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Effects of operational conditions for extraction and sample storage on the structural properties of water-extractable humic substances in soil

Daichi Asakawa[a], Hidemasa Mochizuki[a], Yukiko Yanagi[b,1], Takeshi Suzuki[b], Seiya Nagao[c] and Nobuhide Fujitake[b,*]

[a] The Graduate School of Science and Technology, Kobe University, Kobe 657-8501, Japan
[b] Laboratory of Soil Science, Faculty of Agriculture, Kobe University, Kobe 657-8501, Japan
[c] Graduate School of Environmental Earth Science, Hokkaido University, Sapporo 060-0810, Japan

Abstract

Comparisons of structural properties of water-extractable humic substances (WEHS) obtained from litter layer and mineral soil were examined at different shaking time, soil-to-solution ratio, and storage condition. ^{1}H NMR spectroscopy revealed the effects of operational conditions on the structural properties of WEHS. An increase in shaking time resulted in a significant increase of carbohydrate protons in WEHS and decrease of aliphatic protons, but the variation of soil-to-solution ratio did not significantly affect. No significant differences in the distribution of proton species in WEHS were observed between shaking and soaking extractions. To obtain greater portions of potentially soluble organic matter from Oe/Oa- and A-horizons, soil samples were extracted for shaking time of 12 and 24 h, respectively. Storage of soil samples during 3 days at 1°C caused a remarkable decrease in the amounts of extracted carbon in the WEHS. Although no changes in the distribution of proton species in WEHS during a 3 days storage at 1°C were observed, the proportion of carbohydrate protons in WEHS increased and that of aliphatic protons decreased during a 8 days storage at 1°C. Since proportion of the aromatic protons in WEHS from A-horizon soil significantly increased from 6 to 12% during storage at –30°C, freezing storage should be avoided. Therefore, it is desirable to be stored soil samples in cold conditions and to be extracted within few days.

Introduction

Dissolved organic matter (DOM), which originates from plant litter, soil humus, microbial biomass, or root exudates, is a common constituent in soil and aquatic environments. The hydrophobic acids fraction of DOM is operationally defined as fraction adsorbed to the XAD-8 resin at low pH and desorbed with high pH solution (Leenheer, 1981), and is corresponding to the humic substances (HS) and/or precursors of HS in illuvial soil horizons (Qualls and Haines, 1992; Guggenberger and Zech, 1994). The HS is the main fraction of DOM that bound organic contaminants and reduced the uptake and accumulation of contaminants by biota (McCarthy, 1989; Kukkonen et al., 1990; Haitzer, 1999). Molecular properties (e.g. molecular size, polarity, aromatic content and molecular configuration) of HS can substantially influence the sorptive capacity for contaminants (McCarthy, 1989; Raber and Kögel-Knabner, 1997).

When the HS in DOM investigated, particular attention should be paid to the collection method. The most commonly collection methods of DOM are zero tension lysimetry, vacuum displacement, centrifugation and extraction with water. The use of lysimetry is reasonable for studies that are subject to fluxes of DOM, since DOM collected by lysimeter is dissolved in mobile water. In general, yield of DOM from soils can be strongly influenced by ambient

* Corresponding Author: TEL./FAX: +81-78-803-5847. E-mail address: fujitake@kobe-u.ac.jp (N. Fujitake).
[1] Present address: Faculty of Horticulture, Minamikyushu University, Miyazaki 884-0003, Japan

water content. If soil moisture content is sufficiently high, in situ water could be collected by means of lysimetry, vacuum displacement and centrifugation. Such the collection methods may not be useful for preparative collection of DOM from soils, except for the Podzols or Histosols conditioned by a humid temperate climate. For evaluating the structural properties in soluble HS collected from soils in (semi-)arid regions, extraction with water is better because of its convenience and simplicity for obtaining high yield of samples. Water-extractable organic matter (WEOM), which is organic matter extracted with water, should be distinguished from DOM (Chantigny, 2003; Zsolnay, 2003), because WEOM consists of not only mobile organic matter but also a smaller portion of organic matter in available water (Zsolnay, 2003). Therefore, WEOM could be considered to be a potentially mobile organic matter in soil.

It should be noted that operational conditions prior to analysis influence the chemical properties of WEOM. Many contradictions between the results of several studies for WEOM are due to the use of different operational conditions (Zsolnay, 2003). Therefore, standardization of operational conditions of extraction method of WEOM would help to reduce such contradictions (Chantigny, 2003). For this reason, the effects of operational conditions, such as soil-to-solution ratio, shaking time, extractant, extraction temperature, and sample storage method on the extracted amounts of WEOM and HS in WEOM (water-extractable HS; WEHS) have been investigated (Christ and David, 1994; Zsolnay, 1996; You et al., 1999; Oste et al., 2002). However, structural features for WEHS may be varied by the operational conditions. Nevertheless, no work has focused on the variations of the structural properties of WEHS, which is extracted by a variety of conditions. The structural properties of WEHS may be decisive for the functional properties of WEHS. In the present study, we investigated the effects of operational conditions, such as shaking time, soil-to-solution ratio, shaking methods, and condition of sample storage on the structural properties of WEHS.

Table 1. Selected properties of the studied soil horizons.

Horizon	Depth (cm)	pH* (H_2O)	pH* (KCl)	TOC** (g kg^{-1})	TON** (g kg^{-1})	Al_o+1/2Fe_o† (g kg^{-1})
Oe/Oa	+2-0	4.66	3.77	417	18.9	-
A	0-15	5.19	3.74	25.7	1.7	2.08
B	15-80	5.61	3.89	2.7	0.15	3.11

* Measured potentiometrically at a soil-to-solution ratio of 1:2.5. ** Total organic carbon and total organic nitrogen were determined using a CHN/S analyzer (Perkin Elmer). † Al_o, Fe_o; Oxalate-extractable Al and Fe.

