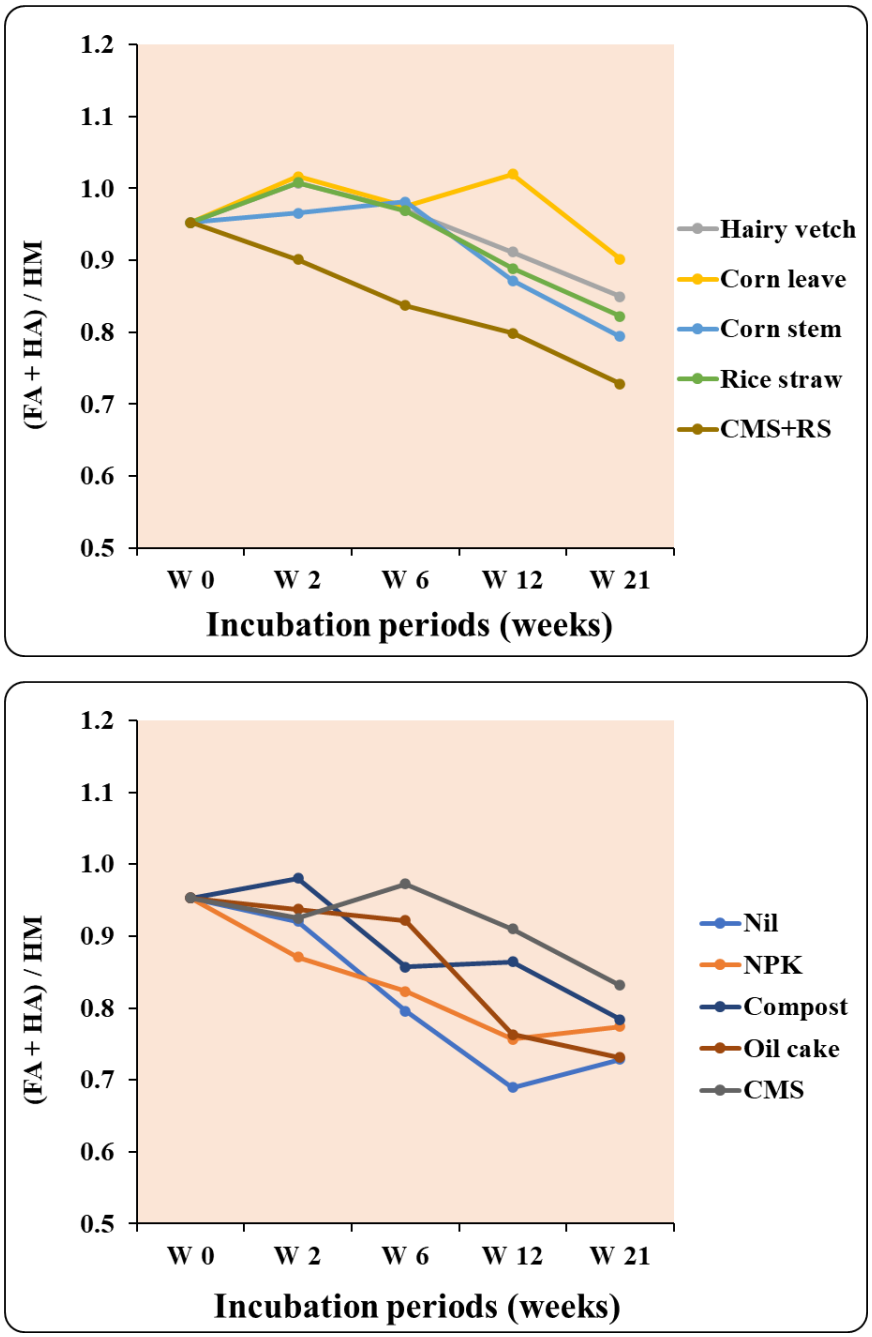

Fig. 4.14b Degree of transformation (DT) in soils amended with organic, inorganic, and combined fertilizers.

4.4.9 Quality of humic substances in soils with different amendments

The C/N ratio in humus fractions (FA, HA, and HM) can be used to assess the effect of organic and inorganic amendments on the formation and stability of SOM, as these fractions are the major C pools in SOM (Stevenson, 1994). The formation of FA, HA, and HM relies largely on the presence of N in the soil or in substrates that are applied into the soil. The greater the presence of N, the more stabilized SOM is formed (See et al., 2005). In general, a decrease in the C/N ratio of FA, HA, and HM indicates well-developed humic matter (Tan, 2014). In our study, we found that the C/N ratio in humus fractions were FA (4-6) < HA (6-8) < HM (8-11) in soils amended with or without fertilizers (Fig. 4.15a and 15b). These results are consistent with See et al. (2005), who found that dissolved organic N was incorporated into low molecular weight humus fractions first, before being polymerized and combined to form larger HA and HM molecules. In addition, the low C/N ratio in FA and HA may be due to the loss of C as gaseous forms during the decomposition. Brady and Weil (2010) and Tan (2014) reported that during the humification process, one-third of C and large proportion of N was incorporated into microbial cells, while two-thirds of C was lost as CO_2 gas. Moreover, we found that the C/N ratio of HM differed among soil amendments. These differences are attributed to the quality of fertilizer materials used. The C/N ratio of HM in soil amended with NPK, CP, CMS, and OC, or without fertilization (Nil), tended to increase during the incubation period. In contrast, the C/N ratio in soils amended with crop residues and combined fertilizer tended to decrease as the incubation progressed. The increase of C/N ratio in HM may indicate the reduction of SOM mobility, which requires N for stabilization. The decrease C/N ratio of HM may be attributed to a considerable amount of N being incorporated, thus stabilizing SOM more effectively (See et al., 2005). Our results suggest that combined fertilization enhances SOM quality more effectively than applying amendments alone.

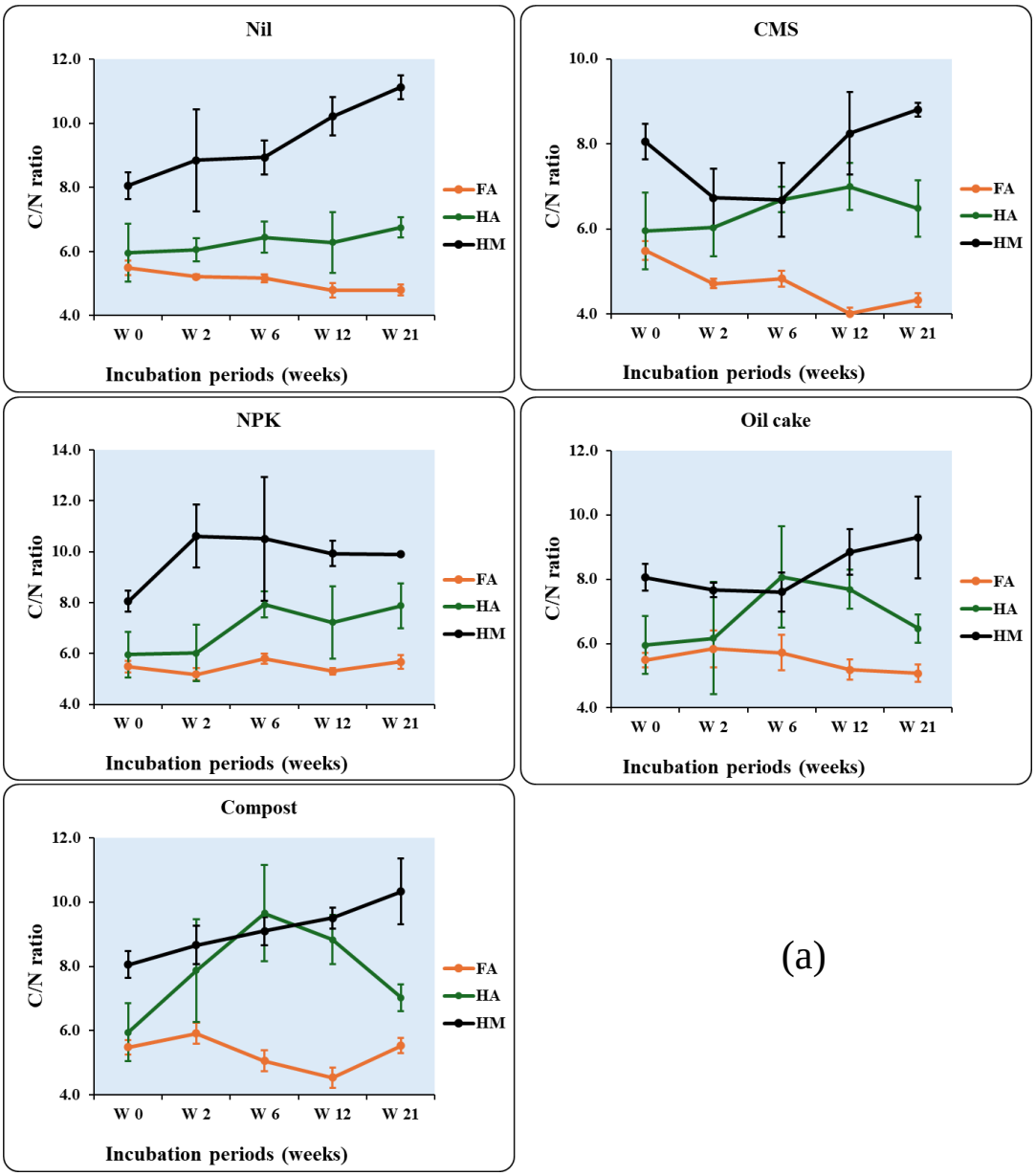

Fig. 4.15a The variability of C/N ratio in fulvic acid (FA), humic acid (HA), and humin (HM) fractions in soil amended with NPK, compost, oil cake, and CMS fertilizers, and without fertilization (Nil).

