

**Review**

# Investigations of environmental changes based on chemical analyses of humic substances

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## Abstract

Humic substances, which are ubiquitously distributed in terrestrial environments, have been characterized using a variety of analytical techniques, in attempts to elucidate the relationship between the structural aspects and environmental factors affecting the decomposition of organic matter and/or humification processes. Research focused on this aspect is discussed in this paper. The composition of chemical structural units in plant litter, humus in surface organic horizons, and humic substances associated with surface mineral soils under Japanese forest stands were determined directly by solid-state CP-MAS <sup>13</sup>C NMR spectra. The early stage of the humification process in the forest floor was explained from vertical changes in the composition of C species. Furthermore, the selective partition of aliphatic constituents into mineral soils was also discussed. A study of a series of humic acid fractions (HAs) separated on the basis of molecular size using a new type of preparative chromatography was introduced. Size fractions of HAs prepared from soils at different stages of vegetation succession showed a correlation between the isotopic fractionation ratio and the color of the HAs as an indicator of humification. Concurrently, the composition of carbon species of the HAs was different depending on the dominant vegetation, indicating that the type of vegetation can have an influence on the components of humic substances within decadal time scales. Variation in the composition of structural units and properties of humic substances obtained from various sources, including natural water was discussed using datasets of C species, apparent molecular weights and elemental composition. The ranges of some parameters were used to differentiate the types of humic substances. Principal component analysis using some parameters extracted two components accounting for a significant rate of variance and can explain humic substances depending on their types. Structural properties obtained under different analytical conditions such as solid or liquid states were successfully interpreted in relation to environmental conditions in these studies.

**Keywords:** Litter, humification, vegetation succession, spectral analyses, chemical structure

## Introduction

Organic substances in terrestrial and aquatic ecosystems are converted into humic substances through physical, chemical and biological transformations under various environmental conditions. Humic substances are ubiquitous on the Earth's surface where organic matter is being supplied mainly by biological and human activity. Because humic substances are produced from various

sources of organic matter, they are highly heterogeneous in composition with polydispersity in sizes. This makes them different from naturally occurring bio-molecules such as proteins, carbohydrates, lipids, and so forth.

The significance of humic substances in soil productivity was addressed in the late 19th century by the great soil scientist, V.V. Dokuchaev. He pointed out relationships between humic acid content and soil productivity or plant nutrition in

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Chernozems distributed in southern Russia (Dokuchaev, 1885; Fukushi translated and published this 1993). Since then, humic substance research from the agronomical or productivity point of view continue to be one of the major topics in modern soil science. Again, in the past few decades, studies of carbon dynamics related to climatic change have shed light over humus carbon quantity as well as the quality of humic substances affecting persistent C reserves in terrestrial environments. Meanwhile, humic substances can also function as carriers of pollutants, thus controlling the fate of xenobiotics and pollutants (McCarthy, 1989). The physical and chemical properties of humic substances are, therefore, considered to be valuable from a variety of environmental aspects.

To understand humic substances as key variables in various ecosystems, studies of humic substances are being actively conducted with respect to their solubilities in aqueous acids and bases (Aiken et al., 1985). This operational definition has played a significant role in handling humic substances in order to characterize and to compare them between different origins. Fractions based on solubility are classified as humic acids (HAs: soluble in alkaline solution and insoluble in acid solution), fulvic acids (FAs: soluble in both alkaline and acid solutions) and humin (insoluble in both alkaline and acid solutions). Within these three fractions, the highest number of

studies has involved HAs followed by FAs, while studies of humin are far smaller than others (Fig. 1).

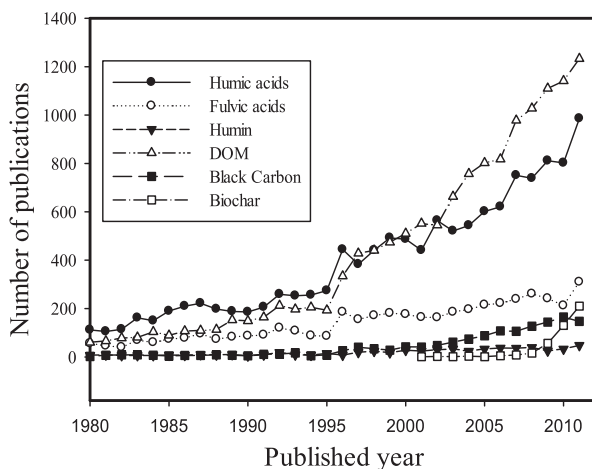
Besides the solubility of humic substances in aqueous media, organic matter in various environments can be obtained or extracted by other methods using the density fractionation technique (e.g. Rodionov et al., 2001; Homma et al., 2002), acid digestion under high temperatures (e.g. Brodowski et al., 2001) and direct collection using lysimeters (e.g. Kalbiz et al., 2000). Char, black carbon and dissolved organic matter have attracted the attention of researchers for the past 20 years despite the low contribution in total C in mineral soils (Fig. 1). Solid state spectroscopic analyses make it possible to obtain structural information by the direct observation of environmental samples without any modification.

