

Amelioration effect of humic acid extracted from solubilized excess sludge on saline-alkali soil

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| 著者別名 | 本嶋 秀子, 入江 光輝, 繁森 英幸, 磯田 博子 |
| journal or publication title | Journal of material cycles and waste management |
| volume | 14 |
| number | 3 |
| page range | 169-180 |
| year | 2012-09 |
| 権利 | (C)Springer 2012. The original publication is available at www.springerlink.com . |
| URL | http://hdl.handle.net/2241/117935 |

doi: 10.1007/s10163-012-0056-y

1 **Amelioration effect of humic acid extracted from solubilized excess**
2 **sludge on saline-alkali soil**

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4 Hideko Motojima · Parida Yamada · Mitsuteru Irie · Masuo Ozaki ·
5 Hideyuki Shigemori · Hiroko Isoda

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7 H. Motojima · P. Yamada · M. Irie · H. Shigemori · H. Isoda (✉)

8 Graduate School of Life and Environmental Sciences, University of
9 Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

10 Tel +81-29-853-5775

11 e-mail: isoda.hiroko.ga@u.tsukuba.ac.jp

12 M. Ozaki

13 Department of Civil and Environmental Engineering, Maebashi
14 Institute of Technology, 460-1 Kamisadorimachi, Maebashi, Gunma
15 371-0816, Japan

16

17 **Abstract** In this study, the main characteristics and soil amendment
18 effect on the saline-alkali soil of humic acid extracted from solubilized
19 excess sludge (SS-HA) were investigated. The excess sludge was
20 solubilized prior to extraction to improve the humic acid recovery rate.
21 The structural features of SS-HA were characterized by an elemental
22 analysis, Fourier transform infrared spectroscopy, and ¹H-nuclear

1 magnetic resonance spectroscopy, and compared with those of HA
2 extracted from non-solubilized excess sludge (ES-HA). The results
3 showed that extraction efficiency of humic acid was enhanced by using
4 solubilization, although structural properties of humic acid extracted
5 from solubilized excess sludge were almost same as those of ES-HA.
6 To study a utilization method of SS-HA, the soil amendment effect on
7 saline-alkali soil by mixture of SS-HA were investigated with model
8 soil-column experiment. SS-HA reduced the pH of the saline-alkali
9 soil, and the effect was faster or immediate observed compared with the
10 case which is only peat added. Moreover, the cation exchangeable
11 capacity of the saline-alkali soil was enhanced by addition of SS-HA.

12

13 **Key words** humic acid, solubilized excess sludge, pH, exchangeable
14 cation, saline-alkali soil

15

16 **Introduction**

17 Activated sludge process has been widely employed for wastewater
18 treatment in many countries. Generation of excess sludge (ES) is one
19 of the drawbacks of this process. Recently, adjustment to sustainable
20 developing society is policy of the Japanese Government in waste
21 management. Organic waste (biomass) is ranked as part of the
22 untouched natural resources, and proactive utilization of those came to
23 be strongly demanded. One possible way for ES to be utilized is to

1 extract some of the beneficial compounds that ES contains for use. It
2 is well known that excess sludge is degraded and humified by
3 microorganisms to become compost etc, and it contains some bioactive
4 compounds such as humic substances (HS).¹ HS is broken down
5 organic components formed by plant decay and other biological
6 activities and is found in almost all terrestrial and aquatic
7 environments. HS have various useful effects due to their functional
8 groups. There are three types of HS, according to their solubility in
9 water: fulvic acid (FA), humic acid (HA) and humin. The fraction that
10 is precipitated by acid is referred to as HA and those that are not
11 precipitated by acid are FA and humin is not soluble in water.² HA
12 is a very important component of soil that affects its physical and
13 chemical properties and improves the fertility of agricultural fields.
14 HA can form complexes with various metal ions and thereby increase
15 the cation exchange capacity (CEC) of the soil.^{3,4} HA also promotes
16 very complex biological activity. For example, HA has many different
17 effects on plant metabolism in the diverse systems that have been
18 tested.⁵⁻⁷ One practical example of soil improvement in response to
19 the addition of HA is the positive effect on the pH of saline-alkali soil
20 after addition of Canadian peat reported by Kawakami *et al.*^{8,9} In the
21 environmental field, FA is known to supply iron nutrients to the sea
22 because of its characteristically strongly bonding to iron.¹⁰ FA also
23 has a role in environmental cleanup operations where its ability to form

1 complexes with metals makes it a useful coagulant for harmful heavy
2 metals and assists in detoxification of carcinogenic hexavalent
3 chromium.¹¹ In healthcare field, physiological action on the human
4 body of HS has been studied in recent years.^{12,13}

5 In this study, to examine whether ES could be a new source of HS,
6 we extracted and purified HS from ES. HS are abundant in peat,
7 weathered coal, and other humified substances.¹⁴ However, the cost of
8 transporting peat from wetlands to arid lands where it might be utilized
9 is very large. Similarly, weathered coal is available only near coal
10 mines. In contrast, ES is an industrial waste that is widely available
11 and continually produced. It thus has the potential to become an
12 environmentally sustainable source of HS. Excess sludge is often
13 chemically reduced by solubilization. Previously, we compared the
14 characteristics of solubilized excess sludge (SS) obtained by using
15 alkalization, microwave irradiation, boiling, and the bead-mill
16 technique, is higher than that of raw excess sludge, or of sludge
17 solubilized by other techniques.¹⁵ We found that the dissolved total
18 organic carbon (TOC) content of SS, particularly sludge solubilized by
19 the bead-mill technique, is higher than that of raw excess sludge, or of
20 sludge solubilized by other techniques. HS constitute an important
21 reservoir of organic carbon in aquatic and terrestrial environmental
22 compartments and account for as much as 50% of the dissolved organic
23 carbon in surface waters.¹⁶ 11% to 66% of TOC was HS in

1 groundwater.¹⁷ It is expected the TOC-rich fraction of SS contains
2 high concentration of HS. Therefore, solubilization using the
3 beads-mill technique is an effective means for their extraction.
4 Compared with the application of raw sewage sludge, the application of
5 extracted and purified HS to agricultural fields is likely to result in
6 fewer adverse effects on the environment such as the addition of heavy
7 metals.

8 Previously, the inhibitory effect of FA extracted from ES or SS on
9 type I allergy using rat basophilic leukemia cells was investigated.^{18,19}
10 In this study, structure of the HA obtained by solubilization (SS-HA)
11 was compared with the structure of HA from non-solubilized excess
12 (ES-HA) with elemental analysis, Fourier transform infrared (FT-IR)
13 spectroscopy and ¹H-nuclear magnetic resonance (¹H-NMR)
14 spectroscopy. As case study for utilization of SS-HA, the application
15 effect of SS-HA on the saline-alkali soil was investigated with modeled
16 column experiment.