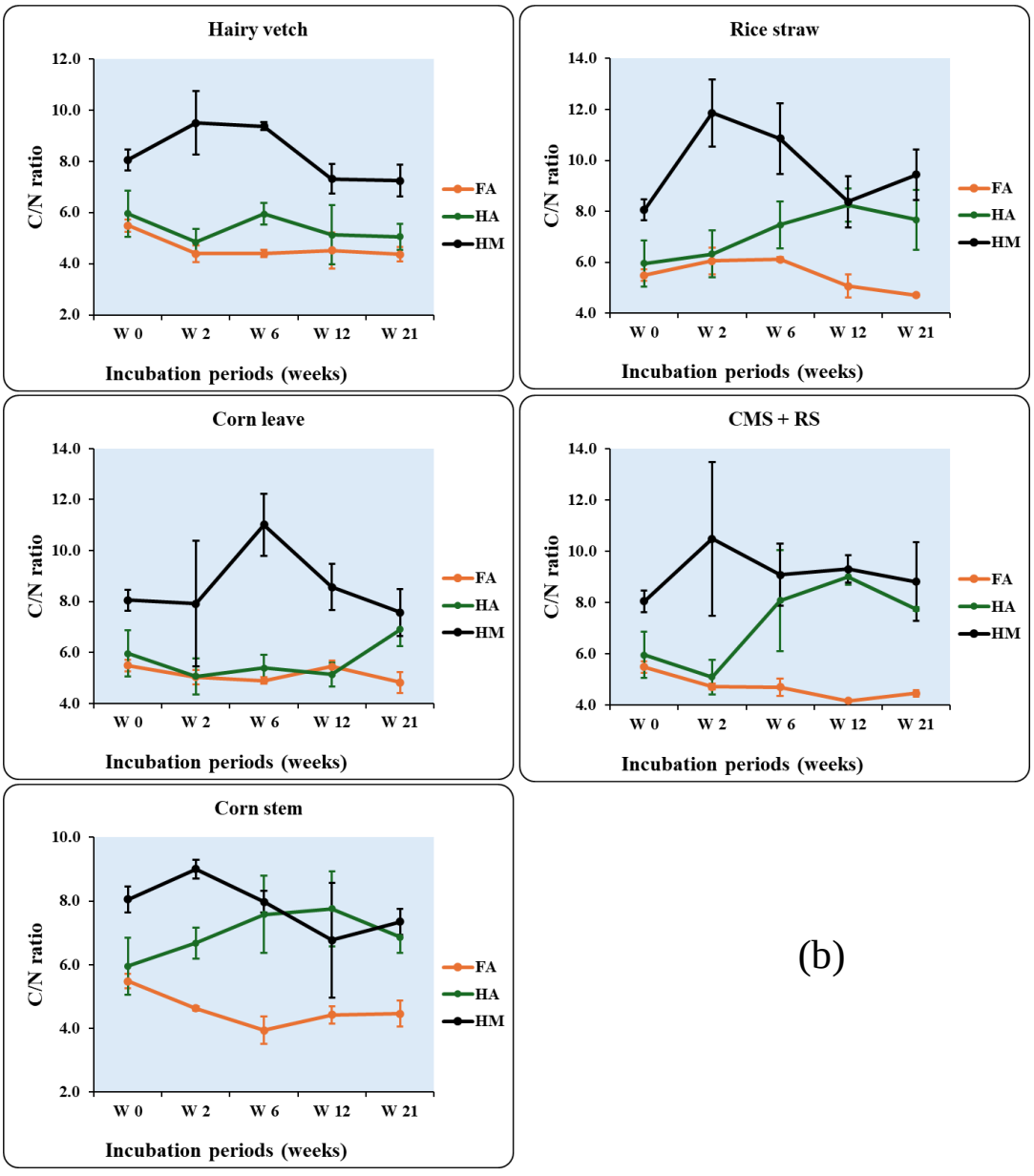

Fig. 4.15b The variability of C/N ratio in fulvic acid (FA), humic acid (HA), and humin (HM) fractions in soils amended with crop residues and combined fertilizers.

4.5. Conclusion

The results of the incubation indicated that organic and inorganic soil amendments elevated CO_2 emission and altered microbial populations during the first 2-3 months of the incubation period. Crop residues (Rice straw, hairy vetch, corn leave, corn stem) and CMS+RS, produced higher CO_2 emission than compost, NPK, and Nil. Regardless of the soil amendments, bacterial populations increased about two- to eightfold within the first 6 weeks compared to the pre-incubation soil (460 x 10^6 CFU g^{-1} soil), then decreased dramatically to a steady state after 12 weeks of incubation. The highest bacterial counts were observed in CL and CS, while the lowest was in Nil treatment. Compared to those in the pre-incubation soil (60 x 10^4 CFU g^{-1} soil and 860 x 10^6 CFU g^{-1} soil), fungi and actinomycetes decreased about fivefold and threefold, respectively, and declined to a steady state after 12 weeks of the incubation.

On the other hand, we observed that soil amended with rice straw and CMS+RS resulted in higher SOC, while soils amended with corn stover led to higher TN. Similarly, soil treated with crop residues and CMS+RS yielded high C and N in debris and FA fractions. However, SOC, TN, C and N in debris and FA fractions decreased dramatically during the incubation period. In contrast, soils amended with organic fertilizers, except for NPK and Nil, increased C and N in HA and HM fractions, indicating that the labile C and N are gradually converted into more stable forms. To confirm the formation of stable SOM, the HI and DT were calculated. We found that the HI increased, and the DT decreased for all studied treatments. However, the HI in most treatments, particularly in Nil and NPK, showed a plateauing increase, whereas that in the CMS+RS treatment exhibited a linear increase. This result clearly demonstrates that soil humic matter occurs mainly in highly polymerized organic

forms, but the combined fertilization promotes SOM stability more effectively than organic or inorganic amendments alone.

Regardless of soil amendments, in addition, the C/N ratio in humus fractions were FA (4–6) < HA (6–8) < HM (8–11). The C/N ratio of HM in soil amended with NPK, compost, CMS, and oil cake, as well as Nil, tended to increase, while the C/N ratio in soils amended with crop residues and CMS+RS, tended to decrease as the incubation progressed. The increase of C/N ratio in HM may indicate the reduction of SOM mobility, which requires N for stabilization. The decrease C/N ratio of HM may be attributed to a considerable amount of N being incorporated, thus stabilizing SOM more effectively. Based on HI, DT, and the C/N ratio in FA, HA, and HM, our results suggest that combined fertilization is more effective in enhancing SOM quality than using organic fertilizer alone, with N playing a key role in controlling its stability.