Comparisons of structural information or the properties of humic substances and/or organic substances between different methodologies can give new insights for humic substance researches. This paper discusses the properties of humic substances observed by different methods and provides their contribution as environmental factors affecting the formation processes that are involved in humic acid formation.

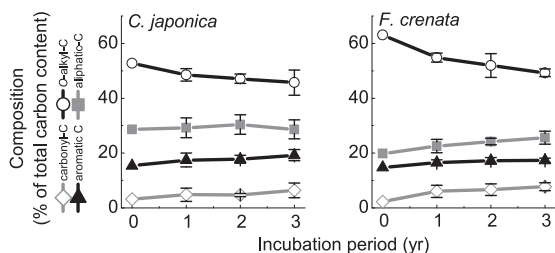
### Forest types affect litter humification processes in forest surface soils.

Carbon dynamics during the humification of litter have been reported for a variety of forest ecosystems and have provided insights into carbon (C) flow in soils. To quantitatively assess how the humification processes of organic matters vary among forest types, solid-state cross-polarization and the magic-angle spinning  $^{13}\text{C}$  nuclear magnetic resonance (CP-MAS  $^{13}\text{C}$  NMR) technique have been widely applied to determine the C compositional changes in various phased-humified plant litter samples and humus-rich soils obtained from forest stands. In this section, differences in early humification processes were compared between conifer (*Cryptomeria japonica*) and hardwood (*Fagus crenata*) litters using research data from prior studies and the effect of forest types on litter humification processes in surface forest soils are summarized.

The study was carried out at two forest stands in the northern Kanto District of Japan: the Tsukuba Experimental site (TKB) and the Ogawa Forest



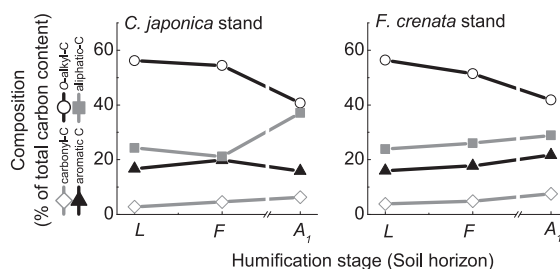
**Figure 1.** Number of publications concerning humic substances or organic matter in environments as the result of a search of the Scopus database using name of substances as a keyword of “article title, abstract, keyword” in papers.



**Figure 2.** Proportions of carbon components in the total carbon determined by solid-state CP-MAS  $^{13}\text{C}$  NMR for humified litter obtained from *Cryptomeria japonica* and *Fagus crenata* during a 3-year incubation. Error bars are standard deviations of the mean for each carbon composition.

Reserve (OFR). The TKB is a 99-year-old *C. japonica* plantation and the OFR is a secondary *F. crenata* forest. Both forest stands are classified as temperate. A litterbag experiment was conducted and solid-state CP-MAS  $^{13}\text{C}$  NMR was used to monitor phased-humified litter litter at both stands. Soil horizon samples were taken from the L and F horizons and the top mineral  $A_1$  horizon of the forest floors using 100-ml cylinder cores. Fine roots and small stones were removed as carefully as possible with tweezers and sieves prior to the chemical analyses. The C compositional changes of humified litters and soils were then examined, and the litter humification processes were compared between both stands.