Materials and Methods

Soil Samples

Soil samples (Oe/Oa-, A- and B-horizons) were taken in November 2003, February and November 2004 from a deciduous broad-leaved forest. The sampling site was located on a east-facing strong slope in Yamashiro, Kyoto prefecture, Japan (34°47.16' N, 135°50.48' E, 188 m above sea level). The dominant tree species were Japanese oak (*Quercus serrata* Thunb.) and Aquifoliaceae (*Ilex pedunculosa* Miq.), and the ground vegetation was dominated by *Sasa* sp. (*Pleioblastus chino* Makino). The mean annual temperature and annual precipitation in 2003 were 15.0°C and 1719 mm, respectively. The soil was classified as a Dystric Cambisols (WRB-classification) derived from granite material. Some data on soil properties are given in Table 1. The soil samples were collected in plastic bags and placed in a cooler with ice for transport back to the laboratory.

Shaking Time and Soil-to-Solution Ratio

A-horizon sample collected in November 2003 was sieved in field-moist conditions (2 mm) to eliminate larger particles. One hundred grams of A-horizon sample stored at 7°C under field-moist conditions for a week were used for extraction with ultra-pure water at a soil-to-solution ratio of 1:5. All extractions were performed on an oven dry mass basis. The suspension was shaken on a reciprocal shaker (110 strokes min^{-1}) for 10 min, 8, 16, 24 and 32 h at 7°C (no replicated). To investigate the effect of soil-to-solution ratio, A-horizon sample was extracted at the soil-to-solution ratio of 1:2, 1:5, 1:10 and 1:20 for 24 h (no replicated). After centrifugation (10,000 g, 30 min), the extracts were filtrated through a pre-washed 0.45-μm pore cellulose acetate membrane filter (Advantec, Tokyo, Japan). Carbon contents in the WEOM (WEOC) in the filtrate were determined by a Shimadzu TOC-V CPH total organic carbon analyzer. The concentration of organic carbon was expressed as the extracted amount of carbon per soil oven dry mass.

Fifty milliliters of ultra-pure water was

added to 2.5 g of fresh Oe/Oa-horizon sample collected in February 2004. The suspensions were shaken at 1°C for 4, 8, 12, 16, 20 and 24 h. All other conditions were given above. The experiments were conducted in triplicate. WEOC in the filtrate was determined by TOC analyzer. For isolation of the WEHS, the WEOM solutions of three replicates were combined.

Water-extractable humic substances

The WEHS was isolated by the method of Thurman and Malcolm (1981) with some modifications. The extracts were acidified to pH 2 with 6 M HCl and passed through a glass column filled with DAX-8 resin. A Supelite DAX-8 resin (Supelco, Bellefonte, PA, USA), which can be used instead of an Amberlite XAD-8 resin (Rohm and Haas, Philadelphia, PA, USA), was employed to fractionate the WEOM. The WEHS was eluted from the resin with 0.1 M NaOH followed by distilled water. Carbon content in the eluate (WEHS-C) was analyzed. The non-WEHS-C, mainly consisting of hydrophilic organic matter, was calculated by subtracting WEHS-C from WEOC. The WEHS fraction was protonated by passing through an ion exchange column (AG MP-50, Bio Rad laboratories, Hercules, CA, USA) and the effluent was then freeze-dried.

Comparison of Shaking and Soaking Extraction

WEOM was extracted by shaking or soaking extraction. For both methods, 10 g of the fresh Oe/Oa-horizon sample (collected in November 2004) was extracted with ultra-pure water at soil-to-solution ratio of 1:20 in triplicate. In shaking extraction, the suspensions were shaken at 1°C for 12 h. In soaking extraction, the suspensions were purged for 5 min with N_2, stirred for 1 min and then allowed to stand at 1°C for 12 h and 72 h. N_2 purge (5 min) and stirring (1 min) were repeated every 12 h. After centrifugation and filtration procedures, WEOC was determined. The WEOM solution was fractionated by DAX-8 resin.

Experiments were replicated for the A-horizon samples (collected in November 2004) from which gravels (>1 cm) were removed manually. Forty grams of fresh A-horizon sample was extracted in a soil-to-solution ratio of 1:5 at shaking time of 24 h, soaking time of 24 h or 72 h.

Storage Conditions

Portions (2.5 g) of fresh Oe/Oa-horizon sample collected in February 2004 were packed into the 100-mL polyethylene bottles. The sample bottles were stored at 1°C for 3, 8, 17 or 24 days, at –30°C for 7 days or freeze-dried. The frozen samples were thawed overnight at 1°C before extraction. Fifty milliliters of ultra-pure water was added to Oe/Oa-horizon samples (soil-to-solution ratio of 1:20), and the suspension was shaken at 1°C for 12 h. Fresh material (control) was extracted within 12 h after sampling. Each treatment contained 4 replicates. The WEHS was isolated using DAX-8 resin.

Experiments were replicated for the A- and B-horizon samples collected in February 2004. Twenty grams of the fresh and stored A- and B-horizon samples (<1 cm) in 200-mL polyethylene bottles were shaken at a soil-to-solution ratio of 1:5 for 24 h (triplicated).

Spectroscopic Analysis

^{1}H NMR spectra of the WEHS solution was obtained at 500 MHz on a Bruker Avance 500 spectrophotometer under homo-gated decoupling conditions. The HOD proton (4.8 ppm) associated with water impurities was irradiated. Approximately 5 mg of WEHS was dissolved in 0.5 mL of 0.1 M NaOH. The hydrogen was exchanged for deuterium according to a method by Fujitake et al. (2003). For the chemical shifts, sodium 3-trimethylsilylpropionate-2,2,3,3, D_4 (TMSP; Euriso-top, Saint Aubin, France) was used as reference. The sample solution was transferred into a 5-mm NMR tube. ^{1}H NMR spectra were recorded under the following conditions: pulse width 14.0 μs (90°), acquisition time 5.4 s, and pulse delay 4.8 s, scan number 64.