17

18 **Materials and methods**

19

20 Collection of sludge samples and solubilization conditions

21

22 The ES used as a source for extraction of HA and FA in this research
23 was collected from the return line of an aeration tank at a wastewater

1 treatment plant in Gunma, Japan. The mixed liquor suspended solid
2 (MLSS) concentration of the sludge was 4200 mg/l. A total of 8 liters
3 of ES was divided into two 4 liters samples. One of these was
4 solubilized to verify improved HS extraction efficiency after
5 solubilization, and HS were extracted from the other sample without
6 solubilization.

7 A rotary drum cylindrical bead mill was used for solubilization of
8 ES. The cylindrical container (139.8 mm × 400.0 mm) (Fig. 1) had
9 four mixing blades, each 2 cm high, on the inside wall of the container.
10 The conditions under which solubilization was carried out were as in
11 the previous study of Motojima *et al.*¹⁵ The procedure for the
12 features analysis of solubilized sludge was described in our previous
13 study.¹⁸

14 15 Extraction of humic acid

16
17 To investigate the improvement of extraction rate and changes to the
18 structure of HA after solubilization, it was extracted from unsolubilized
19 ES as a control. Extraction and isolation procedures were carried out
20 following the protocol of the International Humic Substances Society.

21 22 Analyses of extracted humic acids

23

1 The structural features of ES-HA and SS-HA were characterized by
2 using an elemental analysis, Fourier transform infrared spectroscopy
3 and ^1H -nuclear magnetic resonance spectroscopy. The procedure for
4 the structural features analysis of SS-HA was described in the previous
5 report.¹⁸ Carboxyl group content of ES-HA and SS-HA was
6 determined by the calcium acetate method of Ikeya *et al.*²⁰ Dried
7 ES-HA and SS-HA were placed in 50 ml of 0.5 M aqueous calcium
8 acetate solution. After mixing for 16 h, filtrate was treated with 0.1 M
9 NaOH, with phenolphthalein used as an indicator. The carboxyl group
10 content of HA was calculated by subtracting from the result of the
11 blank test. Measurement of cation exchange capacity (CEC) was by
12 barium acetate saturation according to the standard method of the
13 National Institute for Agro-Environmental Science Japan.²¹

14

15 Modeling of saline-alkali soil

16

17 An artificial saline-alkali soil was prepared by adding a saline solution
18 to sandy material. The saline solution was a mixture of NaCl, KCl,
19 $\text{Ca}(\text{OH})_2$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $(\text{NH}_4)_2\text{SO}_4$, and $\text{KH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$. The sandy
20 material was a mixture of coarse sand (0.2–2.0 mm), fine sand
21 (0.02–0.2 mm) and clay (<0.002 mm) in ratios of 85.5, 13.5, and 1.0
22 wt %, respectively, to form a texture similar to that of Sahara desert
23 soil studied by Yazawa *et al.*²² The particle size distribution of the

1 model soil was determined according to Japanese Industrial Standard
2 (JIS) A 1204 which is an internationally recognized standard. The pH
3 and electrical conductivity (EC) of the model soil were adjusted by
4 addition of saline solution to 8.8 and 3.7 mS/cm, respectively. The pH
5 of the model soil was measured in suspension (soil:pure water = 1:2.5)
6 with a UC-23 pH meter (Central Kagaku Co.) according to the glass
7 electrode method of the Japanese Geotechnical Society (JGS
8 0211-2000), and EC of the soil solution (soil:pure water = 1:5) with a
9 YK-22CT EC meter (Sato-Shoji Co.) following the EC electrode method
10 (JGS 0212-2000). The physical and chemical properties of the model
11 soil are provided in Table 1.

12

13 Batch testing

14

15 The pH buffering ability and pH of the model soil after application of
16 SS-HA were measured by a batch test before the soil-column
17 experiment (described in the next section). The initial pH buffering
18 capacity of SS-HA was determined by the amount (moles) of strong acid
19 or base needed to produce a unit change of pH in the model soil. HCl
20 and NaOH solutions of the required concentrations (0.5, 1.0, 2.0, 5.0,
21 8.0, and 10.0 cmol/kg) were prepared; pure water was used for the
22 neutral point. Freeze-dried SS-HA (0.05, 0.1, and 0.5% of the weight
23 of the base or acid solution) was added to the model soil in NaOH and

1 HCl solution. After shaking for 24 h, the pH of soil solution was
2 measured. The pH change of the model soil in response to SS-HA
3 application was measured as follows. A 1000 mg/l solution of SS-HA
4 was prepared and added to the model soil (0, 0.005, 0.05, and 0.5% of
5 the dry weight of the soil). The mixtures of soil and SS-HA were
6 allowed to stand for three days, after which they were dried at 40 °C for
7 24 h. Each dried soil sample was then shaken in pure water for 2 h
8 (soil:pure water = 1:2.5 for pH, soil:pure water = 1:5 for EC), and pH
9 and EC were measured after filtering through a 1- μ m paper filter.

10

11 Soil-column experiment and parameters measured

12

13 To investigate the ameliorative effect of application of SS-HA to
14 saline-alkali soil, a soil-column experiment was performed at Tsukuba
15 University over 157 days from 30 March to 26 August 2009. The
16 experiment was performed in a cylindrical column of 5.0 cm diameter.
17 Two soil sampling shoes (steel columns; diameter 5.5 cm, length 5.0
18 cm) were connected to the top of a plastic column (length 31.0 cm) (Fig.
19 2). To minimize soil movement, the bottom of the column was covered
20 with glass wool. Experiments were performed for three cases:

21 1. Control 1: Saline-alkali soil only

22 2. Control 2: Saline-alkali soil + 2 wt % peat in the top 10 cm of the
23 column

1 3. Experimental treatment: Saline-alkali soil + 2 wt % peat + 1 wt %
2 SS-HA in the top 10 cm of the column.

3 For the second and third cases, freeze-dried SS-HA was re-dissolved
4 and evenly mixed into the saline-alkali soil with pure water. We
5 assumed that the liquid in the water-SS-HA mixture would have
6 immediately permeated through the top soil layer if the SS-HA had been
7 applied directly to the soil surface. Peat was also added in the second
8 and third cases in order to retain the SS-HA in the top 10 cm of the soil
9 column. Each of the three cases was replicated three times. Peat
10 contains HA, so the second treatment was undertaken as a control to
11 clearly identify the effect of SS-HA. The properties of the peat used
12 are provided in Table 2. The surface of the column was lit with a lamp
13 (57 W) for 8 h each day to accelerate evaporation during the experiment.
14 After 21 days, pure water was poured into the apparatus to cause
15 leaching. The upper surface of the soil column was then dried until
16 day 90, and then held at room temperature until day 157.