Mass loss rates of whole litters and respective C components slowed down exponentially with time for both types of litter. The remaining mass of the *C. japonica* litter was 15% lower than that of the *F. crenata* litter after a 1 year period of incubation. After 3 years, the overall mass loss of *F. crenata* litter was 68%, the same level as that of the *C. japonica* litter (Ono et al., 2013). The carbon compositions of humified litters showed a similar trend in both *C. japonica* and *F. crenata* litters and were relatively constant throughout the litterbag experimental period (Fig. 2). In both litters, the proportions of carbonyl, aromatic, and aliphatic C increased slightly and/or were stable during the 3 years of incubation. Inversely, the proportion of aliphatic C in both litters slightly and gradually decreased during the incubation. The C compositions of the L and F horizons had almost the same trends as those of the litterbag samples (Fig. 3). However,

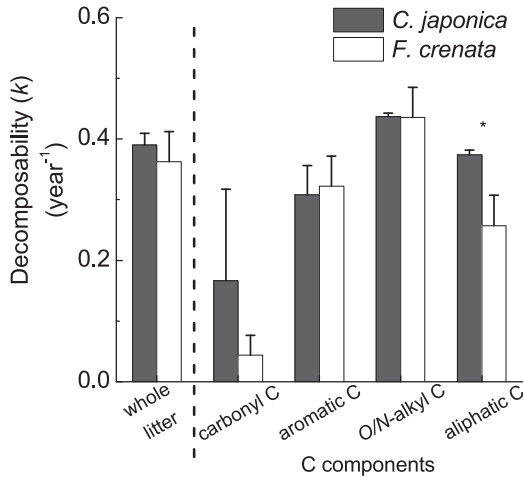


**Figure 3.** Proportions of carbon components in the total carbon determined by solid-state CP-MAS  $^{13}\text{C}$  NMR for the samples of L, F, and  $A_1$  horizons. These data have no field replication.

the C composition of the  $A_1$  horizons, which is a more advanced stage of humification than the L and F horizons, differed between the *C. japonica* and *F. crenata* stands (Fig. 3). O-alkyl C accounted for the majority of the total C in humified litters and L, F, and  $A_1$  horizons at both stands: it accounted for 41–63% of the total C mass. On the other hand, aliphatic C was the opposite with the proportions in humified litters and L and F horizons being stable at a low level ranging from 21 to 31% for both stands (Figs. 2 & 3). However, aliphatic C in the  $A_1$  horizon soil of the *F. crenata* stand was much higher (37%) than that of the *C. japonica* stand (29%). There was no significant difference in the proportions of aromatic and carbonyl C between humified litters and the L, F, and  $A_1$  soil horizons at both stands (Figs 2 and 3).

Previous studies have pointed that the origin of the vegetation influences the distribution of C components in surface soil horizons, because of the variations in the chemistry of C input to soils and in the nature and the magnitude of humification processes by degradative organisms (Krossharvn et al., 1992; Golchin et al., 1995). Are the compositional differences in organic matter accumulated in the top mineral soils of *C. japonica* and *F. crenata* stands caused by difference in forest types? To evaluate the influence of forest vegetation on the distribution of C components accumulated on the surface soils in the present study, the ease of decomposition (Olson's  $k$  values obtained by data fitting to Olson's exponential models: Olson, 1963) of whole litters and C components was compared between *C. japonica* and *F. crenata* litters by the student's  $t$  test ( $\alpha = 0.05$ ). Only the  $k$  value for aliphatic C in the decomposed litter differed

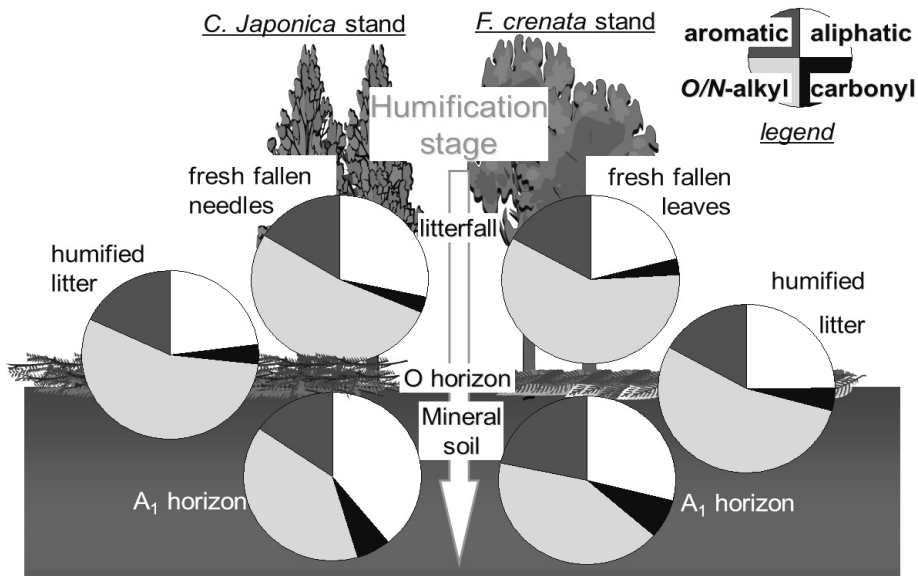
significantly between *C. japonica* and *F. crenata*, although those of the whole litters and the other C components remained unchanged between species (Fig. 4). The decomposabilities of the C components for *C. japonica* were in the following order: O-alkyl