Chemical shift assignments for ^{1}H NMR spectroscopy from Wilson et al. (1983), Wershaw (1985) and Kawahigashi et al. (1996) are summarized in Table 2. The percentage of distribution of aromatic (Har; 6.0-9.0 ppm), methoxyl ($H_{C\text{-}O}$; 3.0-4.3 ppm), and aliphatic (H_{al}; 0.0-3.0 ppm) protons was estimated. The assumption that the proportion of $H_{C\text{-}O}$ is representative for carbohydrates was used (Kalbitz et al, 2003a) on the basis of the report by Kaiser et al. (2002) that a large portion of $H_{C\text{-}O}$ (3.0-4.3 ppm) could be due to carbohydrate H.

Table 2. Proton resonances of humic materials (Wilson et al., 1983; Wershaw, 1985; Kawahigashi et al., 1996)

Chemical shift δ (ppm)	Assignments and interpretations
0.0-0.9	terminal methyl groups attached to saturated aliphatic carbons
0.9-1.6	protons on methylene β attached to olefins or aromatic rings
1.6-3.0	protons on methyl and methylene α attached to aromatic carbons, carbonyl groups, ester groups, and olefins
3.0-4.3	protons on carbons attached to oxygen-sugars, olefins, and methoxyl groups
6.0-9.0	protons attached to carbons of heteroaromatic and aromatic rings, and to carbonyl groups bonded to electronegative groups

Table 3. The amount of C in the water-extractable organic matter (WEOC), the amount of C in the water-extractable humic substances (WEHS-C) and proportion of the WEHS-C in WEOC obtained by different soil-to-solution ratio and shaking time.

	WEOC* (mg C kg^{-1})	WEHS-C (mg C kg^{-1})	WEHS-C/WEOC (%)†
Oe/Oa-horizon *(collected in February 2004)*			
soil-to-solution ratio 1:20			
4 h	882 ± 103	n.d. **	n.d.
8 h	1120 ± 75	511	45.7
12 h	1530 ± 111	714	46.7
16 h	1560 ± 48	701	45.1
20 h	1470 ± 224	706	48.0
24 h	1520 ± 201	n.d.	n.d.
A-horizon *(collected in November 2003)*			
soil-to-solution ratio 1:5			
10 minutes	43.1	20.8	48.2
8 h	57.8	35.7	61.8
16 h	63.4	48.6	76.7
24 h	72.5	60.3	83.3
32 h	67.8	53.6	79.1
soil-to-solution ratio 1:10			
24 h	63.7	49.6	77.9
soil-to-solution ratio 1:20			
24 h	81.6	61.6	75.4

* WEOC from Oe/Oa-horizon is the mean ± S.D. ** n.d. = Not determined. † Percentages of WEHS-C in WEOC

Results and Discussion

Effects of Shaking Time and Soil-to-Solution Ratio

The WEOC and WEHS-C were seriously affected by shaking time (Table 3). For Oe/Oa- and A-horizon soils, 72-80 % of total WEOC and 59-72 % of total WEHS-C were extracted within initial 8 h. The WEHS-C from Oe/Oa- and A-horizon soils increased during initial 12 h and 24 h, respectively, and then became relatively constant. The same trends were observed for WEOC. These results are supported by the observation that WEOC reached steady state within 24 h of shaking time (You et al., 1999; Qualls, 2000). Table 3 also shows that the proportion of WEHS-C from A-horizon increased with increasing shaking time. However, soil-to-solution ratio from 1:5 to 1:20 did not significantly affect WEOC and WEHS-C extracted from A-horizon at a shaking time of 24 h. This result is inconsistent with Kaiser's report (2001), that is, the amounts of WEOC from air-dried soil material increased linearly with an increase in the soil-to-solution ratio from 1:5 to 1:40. The reason for this may be the different sample storage. Since air-drying cause substantial increase in WEOC (Christ and David, 1994; Zsolnay, 1996), soil-to-solution ratio of not less than 1:40 may be necessary to extract total WEOC from air-dried soil.

^{1}H NMR was used to investigate the distribution of aliphatic, carbohydrate and aromatic protons in WEHS. The distribution of proton species in the WEHS was varied by the operational conditions (Fig. 1). As shown in Fig. 1a, the proportion of $H_{C\text{-}O}$ in the WEHS from Oe/Oa-horizon increased from 32.7 to 40.4 % with increasing shaking time from 8 to 12 h, while that of H_{al} decreased from 54.8 to 49.2 %. After a shaking time of 12 h, the opposite effect on the proportions of H_{al} and $H_{C\text{-}O}$ in the WEHS was observed. It appears that the decrease in the proportion of $H_{C\text{-}O}$ after 12 h may be attributed to