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CHAPTER 5

Assessments of soil organic matter quality in a long-term fertilized paddy: Variations in carbon and nitrogen dynamics along with humus formation

5.1 Abstract

A previous study conducted a long-term experiment (over 50 years) aimed at investigating the effect of fertilization on SOC and its fractions in relation to grain yield. The author found that SOC concentration significantly increased in the compost (CP) and NPK+CP treatments, while it decreased in the NPK and Nil treatments. Additionally, only the Nil treatment showed a significant decrease in rice grain yield. The author also measured C contents in different soil particle sizes and found that C accumulated primarily in the < 0.053 mm fraction in the CP and NPK+CP treatments. Despite existing studies on soil C, the impact of long-term fertilization on the quality of humus fractions, which is a major pool of SOM, remains largely underexplored. This study evaluates the long-term effects of different fertilization practices on SOC, TN, and humus dynamics in continuously cultivated paddy soils. The results revealed that SOC and TN increased by about 10% in CP and NPK+CP-treated soils, while decreasing by about 8.5% in NPK and Nil treatments. The debris-C, FA-C, HA-C, and HM-C were significantly higher in CP and NPK+CP treatments compared to those in NPK and Nil treatments. This indicates that continuous cultivation without organic amendments leads to a decrease in SOM fractions, potentially resulting in the degradation of stable organic matter. In addition, the humification index (HI) and the degree of transformation (DT) showed that CP alone contributed to the formation of HA but has no impact on the formation of HM, while NPK+CP enhanced highly polymerized organic forms. Moreover, the C/N ratio of FA, HA, and HM remained stable in the CP and NPK+CP treatments as SOC and humus-C continued to increase,

while the C/N ratio of HA in NPK and Nil treatments decreased with SOC and humus-C contents. These findings showed that continuous addition of CP alone or NPK+CP enhanced the quality of SOM over the years, while continuous application of NPK alone or crop grown without fertilization led to C depletion, resulting in soil degradation and poor soil quality.

Keywords: Hunification indices, Humus fractions, Long-term experiment, Soil degradation, SOM pools, SOM quality indicators.

5.2 Introduction

Soil organic carbon (SOC) and total nitrogen (TN) are widely recognized as key indicators of SOM quantity and quality (Gregorich et al., 1994). SOM plays a crucial role in maintaining soil physical conditions while providing essential nutrients required for successful crop growth. Humus, the largest and most significant component of SOM, is formed through the microbial decomposition of plant and animal residues (Brady and Weil, 2010). It acts as a reservoir of vital nutrients needed for plant development and strongly influences the soil's physical, chemical, and biological properties (Gathala et al., 2007; Tang et al., 2018). Humus contains small portions of non-humic substances and large amount of humic substances (Brady and Weil, 2010). Basically, humic substances can be further chemically separated into fulvic acid (FA), humic acid (HA) and humin (HM), depending on their solubility in acid and alkaline solutions (Guimaraes et al., 2013). Soils in advanced stages of development typically contain higher amounts of HA compared to FA, reflecting the accumulation of heavier aromatic molecules and humin compounds (Amoakwah et al., 2022). Despite their long residence times, the chemical composition of humic substances can undergo changes in response to alterations in land use, management practices, and fertilizations, which differ in C and N concentrations. Tan (2014) reported that the formation of stable humic substances is influenced by microbial N sources. Therefore, a deeper understanding of the changes in the chemical composition of SOC is essential for revealing the long-term dynamics of humus formation, SOM stabilization, and their implications for soil fertility under different fertilization practices.

A long-term fertilizer experiment was initiated in 1967 at the Department of Functional Cereal Crop Research Farm in southeastern Korea, involving continuous

rice cultivation for over 50 years with four treatments: Nil, compost (CP), NPK, and NPK+CP. The study aimed to investigate changes in SOC and its fractions in relation to rice grain yields (Lee et al., 2009). The author found that the SOC concentration significantly increased in the CP and NPK+CP treatments, while it decreased in the NPK and Nil treatments; additionally, only the Nil treatment showed a significant decrease in rice grain yield. The author also measured C contents in different soil particle sizes and found that C was accumulated with the < 0.053 mm fraction mainly in CP and NPK+CP treatments. Despite existing studies on soil C, the impact of long-term fertilization on humus fractions remained largely underexplored. A detailed assessment of how these fractions respond to various fertilization practices is critical for understanding their contribution to soil health and long-term sustainability.

Several indexes can be used to assess the quality of humus fractions. The ratio between concentrations of humic and fulvic acids (HA/FA) indicates the potential mobility of C in the soil system. The proportions in relatively active and resistant HS fractions, expressed as the (HA + FA)/HM ratio, show the degree of HS transformation to the stable C form and SOC illuviation processes. Higher FA-C than HA-C indicates a higher mobility of organic matter with the labile HS fraction predominating, while higher FA-C + HA-C compared to HM-C indicates a lower intensity of organic matter humification associated with less transformation into stable SOM forms (Ukalska-Jaruga et al., 2019; You et al., 2014). Given that most long-term studies have examined the effects of N fertilizer rates on soil C and N contents and pools (Tong et al., 2009), the C/N ratio is commonly used as a parameter to estimate SOM quality. However, very few studies have evaluated the C/N ratio in FA, HA, and HM specifically in terms of SOM quality. Generally, a decrease in the C/N ratio in FA, HA, and HM is indicative of well-developed humic matter (Tan, 2014). Therefore, understanding the

dynamics of the C/N ratio in humic fractions is crucial for assessing SOM quality in long-term management systems.

Therefore, the objectives of the study were: (i) to quantify changes in SOC and TN concentrations in long-term soils amended with CP, NPK, and NPK+CP; (ii) to examine the dynamics of SOM fractions, including debris-C, FA-C, HA-C, and HM-C, and their relative contributions to SOC; and (iii) to assess SOM quality through indices such as HI and DT, as well as the C/N ratios of SOM fractions, to understand the mechanisms driving humification and stabilization.

5.3 Materials and Methods

5.3.1 Ferilization history and soil collection

According to Lee et al. (2009), the long-term experiment was established in 1967 at the Department of Functional Cereal Crop Research Farm, Milyang (36°83'N, 128°84'E, 12 m elevation) in the southeastern part of Korea. The soil is classified as the *Pyeongtaeg* series (somewhat poorly drained, fine silty mixed mesic, Typic Haplaquepts). Four fertility treatments were applied in a randomized complete block design with three replications. Each plot was 10 m by 10 m. The fertility treatments included: Nil (no fertilization), NPK, compost (CP), and NPK + CP.

In the NPK and NPK + CP treatments, inorganic fertilizers were applied at rates of 120-80-80 kg ha^{-1} of N-P_2O_5-K_2O from 1967 to 1972, and at 150-100-100 kg ha^{-1} from 1973 onward, using urea, superphosphate, and potassium chloride. Straw compost mixed with cattle manure, composted for more than six months outdoors, was applied annually at a rate of 10 Mg ha^{-1} in the CP and NPK + CP treatments. In 2007,

the straw compost contained mean values of 431 g kg^{-1} total C, 19.8 g kg^{-1} N, 5.2 g kg^{-1} P, and 29.1 g kg^{-1} K.

Inorganic fertilizers and manure were broadcast by hand onto the surface of each plot before tillage, prior to rice transplanting. Six rice cultivars were used: '*Palkyeng*' (1967–1971), '*Milseong*' (1972–1975), '*Nagdongbyeo*' (1972–1986), '*Palgongbyeo*' (1987–1993), '*Hwanambyeo*' (1994–1996), and '*Hwasambyo*' (since 1997). Soil samples were collected from the Ap horizon (0–15 cm depth) using a sampling auger after each harvest, air-dried, and sieved through a 2 mm sieve for chemical analysis.

Table 5.1 Selected soil samples for the analysis of non-humic and humic substances. Samples were selected 10 years after the onset of the study.

Years	Years after the onset	Treatments				Total
		Nil	NPK	Compost	NPK+CP	
1967						
1977	10	1	1	3		5
1982	15	1	3	3		7
1987	20	3	3	3		9
1992	25	3	3	3		9
1996	29	3	3	3		9
2001	34	3	2	3	3	11
2006	39	3	3	3	3	12
2017	50	3	3	3	3	12
Total	40	20	21	24	9	**74**

Note: CP represents "compost".

5.3.2 Fractionation of non-humic and humic substances

Soil extraction and separation, including NHS, debris, FA, HA, and HM were described in the Chapter 3, section 3.3.3 to section 3.3.5.

5.3.3 Analysis of SOC, TN, and C and N in FA, HA, and HM fractions

The simultaneous analysis of SOC and TN were already desctibed in Chapter 2. Analysis of C and N in FA, HA, and HM were already desbribed in Chapter 3, section 3.3.6 to 3.3.10.

5.3.4 Assessment of SOM quality

The humification index (HI) was calculated as the ratio of HA to FA (HA/FA). An HI ratio lower than the limit of 1.0 indicates the predominance of FA over HA, while an HI ratio higher than 1.0 signifies a high degree of conversion of FA into more stable HA. The degree of transformation (DT) was calculated as the sum of FA and HA over HM (FA+HA)/HM. Lower DT (DT < 1) indicates greater SOM stability. On the other hand, the C/N ratio in the FA, HA, and HM fractions were computed, respectively, to examine the impact of long-term soil amendment on the formation and stability of SOM.