**Figure 4.** Ease of decomposition of whole litter and carbon types between *Cryptomeria japonica* and *Fagus crenata*. Error bars show the standard deviations. Asterisk implies statistical differences determined by Student's t test ( $\alpha = 0.05$ ).

> aliphatic > aromatic > carbonyl; and in the *F. crenata* litter, they were O-alkyl > aromatic > aliphatic > carbonyl. The  $k$  value for aliphatic C in the *C. japonica* litter was higher than that for the *F. crenata* litter, probably owing to differences in lipid leachability and biosynthesis among species. The higher mass loss rate of aliphatic C of the case of the coniferous *C. japonica* litter suggest the existence of high input rates of aliphatic C in top mineral soils from the humified litter compared with those of the hardwood *F. crenata* litter. Results for the C compositions of the A<sub>1</sub> horizons in both forest floors strongly support this possibility (Fig. 4). Namely, the proportion of aliphatic C was 37% in soils under the *C. japonica* stand was higher than that in soils under the *F. crenata* stand (29%). Moreover, a larger content of aliphatic C in the fresh *C. japonica* litter (29%) than in fresh *F. crenata* litter (20%) would also contribute to the high input rate and large accumulation of aliphatic C in the A<sub>1</sub> horizon (Fig. 4).

The differences in the early humification process between coniferous and hardwood forests are shown in Fig. 5. Consequently, the present study indicated that the differences in the litter humification process



**Figure 5.** Summary of the differences in humification processes between *Cryptomeria japonica* and *Fagus crenata* forests. The circle graphs show the proportions of the carbon components in respective samples. The results of the present study suggest that higher mass loss rates of aliphatic carbon of *C. japonica* stands might cause a relatively higher input accumulation of aliphatic carbon into top mineral soils of a *C. japonica* forest in comparison with an *F. crenata* forest. Consequently, initial litter chemistry and litter humification processes would strongly affect the qualities of soil organic matters of top soils.

and initial litter chemistry between coniferous and hardwood forests can be expected to strongly affect the qualities of the soil organic matter of the A<sub>1</sub> horizon.

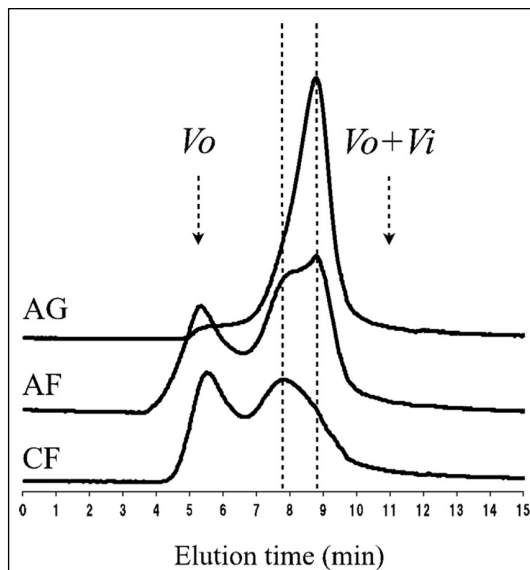
### Analysis of the components of fractionated soil humic acids

Previously, soil humic substances, particularly those found in humic acid fractions, were further fractionated to gain additional insights into their chemical structures. In addition, recent studies have also demonstrated the applicability of fractionation techniques for investigating soil humic acid dynamics in terrestrial ecosystems. In this section, therefore, fractionation techniques that are useful for the characterization of the chemical properties of soil humic acids are discussed. In addition, an example of the application of a fractionation technique to investigate soil humic acid dynamics in a terrestrial ecosystem is described.