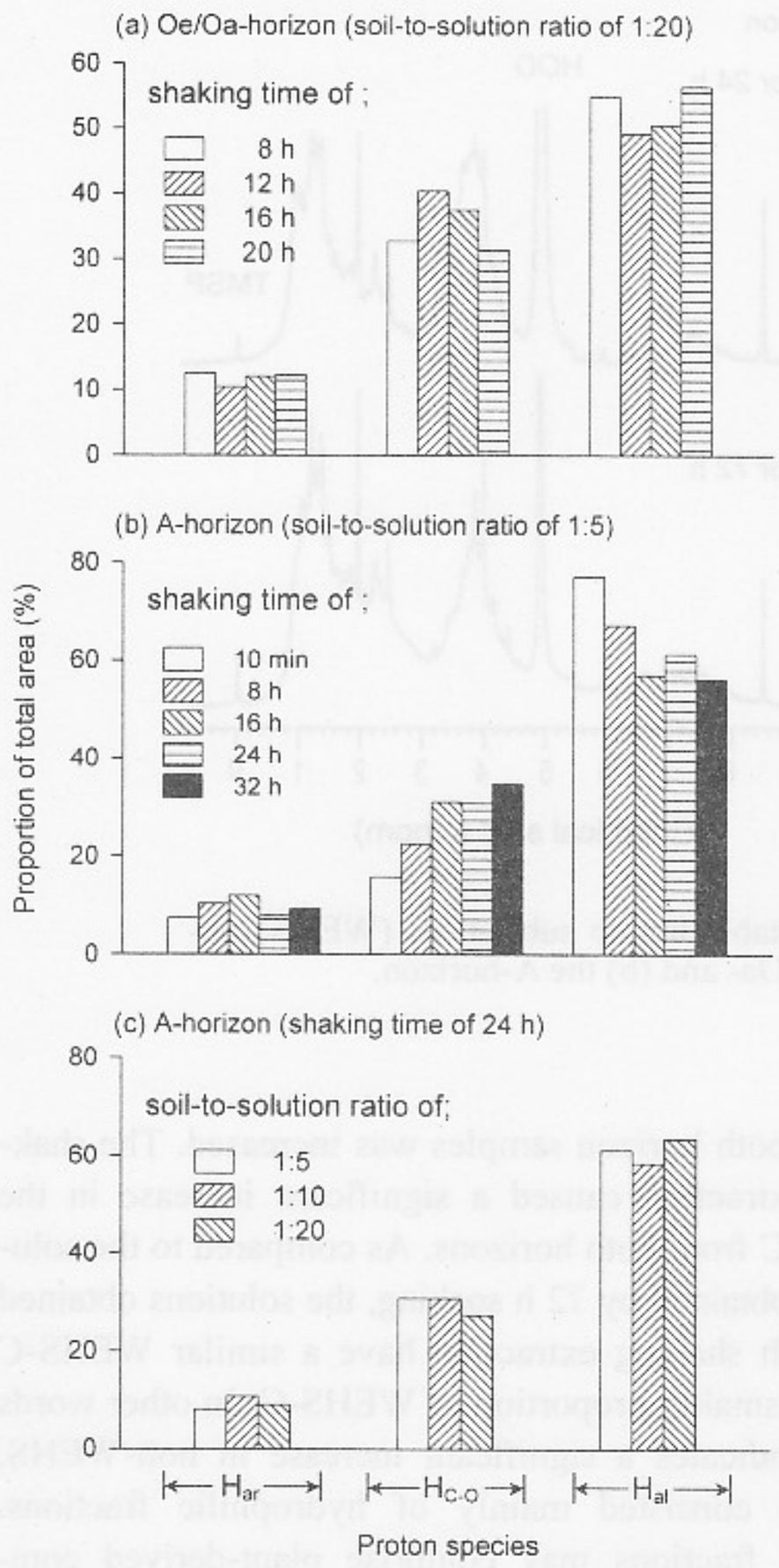

Figure 1. Effect of shaking time on the distribution of the proton species in the water-extractable humic substances (WEHS) from (a) the Oe/Oa- and (b) the A-horizon. (c) Effect of soil-to-solution ratio on the distribution of the proton species in the WEHS from the A-horizon.

preferential utilization of the carbohydrate moiety of WEHS by microorganisms (Kalbitz et al., 2003).

Figure 1b shows that the proportion of H_{al} in the WEHS from A-horizon significantly decreased from 77.1 to 56.9 % and that of H_{C-O} significantly increased from 15.7 to 31.1 % with increasing shaking time during initial 16 h. When shaking time was greater than 16 h, it is assumed that the structural properties of WEHS are independent of shaking time. Moreover, within the soil-to-solution ratio from 1:5 to 1:20, there were no significant changes in the proportions of each type of proton species in the WEHS obtained from A-horizon (Fig. 1c). These results suggested that the soil-to-solution ratio was minor factor for changing the structural properties of WEHS in A-horizon soil sample.

Although WEHS-C from Oe/Oa-horizon reached equilibrium within 12 h, the distribution of proton species in WEHS did not become stable within the experimental time period (Fig. 1a). This could be attributed to the fact that small soil-to-solution ratio was employed. However, a large soil-to-solution ratio is not practical for the preparation of WEHS because of the requirement of large-scale treatment. Therefore, a soil-to-solution ratio of 1:20 and 12 h shaking time was chosen for further study of Oe/Oa-horizon sample. The amount and structural properties of WEHS from A-horizon became relatively stable within 24 h, and were not affected seriously by the soil-to-solution ratio. Thus, A- and B-horizon samples were extracted at a soil-to-solution ratio of 1:5 for 24 h shaking time in further studies.

Table 4. The amount of C in the water-extractable organic matter (WEOC), the amount of C in the water-extractable humic substances(WEHS-C) and proportion of the WEHS-C in WEOC obtained by shaking extraction and soaking extraction.

	WEOC* (mg C kg^{-1})	WEHS-C (mg C kg^{-1})	WEHS-C/WEOC (%)**
Oe/Oa-horizon *(collected in November 2004)*			
soil-to-solution ratio 1:20			
Shaking for 12 h	1950 ± 146	1010	51.9
Soaking for 12 h	810 ± 92	698	86.1
Soaking for 72 h	1250 ± 184	997	80.0
A-horizon *(collected in November 2004)*			
soil-to-solution ratio 1:5			
Shaking for 24 h	82.0 ± 13.5	40.6	49.5
Soaking for 24 h	41.9 ± 1.4	28.3	67.6
Soaking for 72 h	41.1 ± 3.6	35.1	85.4

* Each datum is the mean ± S.D. ** Percentages of WEHS-C in WEOC.