17 Holes at 10-cm intervals along the length of the soil column were used
18 to obtain samples for measurement of the variations of pH and EC with
19 depth (Fig.2). On days 1, 14, 21, and 157, pH and EC were measured
20 using a direct-reading electrode soil pH Tester 30
21 (Takemuradenkiseisakusho Co.) and a soil EC meter HI98331-S
22 (Takemuradenkiseisakusho Co.), respectively. These direct methods
23 were used so that the soil layer in the column would not be disturbed by

1 repeated collection of soil samples for pH and EC measurements.

2 To measure the exchangeable cation concentration (Na^+ , K^+ , Mg^{2+} ,
3 and Ca^{2+}) of the soil, 1.5-g soil samples were collected from the holes
4 in the side of the column (depths of 0, 10, 20, and 30 cm) on day 90,
5 air-dried and passed through a 2-mm sieve and preserved for analysis.
6 A 1-g subsample was extracted from the air-dried soil and shaken for 2
7 h in 10 ml of 0.5 M BaCl_2 , as described by the method of Kamewada *et*
8 *al.*.²³ Exchangeable cation concentrations were determined by ion
9 chromatograph DX-100 (DIONEX) after passing samples through a
10 0.45- μm filter paper.

11

12 Statistical analysis

13

14 The Student's *t*-test was used for comparison between two groups. To
15 compare three groups, statistical significance ($P < 0.05$) was evaluated
16 by one-way ANOVA, and if a significant difference was found, group
17 means were compared using Bonferroni's post hoc test.

18

19 **Results**

20

21 Extraction rate of humic acid

22

23 A comparison of the main properties of the ES and SS which are

1 resource of HAs are shown in Table 3. The results of our MLSS
2 measurement and water-quality analysis show the typical
3 characteristics of SS. The MLSS of SS was lower than that of ES and
4 the soluble fraction was higher (measured as TOC, TN, and carboxylic
5 acid). The HA extraction efficiency was enhanced by an increase of
6 the soluble fraction in response to solubilization (Table 4). The HA
7 recovery rates of ES and SS were 3.7% and 9.1% respectively. As
8 reference, previously reported HA and FA extraction rates from
9 Canadian *Sphagnum* peat (CP), from weathered coals of China, and
10 from soils are presented.^{24,25} The extraction rate obtained for SS-HA
11 approaches that of CP-HA.

12

13 Characterizations of ES-HA and SS-HA

14

15 Knowledge of the elemental composition of HS allows evaluation of the
16 role of those elements in building macromolecules of humus and
17 analysis (in simple terms) of the influence of the origin of humus on its
18 composition and structure. The elemental compositions of ES-HA and
19 SS-HA were similar and showed higher H and N contents than other HA
20 materials such as CP-HA (Table 5).

21 FT-IR spectra can be used to identify the presence of most of the
22 important functional groups in HS, to compare HS of different origins,
23 and to estimate the concentrations of functional groups in HS, and are

1 thus important for the development of structural models. The IR
2 spectra of HS from ES and SS are shown in Fig. 3 with that of CP shown
3 for comparison. These IR spectra were identified based on the study
4 by Stevenson and Goh.²⁶ The broad band at 3400–3200 cm^{-1} is
5 attributed to intermolecular OH stretching of carboxylic and alcoholic
6 groups in different electrostatic environments,²⁷ and the band at
7 2920–2850 cm^{-1} reflects aliphatic C–H stretching vibration, indicating
8 the presence of methyl and methylene groups. The shoulder at 1720
9 cm^{-1} , which is commonly attributed to C=O stretching vibration of
10 COOH groups. The band near 1620 cm^{-1} is attributed to structural
11 vibration of aromatic C=C, H-bonded C=O of quinones, or H-bonded
12 and conjugated ketones. The lack of absorption near 1540 cm^{-1} is
13 attributed to amide groups in the peptide linkages of protein. The
14 absorption band at 1400 cm^{-1} reflects C–H deformation of CH₃ and CH₂
15 groups. The broad adsorption band near 1230–1220 cm^{-1} represents
16 C–O stretching and OH deformation of carboxylic acids, alcohols, and
17 esters. Comparing the spectra of our samples to that of CP-HA, we
18 found some differences in the component ratios of functional groups:
19 the absorption at 2920 cm^{-1} was relatively stronger in ES-HA and
20 SS-HA. ES-HA and SS-HA showed stronger aliphatic characteristics
21 due to the different intensities of oxidation and humification processes
22 they had undergone. Absorption near 1540 cm^{-1} is attributed to amide
23 groups in the peptide linkages of protein. The spectra of ES-HA and

1 SS-HA show absorption of COOH at 1230 cm^{-1} .

2 NMR techniques provide an efficient tool to determine the
3 properties of humus. The $^1\text{H-NMR}$ spectra of HA and FA from ES and
4 SS are shown in Fig. 4. They showed peaks in the region 7.0–8.1 ppm
5 (aromatic H, including quinine phenol), stronger peaks at 3.3–4.6 ppm
6 (carbohydrate H), and weaker peaks in the region 0.8–3.3 ppm
7 (methylene and aromatic CH_2 , CH_3). For a more effective comparison,
8 the proton chemical shift has been quantitatively classified into four
9 regions (I to IV) representing protons in different chemical
10 environments (Table 6).^{28,29} The structure of SS-HA was shown to be
11 similar to that of ES-HA. The results of $^1\text{H-NMR}$ are consistent with
12 the results obtained from elemental analyses and FT-IR.

13

14 Batch experiment

15

16 Before the soil-column experiment, the pH buffering ability of SS-HA
17 was confirmed by batch test. SS-HA showed pH buffering ability in
18 an alkaline environment (Fig. 5), which suggested that application of
19 SS-HA would lower the pH of the saline-alkali soil. This was
20 confirmed, as the pH of the saline-alkali soil decreased as the amount
21 of SS-HA applied increased (Fig. 6). On the other hand, the EC
22 increased rapidly as the pH of the saline-alkali soil decreased.

23

1 Effect of humic substances on pH and EC in soil-column experiment

2

3 The high pH of alkali soil causes phosphate and metal ions to
4 precipitate and thus lowers their availability, causing a serious
5 imbalance in the supply of mineral nutrient ions in the root zone. This
6 may destroy the structure of roots, reducing their activity, and finally
7 causing a reduction or total loss of root function. Moreover, high pH
8 reduces the number of protons outside the roots, which impedes
9 establishment of a transmembrane proton gradient.³⁰ Because more
10 than half of the total root length of maize is within 20 cm of the soil
11 surface, and the maximum root densities of peanut are within the top 30
12 cm soil depth,³¹ and the column length in the soil-column experiment
13 was selected to be 31 cm.