#### Fractionation techniques for soil humic acids

Yonebayashi and Hattori (1990) reported on a valuable fractionation technique using the macroporous, non-ionic Amberlite XAD-8 resin for reducing the complexity of several soil humic acids. An H<sup>+</sup>-saturated humic acid was adsorbed onto the resin at pH 3 and fractionated into four components by stepwise elution using universal buffers adjusted to pH 7 and pH 11, water, and 50% ethanol. The first component consisted of a few structural subunits that were assumed to be condensed aromatic rings with short aliphatic substituents and many carboxyl groups, based on <sup>1</sup>H NMR spectral data. The second component was characterized as being rich in phenolic groups, and the third as containing relatively long aliphatic chains. The fourth component consisted of many structural subunits that were assumed to be aromatic rings with long aliphatic substituents.

We also attempted to fractionate methylated soil humic acids via a fractional precipitation method using methanol–chloroform solutions. High-performance size exclusion chromatography (HPSEC) chromatograms of chloroform-soluble humic acids obtained from three different soil types are shown in Fig. 6. Clear differences in the molecular weight distributions of the humic acids were found for Andosols under grassland (AG) and



**Figure 6.** HPSEC chromatograms of three methylated, chloroform-dissolved humic acids obtained from Andosols under grassland (AG) and deciduous broadleaved forest (AF), and Cambisols under deciduous broadleaved forest (CF). Column, Shodex K-series GPC K-802.5 column with a guard column; column temperature, 40 °C; eluent, chloroform; flow rate, 1.0 mL min<sup>-1</sup>; detection, 280 nm;  $V_0$ , Void volume;  $V_0 + V_i$ , total permeation volume.

Cambisols under a deciduous broad-leaved forest (CF); the molecular size distribution for AG was lower than that for CF. In addition, in the chromatogram of the humic acids for Andosols under a broad-leaved forest (AF), both types of peak forms identified for the AG and CF samples were observed. This result strongly suggests that humic acids from AF may consist of the two major components of the humic acids from AG and CF.

#### Application to ecological research

An interesting example of the application of the fractionation technique is presented, where preparative HPSEC was used to track soil humic acid C dynamics involved in the ecological succession of Japanese pampas grass (*Miscanthus sinensis*) (Iimura et al., 2013). The differences in the degree of darkness ( $A_{600}/C$  values), isotopic ratios ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , and  $\Delta^{14}\text{C}$  values), and <sup>13</sup>C-NMR spectra of size-separated humic acids extracted from Japanese volcanic ash soils were investigated in order to estimate the variations in the polyaromatic structures

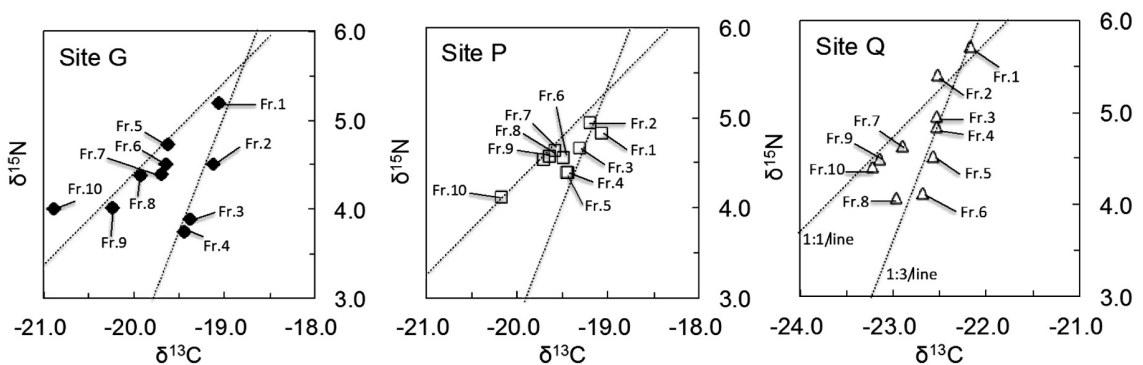
of humic acids during a period of ca. 100 years of natural reforestation of Japanese pampas grassland. For several hundred years, all of the study sites were managed in a similar manner, namely, as grasslands. Subsequently, their management differed; at site G (*Miscanthu ssinensis*: C4 plant), maintenance at the time of this study was still performed by mowing, while at sites P (*Pinus densiflora*: C3 plant) and Q (*Quercus crispula*: C3 plant), maintenance was discontinued ca. 30 and 100 years ago, respectively. For all humic acid size fractions at all sites, the  $\delta^{13}\text{C}$  values correlated positively with the  $\delta^{15}\text{N}$  values and the gradients were much lower for small to medium molecular size fractions than for medium to large molecular size fractions (denoted as *lower-size* and *higher-size* fractions, respectively) (Fig. 7).