Comparison of Shaking and Soaking Extraction methods

Table 4 shows that WEOC obtained from Oe/Oa-horizon by a 72 h soaking time was 1.4 times higher than that obtained by a 12 h soaking time. The WEOC obtained from A-horizon by soaking extraction was independent of the soaking time. When soaking time was increased up to 72 h, the WEHS-C

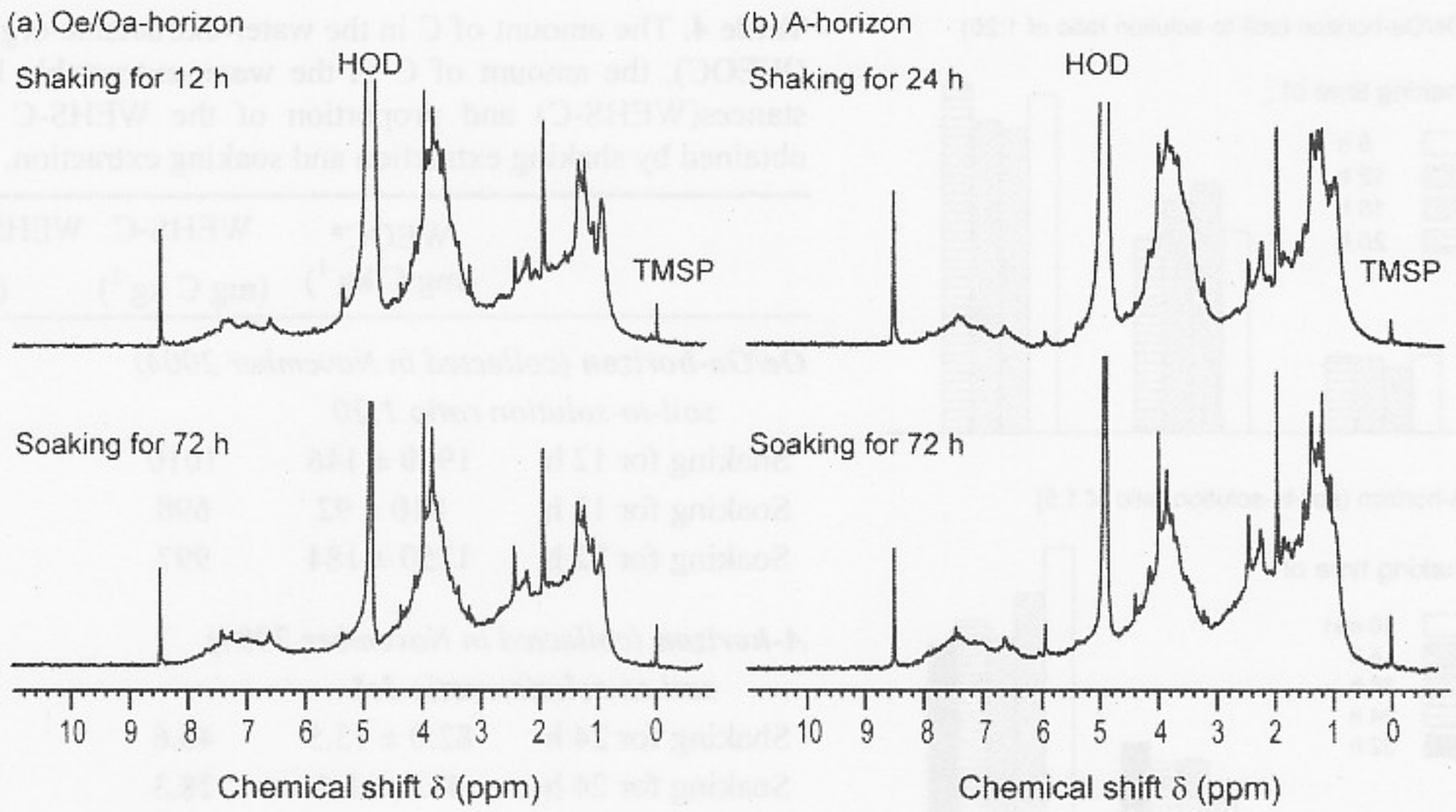

Figure 2. Liquid-state ^{1}H NMR spectra of water-extractable humic substances (WEHS) obtained by shaking and soaking extraction from (a) the Oe/Oa- and (b) the A-horizon.