5.3.5 Statistical analysis

Correlation coefficient (*r*) was tested to examine the relative increase or decrease in SOC and TN in long-term soils amended with CP, NPK+CP, and no fertilization (Nil).

5.4 Results and Discussion

5.4.1 Changes in SOC and TN content in long-term fertilized paddy

The total SOC and TN contents significantly increased in soils amended with CP over the years but continued to decrease in soils amended with NPK and in those without fertilization (Nil) (Fig. 5.1a and 1b). Our findings are consistent with those

reported in previous studies by Lee et al. (2009). After 50 years of continuous cultivation, SOC and TN increased by about 10% in CP-treated soil but decreased by about 8.5% in soils under NPK and Nil treatments. In the NPK and Nil treatments, most of C and N sources can be applied through below ground biomass (mainly from rice root) because large amount of above ground biomass (rice straw) was removed at the harvesting stage. Rice root weight is estimated to make up about 10% of the aboveground biomass (Kiniry et al., 1989), which means that around 1 Mg ha^{-1} of root biomass is returned as organic matter annually in the NPK and Nil treatments. However, total SOC and TN in these treatments significantly and continuously declined. These results showed that continuous rice cropping without organic matter addition can cause C and N depletion, which can subsequently result in soil degradation. There have been numerous reports on the degradation of soil properties due to continuous rice cropping and conventional management practices (Pampolino et al., 2008; Radford et al., 2001, Tong et al., 2009).

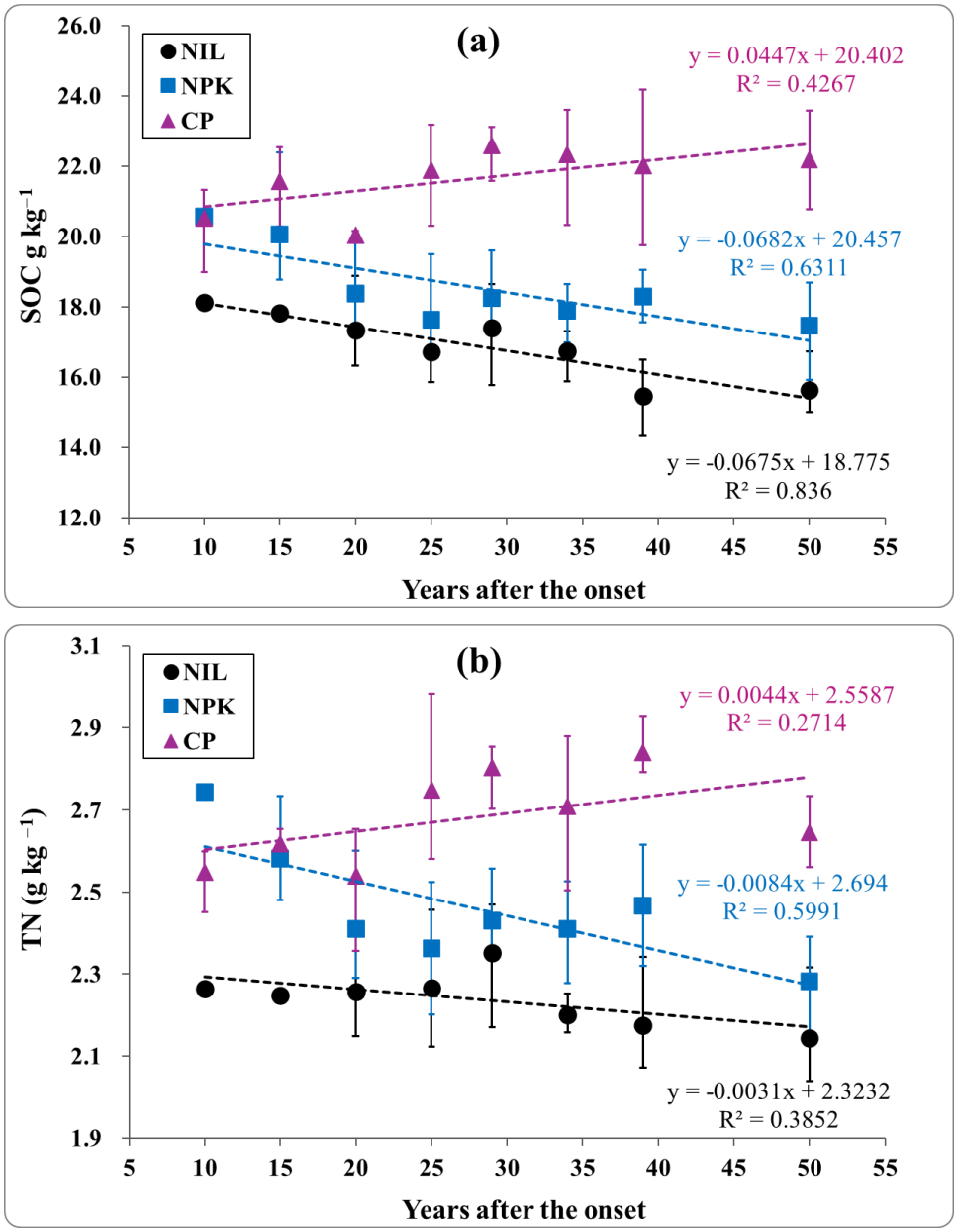

Fig. 5.1 Changes in SOC **(a)** and TN **(b)** contents in paddy soils in relation to the cultivation practice for a period of 50 years. CP represents compost. Data are mean and standard errors (n=3).

5.4.2 Debris-C and its relative proportion to SOC content

On average, C in debris fractions varied from about 1 to 3 g kg^{-1} or 4 to 10% to the total SOC (Fig. 5.2a and 2b). After 50 years of continuous cultivation, debris-C in the CP and NPK+CP treatments increased by about 1% and 16%, respectively, while it decreased by about 8.5% and 19% in the Nil and NPK treatments. Higher debris-C content was observed in NPK+CP (2.3 – 3 g kg^{-1}) and CP (2.0 – 2.7 g kg^{-1}), followed by NPK (1.0 – 1.8 g kg^{-1}), and Nil (1.0 – 1.2 g kg^{-1}) treatments. The higher debris-C content in the NPK+CP and CP treatments compared to the NPK and Nil treatments could be due to the combined or sole application of CP, which not only increased organic debris input but also promoted root growth and biomass accumulation. Donn et al. (2014) conducted an experiment comparing plant root growth between compost-amended soil and untreated soil (Nil) and found that compost application increased plant root biomass by tenfold compared to Nil after 13 weeks. Additionally, the higher debris-C content in CP or NPK+CP treatments may be attributed to the occlusion process, where over time, some debris from compost becomes physically occluded into macro- or microaggregates, forming more decomposed organic material with weaker associations to soil mineral particles (Golchin et al., 1994; You et al., 2014). This may also explain why debris C in the NPK and Nil treatments, although lower than in CP and NPK+CP, did not decrease sharply over a long time. In contrast, the relative proportion of debris-C to total SOC exhibited an opposite trend (Fig. 5.2b). The decreasing proportion of debris-C in both CP and NPK+CP treatments, relative to the increase in SOC, indicates the increased stabilization of SOC into more recalcitrant forms. The annual application of CP provides a considerable amount of labile carbon, which has a fast turnover rate and serves as a nutrient pool in many agricultural soils (Bremer et al., 1994).