Connin et al., (2001) reported that the  $\delta^{15}\text{N}$  value clearly increases relative to the  $\delta^{13}\text{C}$  value with the microbial degradation of litter that is easily decomposed by microorganisms. Therefore, our  $\delta^{13}\text{C}$ : $\delta^{15}\text{N}$  ratio data suggest that components that generally fall along the 1:1 line (lower-size) may be more resistant to microbial attack than those that fall along the 1:3 line (higher-size). We also estimated the C content derived from C3 ( $\delta^{13}\text{C} = -27\%$ ) and C4 ( $\delta^{13}\text{C} = -13\%$ ) plants for size-separated humic acids fractions from each site. The estimated C content derived from C4 plants in the lower-size fraction ranges were determined to be as follows: site G 2.3–3.4 g C kg<sup>-1</sup>, site P 2.2–2.6 g C kg<sup>-1</sup>, and site Q 0.8–0.9 g C kg<sup>-1</sup>. In addition, the mean values for the C content derived from C4 plants of the lower-size components (site G 2.9 g C kg<sup>-1</sup>, site P 2.4 g C kg<sup>-1</sup>, site Q 0.8 g C kg<sup>-1</sup>) decreased much more during natural reforestation than did the estimated C

derived from C3 plants (site G 2.9 g C kg<sup>-1</sup>, site P 2.2 g C kg<sup>-1</sup>, site Q 2.1 g C kg<sup>-1</sup>), particularly from site P to site Q. Furthermore, for the lower-size fractions, the contribution ratio of C4-plant-derived carbon shows a significant positive correlation with the  $A_{600}/\text{C}$  values and a negative correlation with the  $\Delta^{14}\text{C}$  values, and their aromatic characteristics are greater than those of the higher-size fractions within the same humic acid type. The aryl C content of the lower-size fractions was lower, and the *O*-alkyl C content and aliphaticity (alkyl C: *O*-alkyl ratio) were clearly higher for sites P and Q than for site G. These results strongly suggest that stimulating humic acid biodegradation might be achievable with the continuous input of new plant litter during reforestation, even for lower-size humic acid polyaromatic structures with less modern C, despite the fact that lower-size humic acids undergo biodegradation relatively more slowly than higher-size humic acids.

### Chemical structural properties of humic substances and their diversity

The chemical structure of humic substances is a subject of continuous dispute, even today. Although it is generally accepted that the chemical characteristics involve the presence of a hydrophobic part comprised of alkyl and aromatic groups and a hydrophilic part containing various acidic functional groups, humic substances are known to vary with the environment in which they are formed. Indeed, it has been reported that the values in elemental and functional group compositions, in addition to molecular sizes, are extensively distributed according

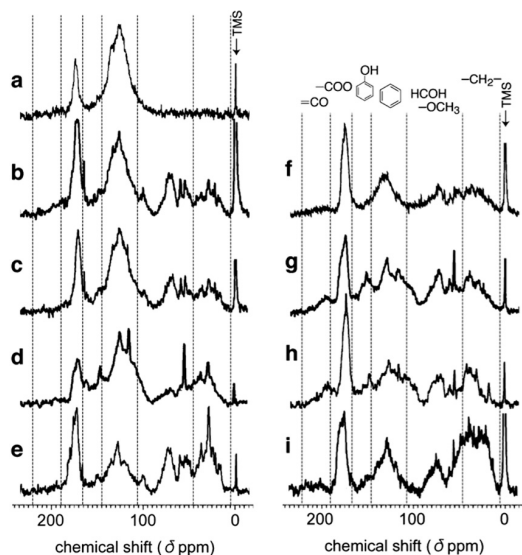


**Figure 7.** Relationship between the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values (‰) for size-separated humic acid fractions taken from the study sites at grassland (site G), coniferous forest (site P), and broad-leaved forest (site Q). The numbers indicate the fraction numbers. The apparent molecular size of each fraction is lower along the fraction numbers from 1 to 10 in all humic acids.