14 The effect of SS-HA on the pH of the soil profile during the
15 soil-column experiments are shown in Fig. 7. For the cases where
16 peat+SS-HA and peat alone (control 2) were added to the saline-alkali
17 soil, the pH in the top 10 cm of the column was lower than that of
18 control 1 on days 1 and 14, and that for peat+SS-HA was lower than
19 that for peat alone. After leaching on day 21, the pH profile of the
20 column in which peat+SS-HA had been added was almost identical to
21 that of control 2 (peat alone), and remained so until day 157.

22 High salt levels, which are indicated by high EC, can reduce
23 water uptake by plants, restrict root growth, cause marginal burning of

1 foliage, inhibit flowering, limit seed germination, and reduce fruit and
2 vegetable yields.³² The changes of soil EC during the column
3 experiments are shown in Fig. 8. Although the batch tests showed that
4 as SS-HA concentrations increased, EC increased and pH decreased, EC
5 during the column experiment showed no significant difference among
6 the three experimental cases.

7

8 Effect of humic substances on exchangeable cations in soil-column
9 experiment

10

11 CEC provides a measure of the capacity of a soil for exchange of
12 cations between soils and soil solutions and gives an indication of the
13 amount and ratios of available plant nutrients in soil. Base saturation,
14 which is the fraction of exchangeable cations that are base cations (K^+ ,
15 NH_4^+ , Ca^{2+} , Mg^{2+}), is closely related to CEC. Therefore,
16 exchangeable cation (NH_4^+ , K^+ , Ca^{2+} , Mg^{2+} , and Na^+) concentrations on
17 day 90 of the soil-column experiments were conformed (Fig. 9).
18 Application of peat+SS-HA showed significantly greater NH_4^+
19 concentrations at the top and bottom of the soil column than was the
20 case for peat alone. The K^+ concentration was also greater in the top
21 layer after application of peat+SS-HA. Application of peat+SS-HA
22 had a significant affect on Na^+ and Ca^{2+} concentrations at the bottom of
23 the soil column. There were no notable differences of Mg^{2+}

1 concentrations after application of peat and peat+SS-HA. Moreover,
2 compared to a single treatment of peat, concentrations of trace element
3 Fe^{3+} were increased by addition of peat+SS-HA. Fe^{3+} concentration
4 after addition of peat alone was 7.5 mmol/kg, whereas addition of peat
5 + SS-HA gave a Fe^{3+} concentration of 9.7 mmol/kg (data not shown).

6

7 **Discussion**

8

9 ES solubilization techniques have been developed, including chemical
10 processes such as ozone oxidation and alkali treatment, physical
11 processes such as supercritical water oxidation and treatment with
12 ultrasonic waves, and aerobic biotreatments using specific
13 microorganisms.^{33,34} From the available solubilization techniques, we
14 chose to use a bead mill. Previously, we showed that sludge
15 solubilized by using a beads mill had a higher TOC concentration than
16 sludge solubilized by alkalization, microwaves, or boiling.¹⁵
17 Moreover, as a mechanical treatment, a beads-mill provides stable
18 solubilization results, and its operation and maintenance is simple.

19 The extraction rate of HA from SS was higher than that of ES-HA
20 (Table 4). The use of solubilization as a pretreatment for extraction
21 of HS increased the extraction efficiency for HA. The extraction rate
22 we achieved for SS-HA approached that of CP-HA.

23 HA can have a wide range of molecular weights and sizes,

1 ranging from a few hundred to several hundred thousand atomic mass
2 units, depending on its elemental composition, structure, and the
3 number and position of functional groups, which differ according to the
4 origin, method of extraction, and natural conditions prevailing during
5 the formation of the HA.^{35,36} The similarities among different HAs,
6 however, are greater than the differences. The physicochemical and
7 biological activities of HA also differ depending on the origin and
8 method of extraction of the HA. For example, HA extracted from peat
9 and that extracted from weathered coal have different physiochemical
10 effects on rice cultivated in a saline-alkali soil.³⁷ For this reason, we
11 investigated the structure of HA extracted from ES after solubilization
12 by using elemental analysis, FT-IR spectroscopy, and ¹H-NMR
13 spectroscopy, and compared it to the structure of HA extracted from ES
14 without solubilization. Then, for reference, we compared the
15 characteristics of SS-HA and ES-HA with those of CP-HA. The results
16 of elemental analysis, FT-IR, and ¹H-NMR showed that the extraction
17 efficiency of HA from ES was enhanced by solubilization, although
18 solubilization had little influence on the structural properties of the HA.
19 The elemental compositions of ES-HA and SS-HA showed higher H and
20 N contents than other HA materials such as CP-HA. The higher H
21 content of ES-HA and SS-HA was probably related to the stronger
22 aliphatic characteristics of these HAs (shown by the FT-IR results), and
23 their higher N content to less humification.

1 HA can form various complexes with metal ions and thus increase
2 the CEC of soils.^{3,4} CEC is the capacity of a soil to exchange cations
3 between the soil and the soil solution, and it is used as a measure of the
4 soil's fertility and nutrient retention capacity. Carboxylate and
5 phenolic groups are primarily responsible for the binding of metals
6 under most natural conditions.³⁸ Our structural analysis showed that
7 SS-HA contains a number of acid functional groups such as the
8 carboxylate and phenolic groups. We measured carboxylate groups'
9 content and CEC of SS-HA for conformation, and those results were
10 compared with that of previous research results.^{39,40} It became clear
11 that carboxyl group exist in SS-HA, and they have cation exchange
12 capacity, although those concentrations were not high compared to
13 CP-HA (Table 7).

14 Prior to column experiment, pH buffering ability of SS-HA was
15 confirmed by batch test. As shown in Fig. 5, SS-HA had pH buffering
16 ability on alkali area. From this result, it was expected that the pH of
17 the saline-alkali soil decreased by SS-HA application. Then, effects
18 of SS-HA application on the pH of the saline-alkali soil were confirmed.
19 As the results, the pH of the saline-alkali soil decreased
20 dose-dependently by SS-HA application (Fig. 6).