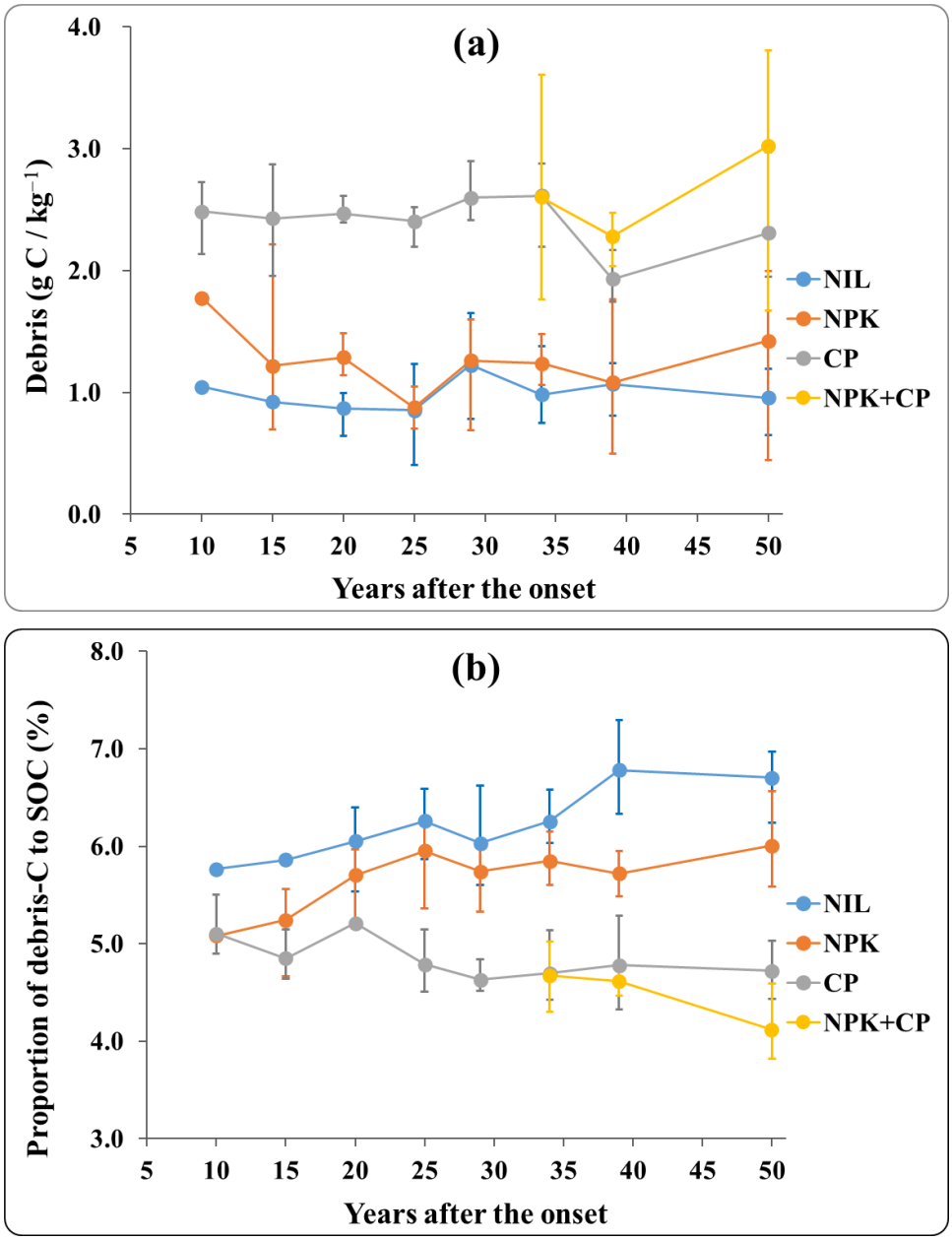

Fig. 5.2 Debris-C **(a)** and its relative proportion to the total SOC content **(b)** in paddy soils in relation to the cultivation practice for a period of 50 years. CP represents compost. Data are mean and standard errors (n=3).

5.4.3 FA-C and its relative proportion to SOC content

Figure 4a and 4b illustrate the changes in FA-C content and its relative proportion to the total SOC over 50 years of continuous cultivation under different treatments: Nil, NPK, CP, and NPK+CP. On average, C in the FA fraction varied from about 4.5 to 7.5 g kg^{-1} or 22 to 34% to the total SOC. The FA-C content in the CP and NPK+CP was higher than in the NPK and Nil treatments. FA-C in the CP and NPK+CP increased by about 7% and 9%, while that in the Nil and NPK treatments decreased by about 8% and 13%, respectively. Higher FA-C in CP and NPK+CP indicate that long-term applications of these fertilizers promoted the formation of more stable humic substances, likely due to the enhancement of microbial activity and soil aggregate stability (Li et al., 2024). FA, composed of simple, water-soluble, low-molecular-weight compounds, is more readily degraded by microbes compared to HA (Amoakwah et al., 2020). The annual additions of CP or NPK+CP provide a considerable amount of labile C, which has a rapid turnover rate and acts as a nutrient reservoir in most agricultural soils (Bremer et al., 1994). Under long-term continuous fertilization, organic materials form non-structural products, with most converting to unstable FA. Over time, microbial activity transforms FA into HA, resulting in its balanced accumulation in the soil (Wang et al., 2022; Li et al., 2024).

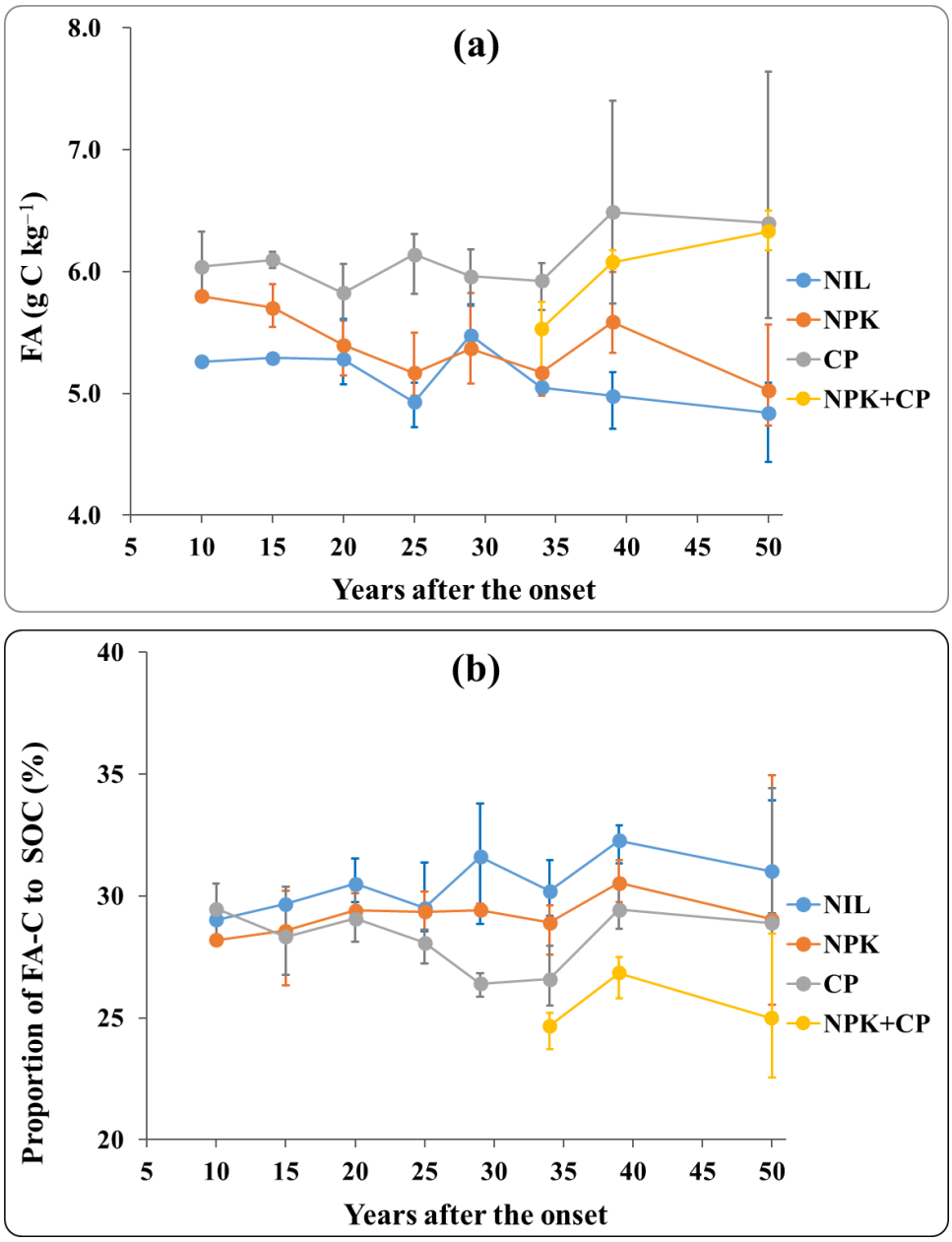

Fig. 5.3 FA-C **(a)** and its relative proportion to the total SOC content **(b)** in paddy soils in relation to the cultivation practice for a period of 50 years. CP represents compost. Data are mean and standard errors (n=3).