to individual humic substances obtained from different environments (e.g. Steelink, 1985; Tsutsuki and Kuwatsuka, 1984; Malcolm, 1990; Yonebayashi and Hattori, 1989; Asakawa et al., 2008; Fujitake et al., 2012). However, such variations in the chemical characteristics provide a significant advantage because information related to the origins or humification processes by which the humic substances are generated can be obtained by analyzing their chemical characteristics. In this work, the nature of information that can be obtained from differences in the characteristics of the chemical structure of humic substances using different analytical techniques such as  $^{13}\text{C}$  NMR, elemental analysis, and high performance size exclusion chromatography (HPSEC) is discussed.

#### Analytical characterization of humic substances.

Figure 8 shows representative  $^{13}\text{C}$  NMR spectra of some humic substances—humic acids (HAs) and fulvic acids (FAs)—obtained from various environments. Different shapes give different spectral patterns. The average values for the quantity of alkyl C calculated from each spectrum are listed in Table 1. The average value was determined to be

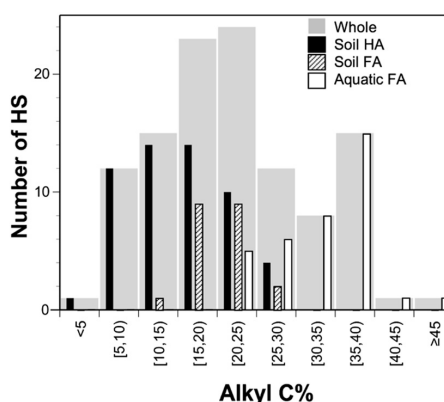


**Figure 8.** Representative spectra of humic substances by  $^{13}\text{C}$  NMR: (a) Buried Andosol HA, (b) Andosol HA, (c) Chernozem HA, (d) Histosol HA, (e) Cambisol HA, (f) Soil FA, (g) Colored water HA, (h) Colored water FA, (i) Clear water FA: Refer to Watanabe and Fujitake (2008) for Measurement procedures and conditions.

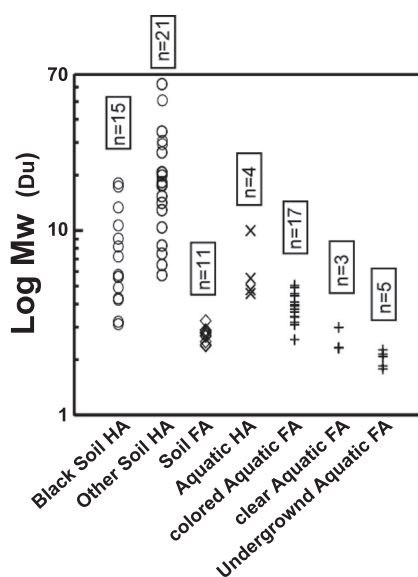
**Table 1** Averaged % of alkyl C content in humic substances.

Humic Sub.	n	average	SD ( $\pm$ )	min	max
Whole	n = 112	22.1	9.8	2.6	45.3
Soil HA	n = 55	15.4	6.3	2.6	28.4
Soil FA	n = 21	20.6	3.2	12.2	25.7
Aquatic FA	n = 36	33.4	6.0	23.0	45.3

22% ( $n = 112$ ); however, a large distribution occurred in the range between 3 and 45%. This provides further evidence to indicate that humic



**Figure 9.** Histogram showing the proportion of Alkyl C% in various humic substances.



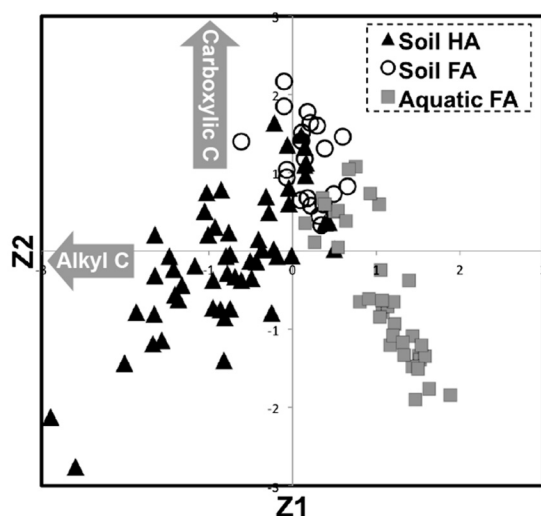
**Figure 10.** The values for the weight-averaged molecular weight of various humic substances, as determined from an HPSEC analysis: Refer to Asakawa et al. (2008) for measurement procedures and conditions.