21 Our soil-column experiment, we confirmed the amelioration
22 effects of SS-HA (Fig. 7). The water potential gradient is affected by
23 gravitational potential, capillary potential, and osmotic potential. In

1 control 1 (soil alone), the vapor phase gradually reached equilibrium
2 with the liquid phase of soil water. While, soil additives peat+SS-HA
3 reduced the pH of the saline-alkali soil, and the effect of additional
4 SS-HA on the pH reduction was faster or immediate observed compared
5 with control 2 (peat alone). After leaching (from 21 to 157 days), the
6 pH of the experimental treatment was not different from that of control
7 2. Thus, application of SS-HA to saline-alkali soil enhanced the pH
8 reduction achieved by the application of peat. The more immediate
9 pH reduction achieved with SS-HA treatment may improve crop
10 germination. It was considered that the mechanism by which the
11 saline-alkali soil was ameliorated by addition of SS-HA was as follows.
12 Carboxyl groups in SS-HA released H^+ , which neutralized the OH^- in
13 the soil solution, thus decreasing soil pH (Fig. 10A). Negatively
14 charged carboxyl groups adsorbed cations from the soil solution,
15 causing the EC of the soil solution to decrease. However, the results
16 of the batch and soil-column experiments were unexpected.
17 Kawakami *et al.*⁸ reported that the EC of saline-alkali soils tends to
18 increase in response to the application of peat. They showed that
19 calcium and magnesium ions, which are poorly soluble in alkaline
20 solution, were released from the soil and dissolved under low pH
21 conditions. However, application of peat+SS-HA in our experiments
22 produced a significant increase in exchangeable cations in the
23 saline-alkali soil by their CEC (Fig. 9). An important property of

1 cations is their attraction to surfaces carrying a negative charge, upon
2 which they adsorb strongly (Fig. 10B). Compared with the results
3 for addition of peat alone, the added SS-HA produced a significant
4 increase in NH_4^+ concentrations at the top and bottom of the soil
5 column. This occurred because the high-N component of SS-HA was
6 broken down to form NH_4^+ . The K^+ concentration also increased in the
7 top layer of the column after application of SS-HA. NH_4^+ , K^+ , and
8 Ca^{2+} are especially important nutrients for plant growth. There was no
9 noticeable change in the Mg^{2+} concentration at the top or bottom of the
10 column after application of either peat alone or peat+SS-HA.
11 Application of peat+SS-HA significantly affected the Ca^{2+}
12 concentration at the bottom of the column. Fe availability in
13 calcareous soils is very low because of the high soil pH of such soils,
14 which reduces the solubility of ferric iron. Our results also show that
15 application of SS-HA to the saline-alkali soil increased Fe content,
16 which is a trace element for plant growth.

17

18 **Conclusion**

19

20 The application of SS-HA was clearly shown to have a beneficial effect
21 by improving the physicochemical properties of the saline-alkali soil.
22 The SS-HA reduced the soil pH, and increased the concentrations of
23 exchangeable cations in the soil. Naturally, higher concentration of

1 exchangeable base cations allow, more acidity to be neutralized and in
2 a shorter time. HS are abundant in peat, weathered coal, and other
3 humified substances. However, exploitation of wetlands as a source
4 of peat has recently been severely restricted by the Ramsar
5 Convention.⁴¹ By comparison, ES is industrial waste that is widely
6 and continually produced, and thus has the potential to be an
7 environmentally sustainable source of HS. We hope that this research
8 will contribute to the growth of the recycling society in the future to
9 minimize raw material consumption and the amount of waste disposal in
10 the environment.

11

12 **Acknowledgments** This research was partially supported by the
13 JST-JAICA Science and Technology Research Partnership for
14 Sustainable Development program (SATREPS).

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1 **References**

- 2 1. Y. Inbar, Y. Chen, Y. Hadar (1990) Humic substances formed during
3 the composting of organic matter, *Soil Sci. Soc. Am. J*
4 54:1316-1323
- 5 2. Stevenson F J (1982) *Humus chemistry*. New York: J. Wiley & Sons,
6 p. 443
- 7 3. Suganya S, Sivasamy R (2006) Moisture retention and cation
8 exchange capacity of sandy soil as influenced by soil additives. *J.*
9 *Appl. Sci. Res* 2:949-951
- 10 4. Stevenson FJ (1994) *Humus chemistry: Genesis, composition,*
11 *reactions*. 2nd. John Wiley & Sons, New York pp 432-434
- 12 5. Maggioni A, Varanini Z, Nardi S, Pinton R (1987) Action of soil
13 humic matter on plant roots: Stimulation of ion uptake and effects
14 on (Mg²⁺⁺ K⁺) ATPase activity. *Sci. Total Environ* 62:355-363
- 15 6. Albuzio A, Dell'Agnola G, Dibona D, Concheri G, Nardi S (1993)
16 Humic constituents of forest soils as plant growth regulating
17 substances. In: Paoletti MG, Foissner W, Coleman DC (ed) *Soil*
18 *biota, nutrient cycling, and farming systems*. Lewis Publ, Boca
19 Raton, FL pp. 15–257. Nardi S, Pizzeghello D, Muscolo A,
20 Vianello A (2002) Physiological effects of humic substances in
21 higher plants. *Soil Biol. Biochem.* 34:1527-1537
- 22 8. Kawakami H, Niijima Y, Wang Z (2002), Change in
23 physicochemical properties of the saline alkali soil through

- 1 improvement of the soil by applying peat, Part 1. pH (in Japanese).
2 Journal of Japan Peat Society 1:16-24
- 3 9. Kawakami H, Niijima Y, Yoshida A, Wang Z, Ota Y (2003) Effect
4 of peat applied to the desert soil of Xinjiang, China, on the growth
5 of wheat, Part 1. Pot culture (in Japanese). Journal of Japan Peat
6 Society 2:16-25
- 7 10. Kuma K, Tanaka J, Matsuyama K (1999) Effect of natural and
8 synthetic organic-Fe (III) complexes in an estuarine mixing model
9 on iron uptake and growth of a coastal marine diatom. *Chaetoceros*
10 *Sociale*, Mar. Biol 134:761–769
- 11 11. Zhilin DM, Schmitt-Kopplin P, Perminova VI (2004) Reduction of
12 Cr (VI) by peat and coal humic substances, *Environ Chem Lett*
13 2:141–145
- 14 12. Tachibana Y, Hiribe S, Tawa R (2004) Studies of antioxidative
15 activity of humic substances in peat (1) (in Japanese). *Trace*
16 *Nutrients Research* 23:104-108
- 17 13. Yang HL, Hseu Y, Lu FE, Lin JL (2004) Humic acid induces
18 apoptosis in human premyelocytic leukemia HL-60 cell, *Life*
19 *Sciences* 75:1817-1831
- 20 14. Igor AS, Andrei IK, Shin YAH, Sang BW, Choon SJ, Olesya NK,
21 Byong SK (2003) Characterization and biological activity of humic
22 substances from mumie. *J. Agric. Food. Chem.* 51:5245-5254
- 23 15. Motojima H, Ozaki M, Shibata T, Abe Y, Isoda H (2005) Excess