5.4.4 HA-C and its relative proportion to SOC content

The HA-C content varied from approximately 2.3 to 6.3 g kg^{-1}, or about 15 to 25% of the total SOC (Fig. 5.4a and 4b). Unlike FA-C, HA-C in CP and NPK+CP significantly increased over 50 years of continuous cultivation, whereas it decreased over time in the Nil and NPK treatments. HA-C in the CP treatment increased by about 2.0 g within 50 years, equivalent to a 46% increase compared to the initial year. Similarly, HA-C in the NPK+CP treatment increased by about 0.64 g within 15 years, corresponding to a 13% increase compared to the initial year. Additionally, the increase of HA in these treatments is proportional to the increase of SOC. The increase in HA indicates a rise in more recalcitrant organic molecules. In general, the increase and formation of the heavy fraction are caused by microbial polymerization of simpler compounds with one another and with complex residual products, forming long, complex chains that resist further decomposition (Brady and Weil, 2010). These high-molecular-weight compounds interact with N-containing amino compounds, giving rise to a significant component of resistant humus (Brady and Weil, 2010; Tan, 2014). There have been many reports about long-term addition of compost or the combination effect on HA fractions (Amoakwah et al., 2020; Li et al., 2023; Li et al., 2024). In contrast, HA-C content in the Nil and NPK treatments decreased over time, with average reductions of 17% and 26%, respectively, over 50 years of continuous cultivation. The decrease in HA-C content in both the NPK and Nil treatments can be attributed to nutrient limitations caused by long-term cultivation without the addition of organic materials.

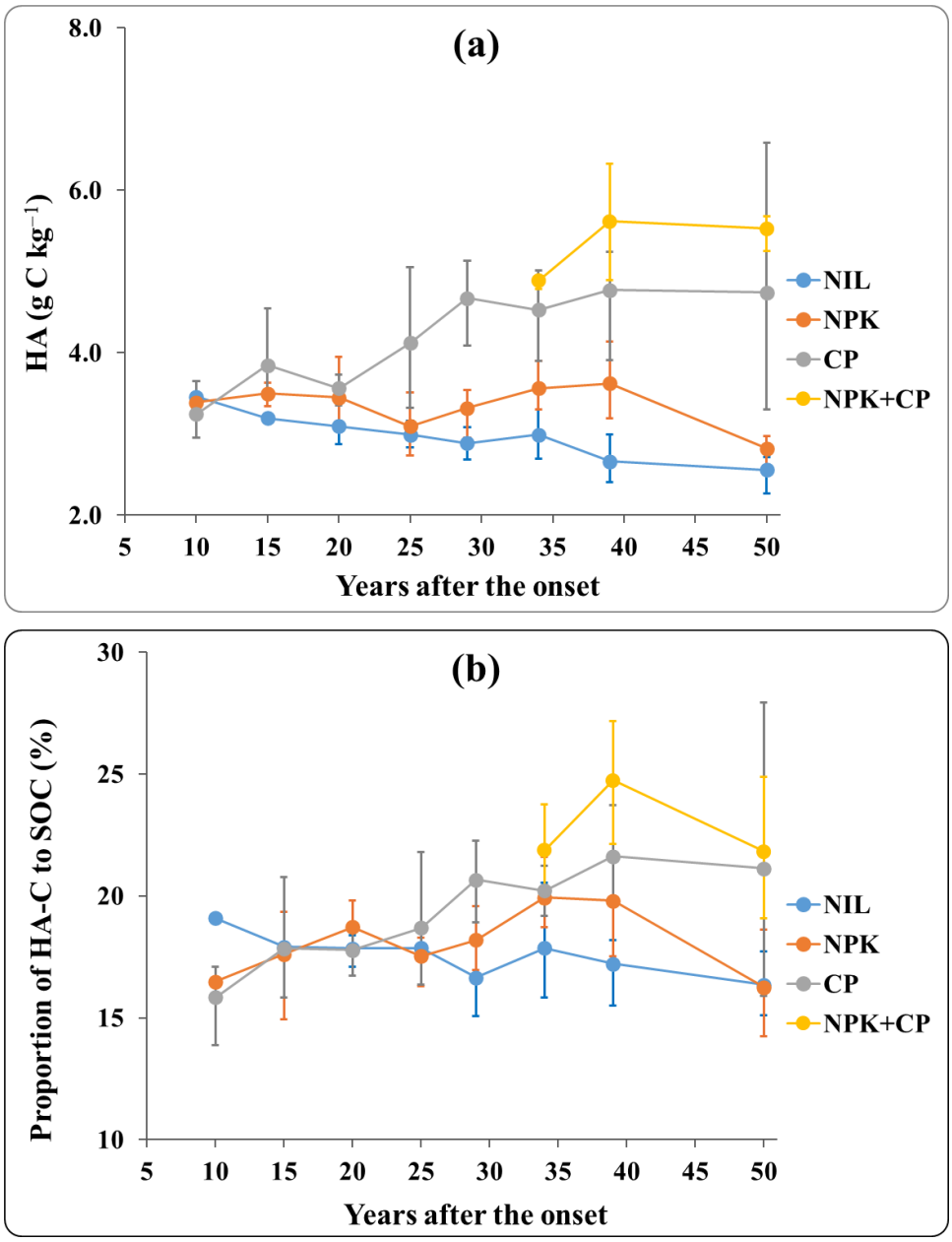

Fig. 5.4 HA-C **(a)** and its relative proportion to the total SOC content **(b)** in paddy soils in relation to the cultivation practice for a period of 50 years. CP represents compost. Data are mean and standard errors (n=3).

5.4.5 HM-C and its relative proportion to SOC content

The HM-C content varied from about 6.0 to 12.0 g kg^{-1}, or about 37 to 47% of the total SOC (Fig. 5.5a and 5b). HM-C in CP treatment remained relatively stable for a period of 50 years of continuous cultivation practices, indicating that addition of CP alone could maintain HM-C, but may not promote HM-C to a higher level. In contrast, HM-C in NPK+CP increased by about 13% in the last 15 years, suggesting that the combination of CP and NPK could increase and enhance the stability of HM in the soil. This result highlighted the importance of N in the formation of stable organic fraction. Brady and Weil (2010) and Tan (2014) reported that when incorporating organic and inorganic fertilizers, microbes capable to consume inorganic N and with enough energy (carbon), they convert these N forms into organic N or fix them through N-fixing bacteria. Because of this, the N content of the humic matter tended to increase. In contrast, the HM-C in the Nil and NPK treatments decreased by about 14.0% and 13.0%, respectively, after 50 years of continuous cultivation. This result clearly indicates that continuous cultivation without addition of organic materials caused a potential degradation or reduction of stable organic fraction, which may limit the long-term storage of C in the soil.