substances have a wide diversity of structures. Moreover, when the amount of alkyl C of each humic substance is plotted in the form of a histogram (Fig. 9), the distribution range of the alkyl C quantities in the different humic fractions followed the order: aquatic FAs > soil FAs > soil HAs. As shown in Fig. 10, the values for the weight-averaged molecular weight of the humic substances ( $n = 76$ ) calculated using data obtained from HPSEC analyses also appear to be dependent on the type of humic substance.

These results show that the ranges of the characteristic values obtained using the analytical techniques differed according to the humic fractions—aquatic FAs, soil FAs, or soil HAs, although these analytical values were within the typical expected range for humic substances.

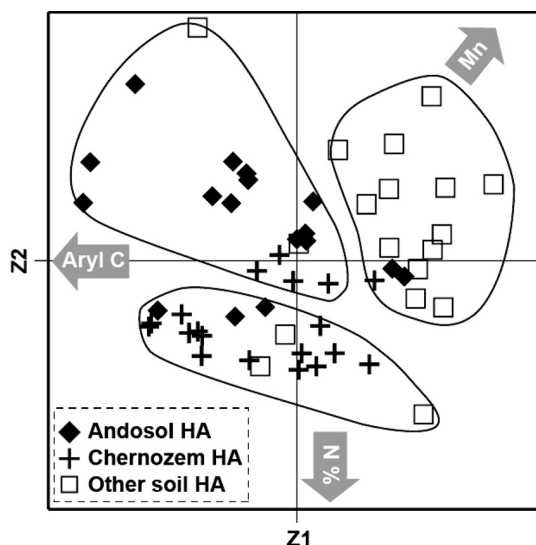
### Grouping of humic substances

A principal-component-analysis plot produced using the values of all the carbon functional group rates determined from  $^{13}\text{C}$  NMR spectra as a parameter is shown in Fig. 11. As shown in the plot, there is clear localization in the distributions of soil HAs and water FAs. Cluster and



**Figure 11.** Plot of the factor score for the two principal components of various humic substances ( $n = 112$ ), based on combinations of the variable parameters obtained from  $^{13}\text{C}$  NMR analysis. The cumulative explained variance with the first two principal components was 65.2% of the total variance. The first component was mainly related to aryl C%, and the second component was mainly related to carboxylic C%.

principal-component analysis from the data obtained from the  $^{13}\text{C}$  NMR, HPSEC, and elemental analysis data, were utilized in order to reveal either differences or similarities between various soil HAs ( $n = 54$ ) in a more objective manner. The score plot for the two principal components from the principal-component analysis is shown in Fig. 12. This plot could be classified based on the three clustered groups: (i) the scores for the mainly Andosol HAs were plotted in the direction of high aryl C values; (ii) the scores for the mainly Chernozem HAs were plotted in the direction of a high N quantity; and (iii) the scores for the HAs of other soil types with high values of alkyl C and number of averaged molecular weight. It was speculated that the grouping might depend on the differences in the quantities of aryl and alkyl C or on the quantity of N caused by the origin or land-use type, respectively. The aquatic FAs ( $n = 13$ ) were classified into two clear water groups and one brown water group based on the differences in their aryl and O-alkyl C contents, as reported in Tsuda *et al.*, (2012). This grouping might depend on the differences in the aquatic microbial activity caused by the variations in the residence time of the water.



**Figure 12.** Plot of the factor score for the two principal components of various soil humic acids ( $n = 56$ ), based on combinations of the variable parameters obtained from  $^{13}\text{C}$  NMR, elemental and HPSEC analysis: The areas inside the solid line are large clusters, based on the cluster analysis. The cumulative explained variance with the first two principal components was 64.0% of the total variance.



On the other hand, it was found that the differences in the chemical characteristics of the different types of soil FAs ( $n = 36$ ) were small; they were substantially smaller than the HAs (Iimura et al., 2012). Further studies will be needed to completely understand characteristics of the chemical structure of humic substances in order to obtain accurate information regarding the origins or the processes involved in the generation of the organic matter contained within them.

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