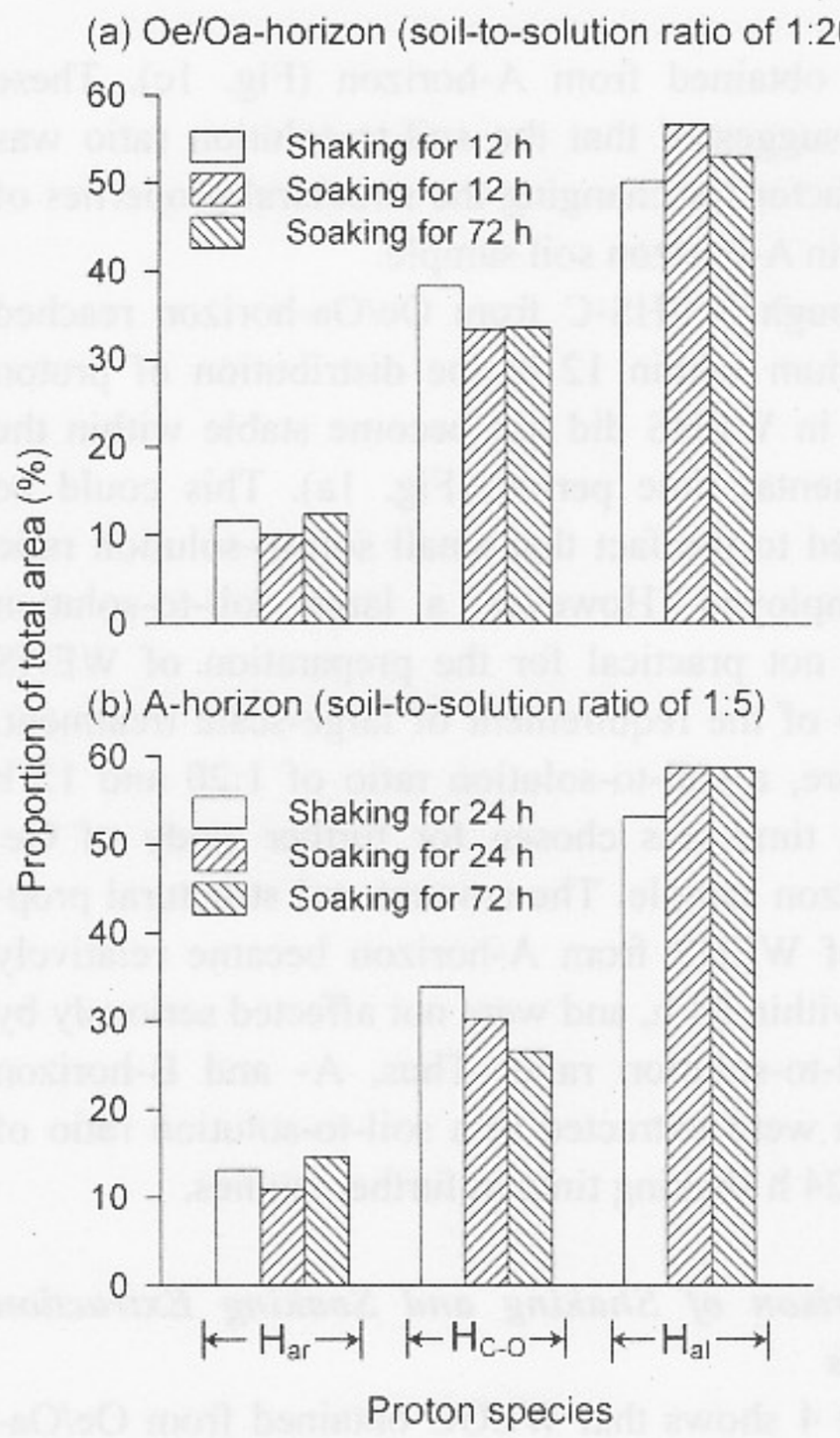

Figure 3. Difference in the distribution of the proton species in the water-extractable humic substances (WEHS) from (a) the Oe/Oa- and (b) the A-horizon between shaking extraction and soaking extraction and between soaking times.

from both horizon samples was increased. The shaking extraction caused a significant increase in the WEOC from both horizons. As compared to the solutions obtained by 72 h soaking, the solutions obtained by 24h shaking extraction have a similar WEHS-C and a smaller proportion of WEHS-C. In other words, this indicates a significant increase in non-WEHS, which consisted mainly of hydrophilic fractions. These fractions may comprise plant-derived compounds and microbial metabolites (Guggenberger et al., 1994). Since shaking procedure disrupts cells, a part of the increase in non-WEHS-C should be due to the cell lysis.

The ^{1}H NMR spectra for the WEHS obtained by shaking and soaking extractions are given in Fig. 2. This ^{1}H NMR spectrum of WEHS obtained by shaking extraction was very similar to that by soaking extraction. However, the signal intensity of $H_{C\text{-}O}$ in WEHS obtained by shaking extraction was slightly larger than that by soaking extraction. As shown in Fig. 3, the proportions of $H_{C\text{-}O}$ in the WEHSs from Oe/Oa and A-horizon obtained by shaking extraction were somewhat higher than those obtained by soaking extraction. It appears that differences in structural properties in WEHS obtained by shaking and soaking extraction were relatively minor, but the WEHS was efficiently extracted by shaking extraction than soaking extraction.

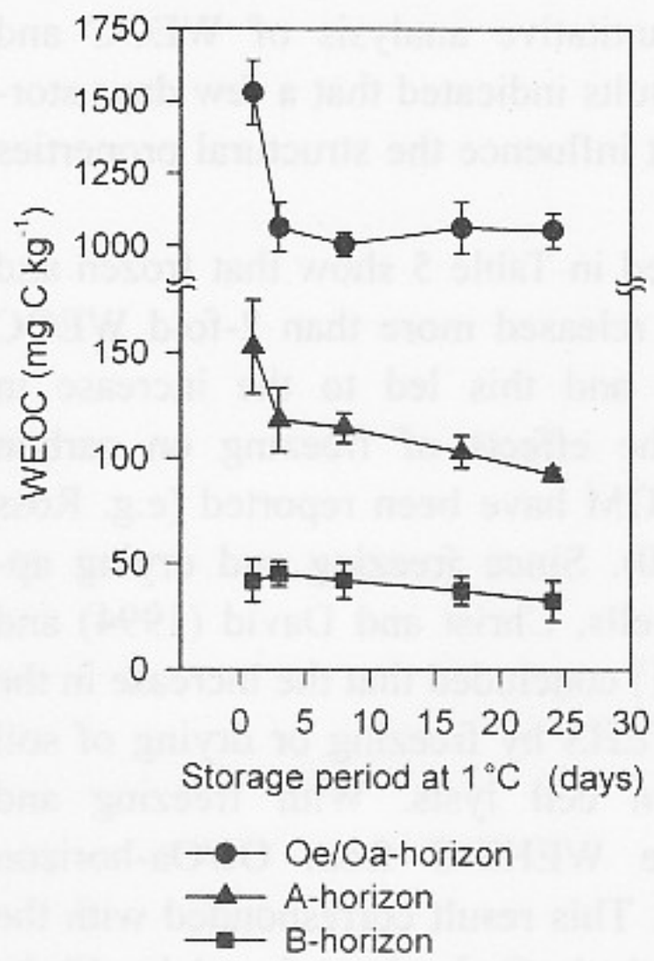

Figure 4. Effect of storage period at 1°C on the amount of C in the water-extractable organic matter (WEOC) from the Oe/Oa-, the A-, and the B-horizon. Error bars represent standard deviation (n = 4 or 3).