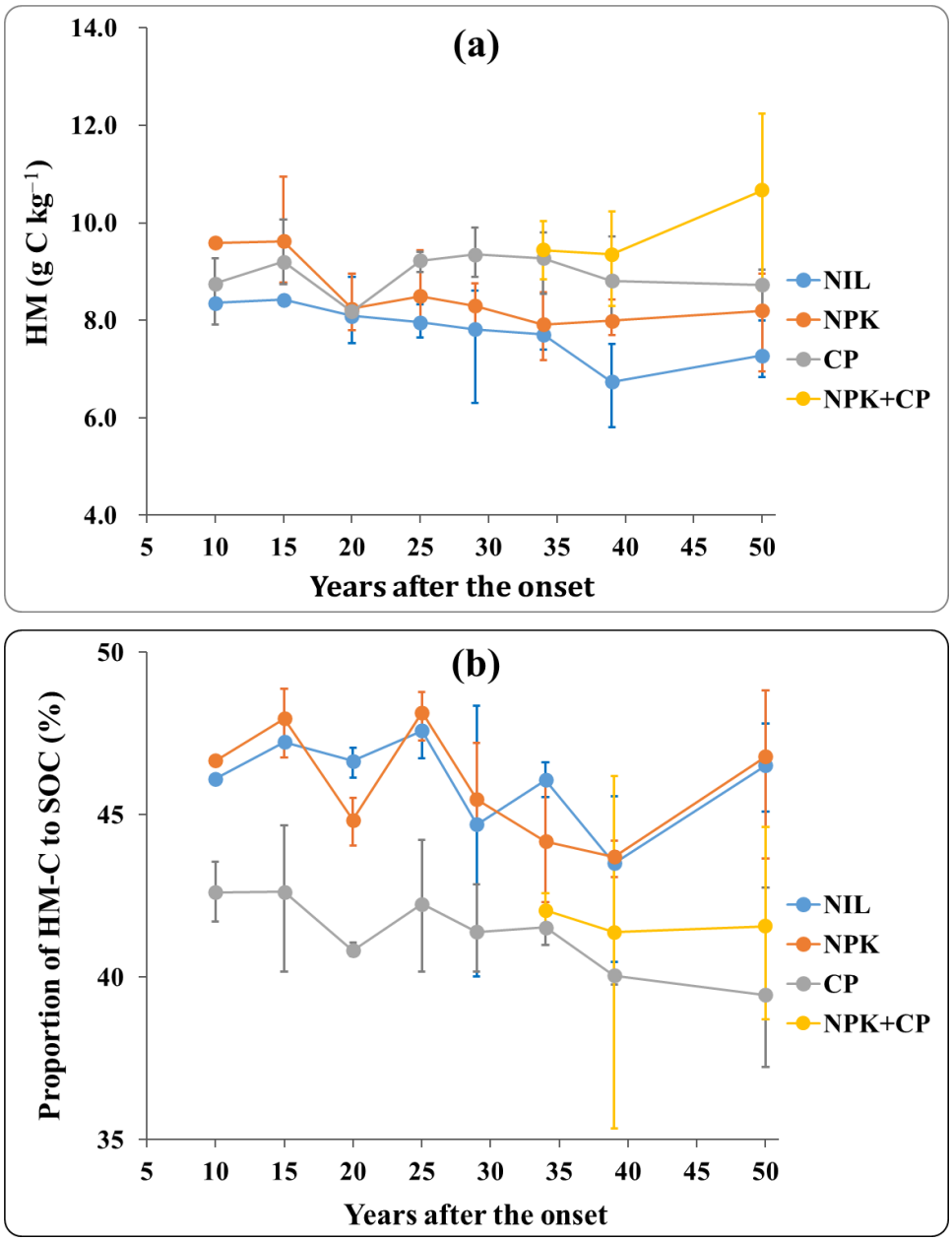

Fig. 5.5 HM-C **(a)** and its relative proportion to the total SOC content **(b)** in paddy soils in relation to the cultivation practice for a period of 50 years. CP represents compost. Data are mean and standard errors (n=3).

5.4.6 Formation of stable humus

The ratio between HA-C and FA-C (HA/FA) expressed by the humification index (HI) indicates the quality of organic material that could enhance soil physical properties and improve plant growth (Ukalska-Juagar et al., 2019). The higher HI than 1.0 is indicative higher humification rates (conversion of FA to HA) or HA persisted longer in the soil (Moraes et al., 2011; Watanabe et al., 2001). On the other hand, a low degree of transformation (DT), (FA+HA)/HM, can be related to a strong interaction between the SOC and the soil mineral phase, resulting in high SOC stability in the more recalcitrant HM fraction (Guimaraes et al., 2013; Ukalska-Juagar et al., 2019). The main factors affecting HI and DT are the amount of organic C inputs and time of active plant residue transformation in soil affected by microbial activity, climate and soil texture (Feng-bo et al., 2015). In our study, we found that HI was higher in the CP and NPK+CP treatments and increased gradually, while that in Nil and NPK treatments tended to decrease over the period of 50 years. Similar patterns were also observed for DT, but DT in NPK+CP treatment decreased greatly after the last 10 years. The higher HI and DT in the CP treatment showed that CP contributed to the formation of HA but had no impact on the formation of HM. The NPK+CP treatment had higher HI and lower DT, suggesting that continuous applications of NPK+CP in paddy soils resulted in a greater increase in highly polymerized organic forms, compared to soils treated with CP, NPK alone.

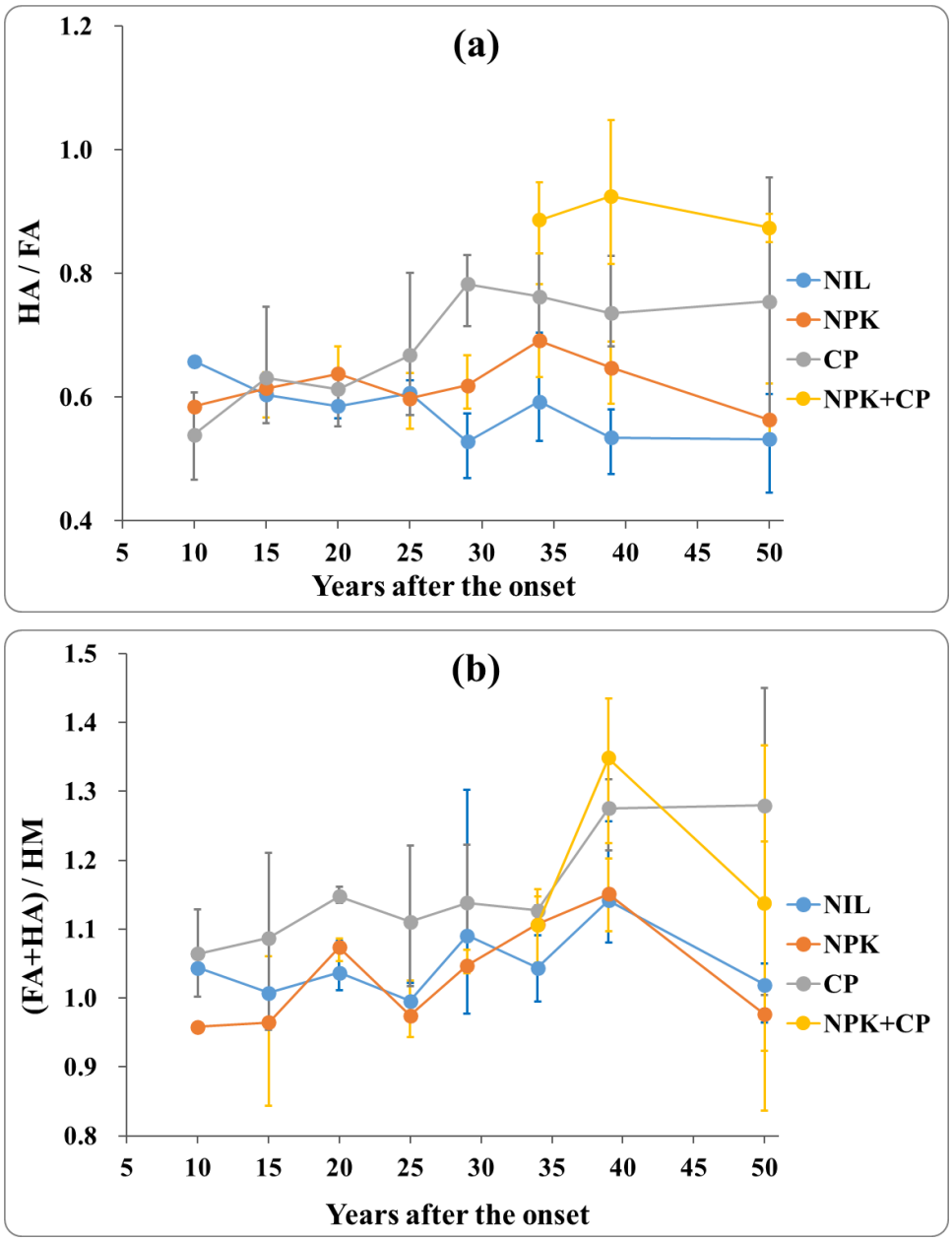

Fig. 5.6 Humification index (HI) **(a)** and degree of transformation (DT) **(b)** in paddy soils in relation to the cultivation practice for a period of 50 years. CP represents compost. Data are mean and standard errors (n=3).

5.4.7 SOM quality in long-term fertilized and unfertilized paddy soils

The C/N ratio in FA, HA, and HM can be used to assess the effects of soil amendments on the formation and stability of SOM (Stevenson, 1994). In general, a decrease in the C/N ratio of FA, HA, and HM indicates well-developed humic matter (Tan, 2014). In all studied treatments, the C/N ratio increased from FA (~9.0) to HA (~15.0) and decreased from HA to HM (~9.0). The C/N ratio of FA, HA, and HM remained stable in CP and NPK+CP treatments as SOC (Fig. 5.1) and HA-C (Fig. 5.4) continued to increase. In contrast, the C/N ratio of HA in NPK and Nil treatments decreased with SOC and HA-C contents. These findings showed that continuous addition of CP or NPK+CP enhanced the quality and stability of SOM over years, while continuous application of NPK alone or no fertilization led to C depletion, resulting in soil degradation. The increase in the C/N ratio from FA (~9.0) to HA (~15.0) indicates the conversion of labile C into more stable forms. The C/N ratio in FA is around 9, indicating that FA is less aromatic and more chemically active. FA typically contains more oxygen-containing functional groups (e.g., carboxyl and hydroxyl) and has a lower molecular weight, which facilitates microbial decomposition and nutrient cycling (Brady and Weil, 2010). The higher C/N ratio in HA indicates a more C-rich composition, which could signify a greater degree of humification and polymerization. This aligns with the finding of See et al. (2005), who observed that humic substances with higher C/N ratio tend to have more aromatic and stable structure, and when N is available, these high molecular weight compounds interact with N-containing amino compounds, transforming HA into more resistant HM, causing C/N ratio of HM to decrease. Unlike FA, HM is tightly bound to the mineral matrix, which protects it from microbial degradation. Thus, its relatively low C/N ratio could be attributed to the presence of mineral-associated N or other chemically condensed forms of N (See et al., 2005; Brady and Weil, 2010).