- 1 Sludge Treatment using Solubilization. TJASSST' 6, November
2 7-12, Tunisia, Sousse, The Society of Borj Cedria Science and
3 Technology Park, Tunisia and The Alliance for Research on North
4 Africa (ARENA), University of Tsukuba, Japan Organize Sousse,
5 94-95
- 6 16. Schmitt P, Kettrup A, Freitag D, Garrison AW (1996) Flocculation
7 of humic substances with metal ions as followed by capillary zone
8 electrophoresis. *Fresenius J. Anal. Chem* 354:915
- 9 17. Nagao M (1995) Characteristics of humic substances and
10 TRU-humate complexes in groundwater (in Japanese).
11 *Houshyaseihaikibutsukenkyu* 1:231-242
- 12 18. Motojima H, Yamada P, Han J, Ozaki M, Shigemori H, Isoda H
13 (2009) Properties of Fulvic Acid Extracted from Excess Sludge and
14 Its Inhibiting Effect on β -Hexosaminidase Release. *Biosci.*
15 *Biothechnol. Biochem* 73:2210-2216
- 16 19. Motojima H, Villareal M. O, Han J and Isoda H (2011) Microarray
17 Analysis of Immediate-type allergy in KU812 Cells in Response to
18 Fulvic Acid. *Cytotech* 63(2):181-190
- 19 20. Ikeya K, Kawahigashi M, Sumida H, Tsutsuki K, Nagao S,
20 Fukushima M, Fujitake N, Maie N, Yamamoto S, Watanabe A
21 (2007) *Genso anteidouitaibunseki*. (in Japanese) In: Watanabe A,
22 Fujitake N, Yamamoto S (ed) *Fushokubushitsu Bunseki*
23 *Handobukku*, Sankensha. Tokyo, pp 64-67

- 1 21. National Institute for Agro-Environmental Science Japan (1992)
2 Youion koukanyouryou (in Japanese). In: Kubota et al. (ed)
3 Hiryoubunsekihou, Japan fertilizer and feed inspection association,
4 Tokyo, pp 148-154
- 5 22. Yazawa Y, Shinoda Y, Yazaki F, Yamaguchi T (1997) Controlling
6 permeability and salinity in sandy soils with ammonium humate. J
7 Arid Land Studies 7:23-33
- 8 23. Kamewada K (1997) Dojoukagaku (in Japanese). In:
9 Dojoukankyoubunsekeihou editorial comity (ed)
10 Dojoukankyoubunsekihou, Hakuyoushya, Tokyo, pp 215-222.
- 11 24. Yamada P, Akiba T, Yazawa Y, Yamaguchi T (2002) Characteristics
12 of humic and hulvic acids extracted from weathered coal of China
13 (in Japanese). Nihonlagakukaishi, 3: 351-357
- 14 25. Watanabe A (2007) tyu-shutu/seiseihou. In: Watanabe A., Fujitake
15 N and Ngao S, Sankeisya (eds.) Fusyokubusitubunsekihandobukku.
16 Sankeisha, Nagoya Japan, pp.8-13.
- 17 26. Stevenson F.J, Goh KM (1971) Infrared spectra of humic acid and
18 related substances. Geochim. Cosmochim. Acta 35:471-483
- 19 27. Bellamy, J (1975) The infrared spectra of complex molecules.
20 Chapman and Hall, London
- 21 28. Asakawa D, Mochizuk H, Yanagi Y, Suzuki T, Nagao S, Fujitake N
22 (2006) Effects of operational conditions for extraction and sample
23 storage on the structural properties of water-extractable humic

- 1 substances in soil. *Humic Substances Research* 3:15-24
- 2 29. Kang K, Shin HS and Park H (2002) Characterization of humic
3 substances present in landfill leachates with different landfill ages
4 and its implication. *Water Res* 36:4023-4032
- 5 30. Guo R, Shi L and Yang T (2009) Germination, growth, osmotic
6 adjustment and ionic balance of wheat in response to saline and
7 alkaline stresses. *Soil Sci Plant Nutr* 55:667-679
- 8 31. Fageria NK, Baligar VC, Ralph B, Clark R, Clark B. (2006), Root
9 distribution in soil. In: N. K. Fageria, V. C. Baligar, R. B. Clark
10 (ed) *Physiology of crop production (crop science)*, Routledge, New
11 York, pp 32-33
- 12 32. Colorado State University Extension, *Managing Saline Soils*,
13 <http://www.ext.colostate.edu/pubs/crops/00503.pdf> (2009).
- 14 33. Kim S, Kramer RW, Hatcher PG (2003) Graphical method for
15 analysis of ultrahigh-resolution broadband mass spectra of natural
16 organic matter, the Van Krevelen diagram. *Anal. Chem*
17 75:5336-5344
- 18 34. Stendahl K and Järfverström (2003) Phosphate recovery from sewage
19 sludge in combination with supercritical water oxidation. *Water Sci*
20 *Tech* 48:185-191
- 21 35. Kam SK and Gregory J (2001) The interaction of humic substances
22 with cationic polyelectrolytes. *Water Res* 35:3557-3566
- 23 36. Norden M and Dabek-Zlotorzynska E (1996) Study of metal-fulvic

- 1 acid interactions by capillary electrophoresis. *J Chromatogr A* 739:
2 421-429
- 3 37. Yamada P, Oosawa N, Yazawa Y, Nishizaki Y and Yamaguchi T
4 (2002) Improvement of physicochemical properties of alkali soil by
5 humic materials: addition effect of the local peat and weathered
6 coal on the paddy field rice cultivation on the saline arid land in
7 Keerquin desert, China (in Japanese). *Journal of Arid Land Studies*,
8 12:203-213
- 9 38. Ouattmane A, Hafidi M, Gharous MEI, Revel JC (1999)
10 Complexation of calcium ions by humic and fulvic acids Analysis.
11 *Humic substances* 27:428-431
- 12 39. Yamaguchi T, Hayashi H, Yazawa Y, Uomori M, Yazaki F,
13 Bambalov NN (1998) Comparison of basic characteristics of humic
14 acids extracted from peats and other sources. *Int Peat J* 8:87-94
- 15 40. Yamada P, Isoda H, Han JK, Talorete TPN, Yamaguchi T, Abe Y
16 (2007) Inhibitory effect of fulvic acid extracted from Canadian
17 sphagnum peat on chemical mediator release by RBL-2H3 and
18 KU812 cells. *Biosci Biotechnol Biochem* 71:1294-1305
- 19 41. Matthews GVT (1993) The Ramsar convention on wetlands: its
20 history and development, In: Erika Luthi Ramsar (ed), *Ramsar*
21 *Convention Bureau*, Gland, Switzerland, p. 122

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1 **Figure legend**

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3 **Fig. 1.** Laboratory equipment used for solubilization of excess sludge

4

5 **Fig. 2.** Apparatus used for the saline-alkali soil-column experiment

6 Soil additives were mixed in the top 10-cm of the soil. The surface of
7 the column was exposed to heat from a 57-W lamp for 8 h each day to
8 promote evaporation.