Table 5. The amount of C in the water-extractable organic matter (WEOC), the amount of C in the water-extractable humic substances (WEHS-C) and proportion of the WEHS-C in WEOC obtained from differently stored soil material.

	WEOC* (mg C kg^{-1})	WEHS-C (mg C kg^{-1})	WEHS-C/WEOC (%)†
Oe/Oa-horizon *(collected in February 2004)*			
soil-to-solution ratio 1:20			
Field-fresh**	1530 ± 111	714	46.7
3 days at 1°C	1060 ± 85	475	44.7
8 days at 1°C	1000 ± 41	515	51.2
Freezing	2870 ± 272	750	26.1
Freeze-drying	3910 ± 395	931	23.8
A-horizon *(collected in February 2004)*			
soil-to-solution ratio 1:5			
Field-fresh**	153 ± 22	80.9	52.9
3 days at 1°C	118 ± 15	66.0	55.9
8 days at 1°C	115 ± 7	57.6	50.3
Freezing	181 ± 28	35.9	29.1
Freeze-drying	296 ± 22	50.0	16.9

* Each datum is the mean ± S.D. ** Field-fresh soil materials were extracted within 12 h after sampling. † Percentages of WEHS-C in WEOC.

Effects of Storage Conditions

As shown in Fig. 4, within 3 days of storage at 1°C, the WEOC from Oe/Oa- and A-horizons decreased by 34% and 23%, respectively. This effect is similar to the observation by Ross and Bartlett (1990), who compared the properties of soil solutions obtained from the field-fresh Spodosols (within 3 h after sampling) with those from differently stored samples. They observed that the storage of Oa-horizon soil for 4 days at 3°C resulted in a decrease of soluble organic carbon by 16-30%. Since microbial degradation of extracted organic carbon takes place within a few days (Jandl and Sollins, 1997; Kalbitz et al., 2003b), rapid decrease in the WEOC may be due to microbial degradation in the collected solid soil phase. After 3 days, the amount of WEOC from A-horizon gradually decreased (Fig. 4). The effect of 24 days storage at 1°C on the WEOC from B-horizon was not significant. In contrast, some investigators reported that long-term storage of soil materials at a low temperature increased the dissolved organic carbon content (Ross and Bartlett, 1990; Christ and David, 1996; Kaiser et al., 2001). Such the difference is probably due to the use of different soil materials, different extraction conditions or different periods of time to extract control samples. Indeed, Pérez et al. (2004) observed that dissolved organic carbon content from two surface horizon samples increased or decreased during a 15 days storage at 4°C. There were no significant effects of soil sample storage at 1°C on the proportions of WEHS-C in WEOC from Oe/Oa- and A-horizon samples (Table 5).

Figure 5 shows that the storage of field-moist Oe/Oa- and A-horizon samples for 3 days at 1°C had little influence on the distribution of proton species for the WEHS. However, after 8 days, the proportion of H_{al} in the WEHS from both horizons decreased and that of $H_{C\text{-}O}$ increased. It appears that the WEHS from A-horizon is more susceptible to storage at 1°C than that from Oe/Oa-horizon.

It was considered that microbial degradation of soil organic matter occurred during the storage of field moist soil material even at low temperature. Many studies show that microbial degradation of soil organic matter is related to its molecular properties, that is, the hydrophobic fraction in the WEOM is less degradable than the hydrophilic fraction (Jandl and Sollins, 1997; Kalbitz et al., 2003a), and selective degradation of carbohydrate components in the soil organic matter in liquid phase and solid soil phase was observed (Baldock et al., 1992; Almendros and Dorado, 1999; Yanagi et al., 2002; Kalbitz et al.,