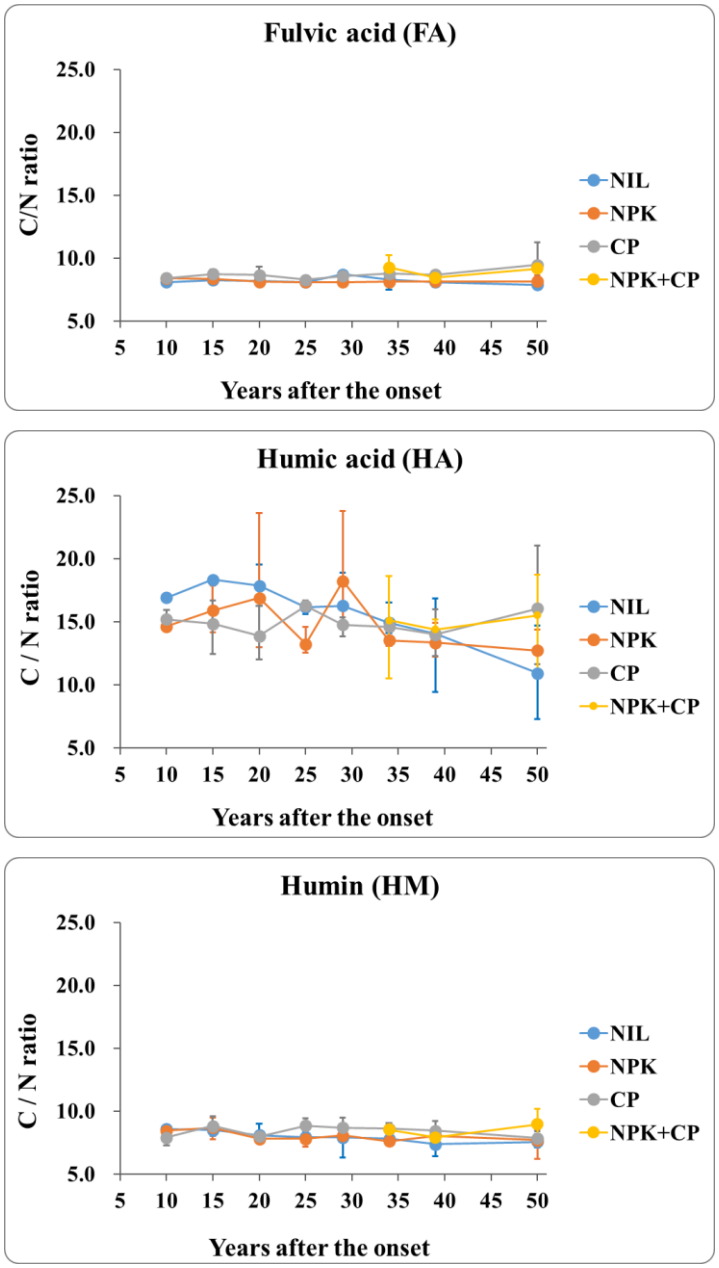

Fig. 5.7 C/N ratios of FA, HA, and HM fractions of paddy soils amended with different fertilizers under continuous cultivation for 50 years. CP represents compost. Data are presented as means ± standard errors (n = 3).

5.5. Conclusion

The results of the long-term study showed that SOC and TN increased by about 10% in soil amended with CP or NPK+CP and decreased by about 8.5% in soil amended with NPK alone as well as that in the Nil treatment, over 50 years of continuous cultivation of rice crop. Similar patterns were also observed in the SOM fractions. Debris-C, FA-C, HA-C, and HM-C in CP and NPK+CP treatments increased by about 1–16%, 7–9%, 46%, and 13%, and by 0.2–13%, respectively, compared to the initial year (year 10 vs. year 50). The CP alone increased higher HA-C pool (46%) than that of NPK+CP (13%), but CP (0.2%) had no effect on the increase of HM-C, unlike NPK+CP (13%). This result suggests that applying CP alone is not an effective strategy for enhancing SOM quality or stability compared to NPK+CP. In contrast, C content in the NPK and Nil treatments decreased by about 8.5–19%, 8–13%, 17–26%, 13-14%, respectively, after 50 years of rice cultivation. This result clearly indicates that continuous cultivation without addition of organic materials can cause a potential degradation or reduction of stable organic matter, which may limit the long-term storage of C in the soil.

On the other hand, the humification index (HI) was higher in the NPK+CP and CP treatments, with a gradual increase observed in the CP treatment compared to the NPK and Nil treatments. Similarly, the degree of transformation (DT) increased steadily in the CP treatment, while it fluctuated but remained stable in the other treatments over the 50-year period. The higher HI and DT in the CP treatment indicates that CP contributed to the formation of HA but had no impact on the formation of HM. In contrast, the NPK+CP treatment had higher HI and lower DT than CP treatment. This suggested that continuous applications of NPK+CP in paddy soils resulted in a greater increase in highly polymerized organic forms.

The C/N ratio increased from FA (~9.0) to HA (~15.0) and decreased from HA to HM (~9.0). The C/N ratio of FA, HA, and HM remained stable in the CP and NPK+CP treatments as SOC and humus-C continued to increase. In contrast, the C/N ratio of HA in NPK and Nil treatments decreased with SOC and humus-C contents. These findings showed that continuous addition of CP alone or NPK+CP enhanced the quality of SOM over the years, while continuous application of NPK alone or crop grown without fertilization led to C depletion, resulting in soil degradation.

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CHAPTER 6

Summary of the present study and future perspectives

6.1 Summary

This study developed and validated a novel alkaline persulfate digestion method for the simultaneous determination of SOC and TN in air-dried soil samples. By using $Na_2S_2O_8$ and NaOH as oxidizing agents and AYR with K_2CO_3 as pH indicator to absorb CO_2 released during soil oxidation. The method achieved high precision, low detection limits, and strong reliability compared to elemental analyzers. Optimized digestion conditions ensured accurate measurements, making this approach cost-effective and suitable for high-throughput analysis. The method can be extended for determining of C and N in FA, HA, and HM with slight modification. This advancement offers a valuable tool for efficiently analyzing SOC and TN, particularly in studies focused on soil nutrient dynamics.

The study also investigated SOC content and SOM fractions across various land uses, including orchard, paddy, upland, greenhouse, volcanic ash and reclaimed soils. Results showed that volcanic ash soils contained significantly higher SOC levels compared to common and reclaimed soils. The relative contributions of SOM fractions indicated that HA and HM were the major contributors to SOC stabilization, whereas NHS, debris, and FA decreased with increasing SOC. These findings underscore the importance of HA and HM in long-term carbon storage and stabilization processes.

Long-term incubation studies revealed that organic and inorganic soil amendments, particularly combined treatments like CMS+RS, enhanced SOM quality by promoting microbial activity, increasing humification indices (HI), and stabilizing labile fractions into more recalcitrant forms. Crop residues, such as rice straw, further

improved SOC and TN in debris and FA fractions during the early incubation stages. However, these fractions declined over time as labile carbon transformed into more stable HA and HM fractions. Combined amendments were more effective in improving SOM stability than single organic or inorganic fertilizers, emphasizing their critical role in soil management.

The long-term fertilization experiment demonstrated that combined compost and NPK treatments (NPK+CP) significantly increased SOC, TN, and stable humus fractions, enhancing SOM quality over 50 years of continuous rice cultivation. In contrast, soils receiving only NPK or no fertilization (Nil) experienced SOC depletion and degradation of SOM quality. The higher humification index and stable C/N ratios observed in NPK+CP treatments indicate that combined fertilization promotes the formation of highly polymerized organic matter, ensuring long-term soil health and fertility.

6.2 Future perspectives

Future studies should expand the application of the developed digestion method to diverse soil types and environmental conditions to enhance its utility in global soil studies. Further research is needed to explore the long-term effects of various fertilization strategies on SOM dynamics in other ecosystems, such as grasslands and forest soils. Incorporating advanced analytical techniques, such as isotopic tracing or spectroscopy, could provide deeper insights into the transformation pathways of SOM fractions and their implications for C sequestration. Additionally, evaluating the interaction of soil amendments with climate variables could inform adaptive soil management practices to mitigate the impacts of climate change while improving soil health and productivity.

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-Khok Pros