9

10 **Fig. 3.** FT-IR spectra of humic acid extracted from non-solubilized

11 excess sludge (ES-HA) and solubilized excess sludge (SS-HA)

12 CP-HA, humic acid extracted from Canadian *sphagnum* peat

13

14 **Fig. 4.** $^1\text{H-NMR}$ spectra of humic acid extracted from excess sludge

15 without solubilization (ES-HA) and humic acid extracted from

16 solubilized excess sludge (SS-HA)

17

18 **Fig. 5.** pH buffer curves of SS-HA obtained by batch tests

19 The pH buffering capacity of the model soil after application of
20 different amounts of SS-HA was determined by as the number of moles
21 of strong acid or base needed to produce a unit change of pH of the
22 model soil.

23

1 **Fig. 6.** Effects of SS-HA on pH and EC in a saline-alkali soil
 2 determined by batch tests (3 days after addition of SS-HA)

3

4 **Fig. 7.** Effects of humic substances on pH of the saline-alkali soil in
 5 the column experiments

6 (■) Peat (2 wt %) added in the top 10-cm of soil; (□) peat (2 wt%) +
 7 SS-HA (1 wt %) added in the top 10-cm of soil (peat 2%/soil plus
 8 SS-HA 1%/peat); (○) control (without humic substances added).

9 Statistical significance ($P < 0.05$) was evaluated by one-way ANOVA,
 10 and then, group means that were significantly different were compared
 11 using Bonferroni's post hoc test. $*P < 0.05$, peat alone and
 12 peat+SS-HA versus control 1; $**P < 0.01$, peat alone and peat+SS-HA
 13 versus control 1; ^aSignificant difference between SS-HA+peat and peat
 14 alone (control 2).

15

16 **Fig. 8.** Effect of humic substances on EC of the saline-alkali soil in
 17 the column experiments

18 (■) Peat (2 wt %) added in the top 10-cm of soil; (□) peat (2 wt %) +
 19 SS-HA (1 wt %) added in the 10-cm of soil; (○) control (without humic
 20 substances added). Statistical significance ($P < 0.05$) was evaluated
 21 by one-way ANOVA and then, group means that were significantly
 22 different compared using Bonferroni's post hoc test. $*P < 0.05$, peat
 23 alone and peat+SS-HA versus control 1; $**P < 0.01$, peat alone and

1 peat+SS-HA versus control 1.

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3 **Fig. 9.** Effects of humic substances on exchangeable cation
4 concentrations on day 90 of the column experiments

5 $P < 0.05$, peat+SS-HA versus peat alone.

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7 **Fig. 10.** Mechanism of amelioration of saline-alkali soil by addition
8 of humic substances extracted from solubilized excess sludge

9 (A) pH reduction. (B) Increase of exchangeable cation concentration.

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Table 1. Properties of the experimental soil (model saline-alkali soil)

(1) Physical properties

| ρ_b | Particle size distribution | | | | |
|----------|----------------------------|--------------------|-----------------------|------------------------|----------------|
| | Clay ~0.002mm | Silt 0.002~0.02 | Fine sand 0.02~0.2 | Coarse sand 0.2~2.0 | Gravel 2.0~ |
| g/cm | % | | | | |
| 1.54 | 1.00 | 0.00 | 13.47 | 85.53 | 0.00 |

(2) Chemical properties

| pH ¹⁾ | Ash | EC ²⁾ | CEC | ESP ³⁾ | Exchangeable bases | | | |
|------------------|-------|------------------|---------|-------------------|--------------------|----------------|------------------|------------------|
| | | | | | Na ⁺ | K ⁺ | Ca ²⁺ | Mg ²⁺ |
| | % | mS/cm | cmol/kg | % | cmol/kg | | | |
| 8.81 | 99.35 | 3.69 | 12.02 | 54.10 | 6.50 | 0.99 | 2.97 | 4.28 |

1) Soil-water suspension (2:5) 2) Soil-water suspension (1:5)

3) ESP, exchangeable_sodium_percentage (Na⁺ / CEC)

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Table 2. Properties of the peat

| Property | Value |
|----------------------------------|-------|
| Moisture content (% dry w/w) | 64.0 |
| Ash | 1.5 |
| Elemental composition (% d.a.f.) | |
| C | 48.0 |
| H | 5.8 |
| N | 0.5 |
| O | 45.8 |
| Extraction rate (% d.a.f.) | |
| Humic acid | 3.6 |
| Fulvic acid | 5.2 |

d.a.f., dry ash free

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Table 3. Main Properties of non-solubilized excess sludge (ES) and solubilized excess sludge (SS)

| Sample | Sludge concentration | TOC | IC | TN | Acetic acid | Propionic acid |
|--------|-------------------------|--------|------|-------|------------------------------------|----------------|
| | | (mg/l) | | | (mg-soluble fraction/g-dry sludge) | |
| ES | 4200 | 5.2 | 13.6 | 8.8 | ND | ND |
| SS | 3108 | 547.5 | 38.0 | 144.5 | 11.4 | 3.5 |

Each value of represents the mean of two measurements.

TOC, total organic carbon; IC, inorganic carbon; TN, total nitrogen; ND, not detected

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Table 4. Extraction rates of humic acid from excess sludge (ES) and solubilized excess sludge (SS) compared with rates reported in the literature

| | HA | |
|--------------------------------|------|---|
| | % | Reference |
| ES | 3.7 | |
| SS | 9.1 | |
| <i>Sphagnum</i> peat (Canada) | | |
| | 12.8 | |
| Weathered coal (Menggu, China) | 26.4 | Yamada <i>et al.</i> , 2002 ²⁴ |
| Weathered coal (Urumqi, China) | 7.2 | |
| Inokashira soil (Japan) | 2.1 | |
| Dando-soil (Japan) | 1.5 | Watanabe <i>et al.</i> , 2007 ²⁵ |