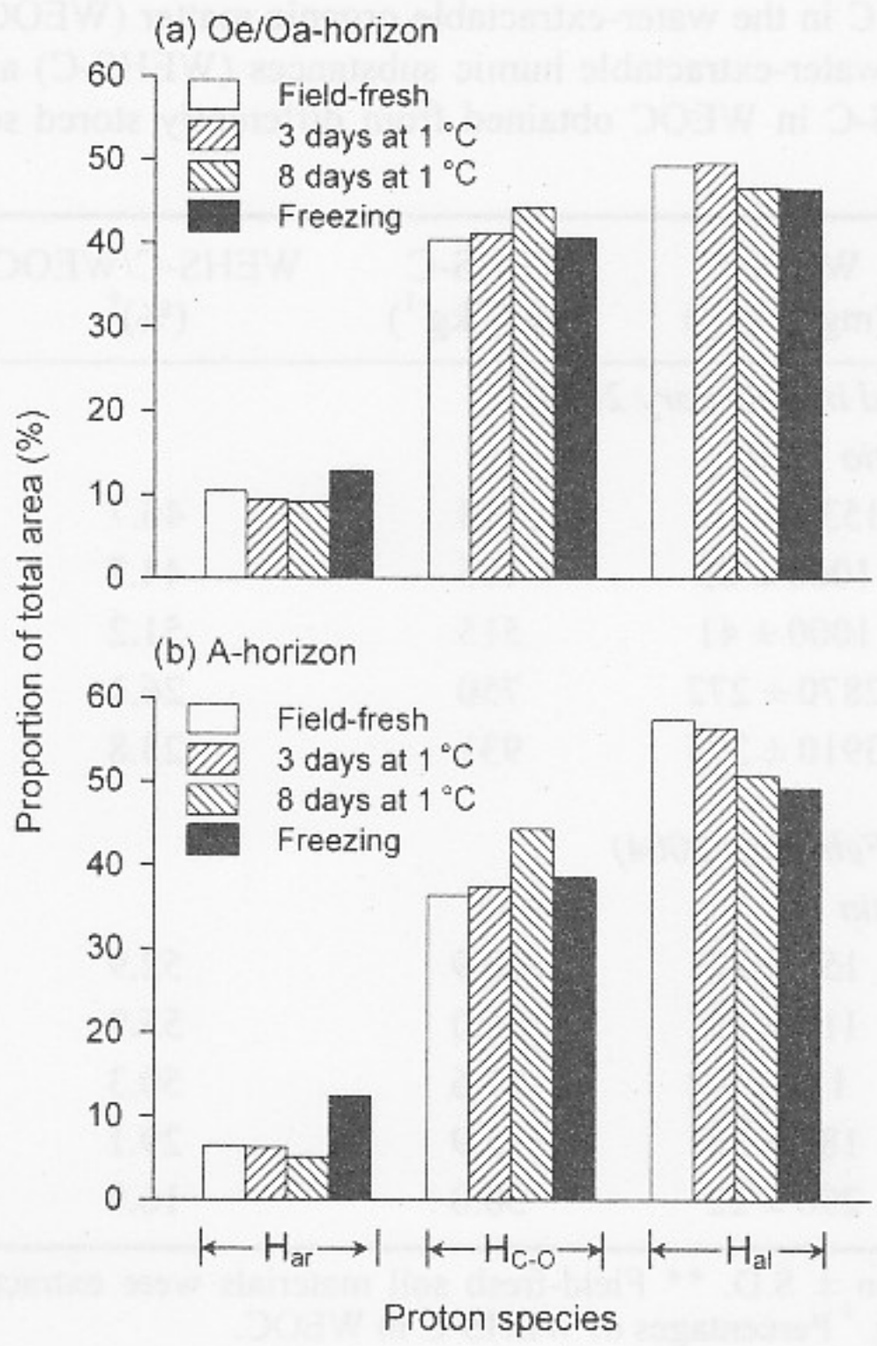

Figure 5. Effect of soil sample storage condition on the distribution of the proton species in the water-extractable humic substances (WEHS) from (a) the Oe/Oa- and (b) the A-horizon. Field-fresh soil materials were extracted within 12 h after sampling.

2003b). In this study, the degree of decrease in the WEHS-C was similar to that of non-WEHS-C (Table 5) and the selective decreases in the carbohydrate components in the WEHS (Fig. 5) was not observed. Therefore, the decreases in WEOC and WEHS-C and changes in the distribution of proton species in the WEHS during storage at 1°C may not be due to microbial degradation. The WEOM, instead, seems to be insolubilized by oxidative degradation and/or a combination of precipitation and complexation with mineral materials. In fact, speciation of metals in soil solution, objectively the fraction complexed by DOM, remarkably changed with soil sample storage at 4°C (Pérez et al., 2004). The complexation of metals and DOM might have caused the changes in the distribution of proton species in WEHS from A-horizon by storage at 1°C for 8 days.

As recommended by many investigators (Ross and Bartlett, 1990; Pérez et al., 2004), field-moist soil samples should be extracted as soon as possible after sampling for quantitative analysis of WEOC and WEHS-C. Our results indicated that a few days storage at 1°C did not influence the structural properties of WEHS.

The results listed in Table 5 show that frozen and freeze-dried soils released more than 2-fold WEOC from fresh soils, and this led to the increase in non-WEHS-C. The effects of freezing on carbon contents in the DOM have been reported (e.g. Ross and Bartlett, 1990). Since freezing and drying apparently disrupt cells, Christ and David (1994) and Kaiser et al. (2001) concluded that the increase in the amount of non-WEHS by freezing or drying of soil could result from cell lysis. With freezing and freeze-drying, the WEHS-C from Oe/Oa-horizon slightly increased. This result corresponded with the effect on the Spodsols Oa-horizon found by Christ and David (1994). In contrast, 56% and 38% of WEHS-C from A-horizon were decreased by freezing and freeze-drying, respectively. This may be due to abiotic insolubilization.

As shown in Fig. 5, freezing treatment caused a remarkable increase in the proportion of H_{ar} in the WEHS and decrease in that of H_{al}, especially from the A-horizons. Freezing might cause abiotic insolubilization of WEHS, which has low proportion of H_{ar} and high proportion of H_{al}.

Conclusions

This study demonstrated that operational conditions for extraction and sample storage can significantly affect the structural properties of WEHS, although differences in the effects on other types of soil remain unknown. The proportions of H_{al} in the WEHS from Oe/Oa- and A-horizons decreased and those of H_{C-O} increased with increasing shaking time. The WEHS from Oe/Oa-horizon possibly be degraded after a shaking time of 12 h. To obtain greater portions of potentially soluble organic matter from Oe/Oa- and A-horizons, soil samples were extracted for shaking time of 12 and 24 h, respectively. Storage at 1°C resulted in the remarkable decreases in the WEOC from Oe/Oa- and A-horizons within 3 days. Consequently, field-moist soil samples should be extracted as soon as possible after sampling for quantitative analysis of WEOC and WEHS-C. Our results also indicated that a 3 days storage at 1°C did not influence the distribution of proton species in the WEHS, while 8 days storage resulted in increase in

the proportion of H_{C-O} and decrease in H_{al} in the WEHS. Freezing storage should be avoided, since it caused increase in the proportion of H_{ar} in the WEHS.

It is difficult to standardize a collection method of WEHS and/or WEOM for practical reasons such as variety in the types of soils, laboratory equipments, and distance from laboratory to sampling site. Accordingly, comprehensive understanding of the methodological artifacts has become increasingly important. To compare with other studies of WEHS and/or WEOM, each investigator should precisely describe their extraction method and storage condition.

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