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Table 5. Composition of humic acid extracted from excess sludge (ES) and solubilized excess sludge (SS) compared with values from the literature

| Sample | Ash (% dry w/w) | Elemental composition (% d.a.f.) | | | | | | |
|-------------------------------|--------------------|----------------------------------|-----|-----|-----|------------|-----|-----|
| | | C | H | N | S | O | H/C | O/C |
| Humic acid | | | | | | | | |
| ES | 1.9 | 47.2 | 7.0 | 9.4 | 1.4 | 35.1 | 1.8 | 0.6 |
| SS | 2.1 | 50.6 | 7.4 | 8.2 | 1.9 | 31.9 | 1.8 | 0.5 |
| <i>Sphagnum</i> peat (Canada) | 1.0 | 56.5 | 5.1 | 1.9 | — | 36.6 (O+S) | 1.1 | 0.5 |
| Weathered coal (Menggu China) | 11.0 | 59.8 | 3.7 | 1.1 | — | 35.4 (O+S) | 0.7 | 0.4 |
| Weathered coal (Urumqi China) | 2.1 | 62.5 | 4.7 | 2.9 | — | 29.9 | 0.9 | 0.4 |
| Inokashira soil (Japan) | — | 54.8 | 4.3 | 4.0 | 0.3 | 36.6 (O+S) | 0.9 | 0.5 |
| Dando-soil (Japan) | — | 53.0 | 5.3 | 4.5 | 0.3 | 37.0 | 1.2 | 0.5 |

—, Not decided

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Table 6. Hydrogen distribution calculated from ^1H -NMR results for humic acid extracted from excess sludge (ES) and solubilized excess sludge (SS)

| Chemical shift region δ (ppm) | Assignment | Relative contribution (%) | |
|--------------------------------------|---|---------------------------|-------|
| | | ES-HA | SS-HA |
| I. (0.4-1.7) | Terminal CH_3 , CH_2 , and CH of methylene chains, etc | 28.55 | 30.93 |
| II. (1.7-3.3) | CH_3 , CH_2 , and CH proton α to aromatic or carboxyl groups, etc | 25.40 | 23.34 |
| III. (3.3-4.6) | Protons on carbon α to oxygen, carbohydrate, etc | 36.06 | 32.85 |
| IV. (6.5-8.1) | Aromatic protons (including quinone, phenol, etc) | 9.99 | 12.88 |

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Table 7. Carboxyl group content and cation exchange capacity of humic substances extracted from solubilized excess sludge (SS) compared with values from the literature

| | | CEC | Carboxyl group (cmol/kg) | Reference |
|----|---------------------------|-----|-----------------------------|--|
| HA | SS | 140 | 98 | |
| | Sphagnum peat (Canada) | — | 192 | |
| | Reed-sedge peat (Belarus) | — | 186 | Yamaguchi <i>et al.</i> , 1998 ³⁹ |
| | Reed peat (Japan) | — | 68 | |
| | Woody peat (Indonesia) | — | 25 | |
| FA | SS | 175 | 159 | |
| | Sphagnum peat (Canada) | — | 398 | Yamada <i>et al.</i> , 2007 ⁴⁰ |

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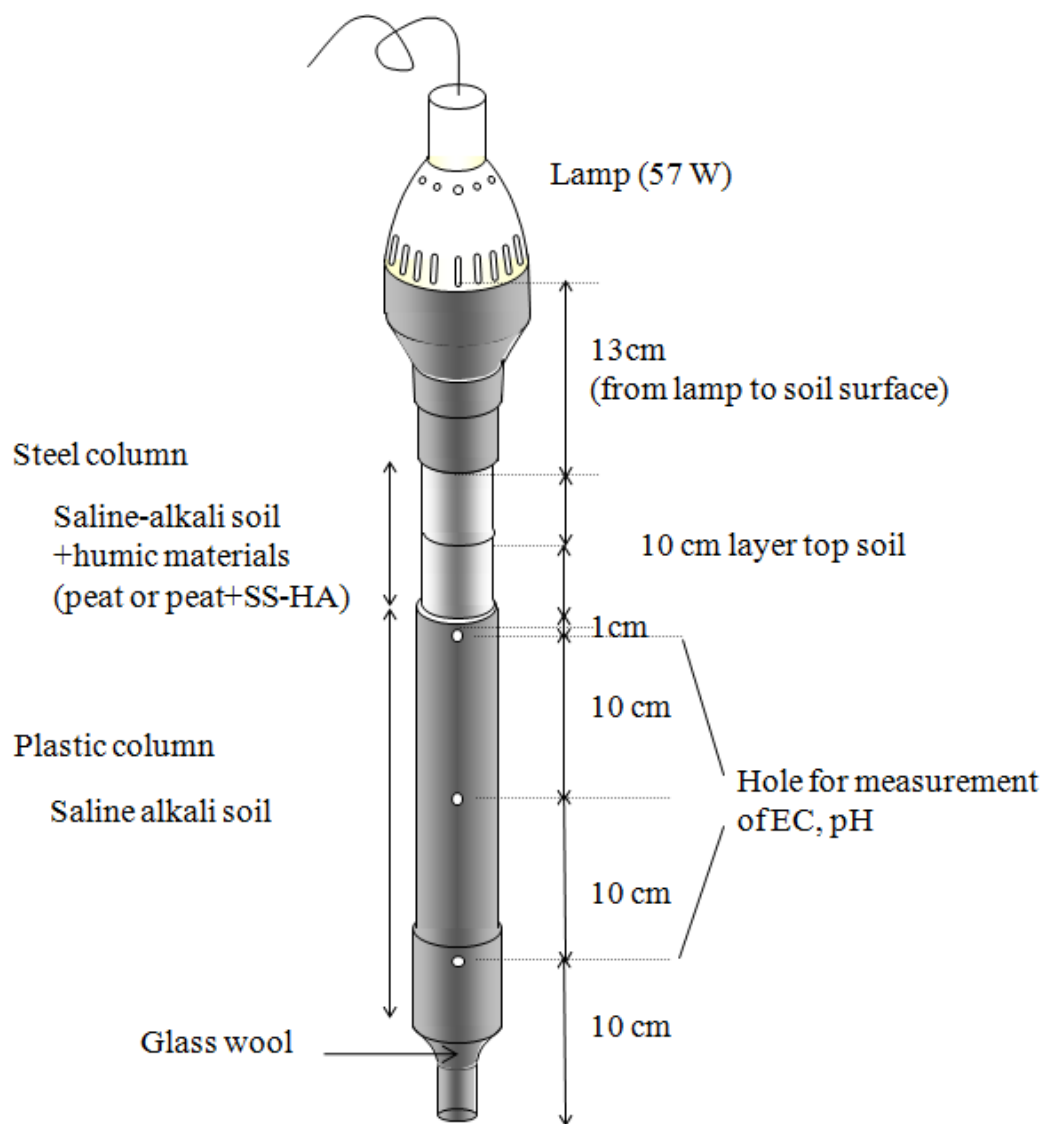
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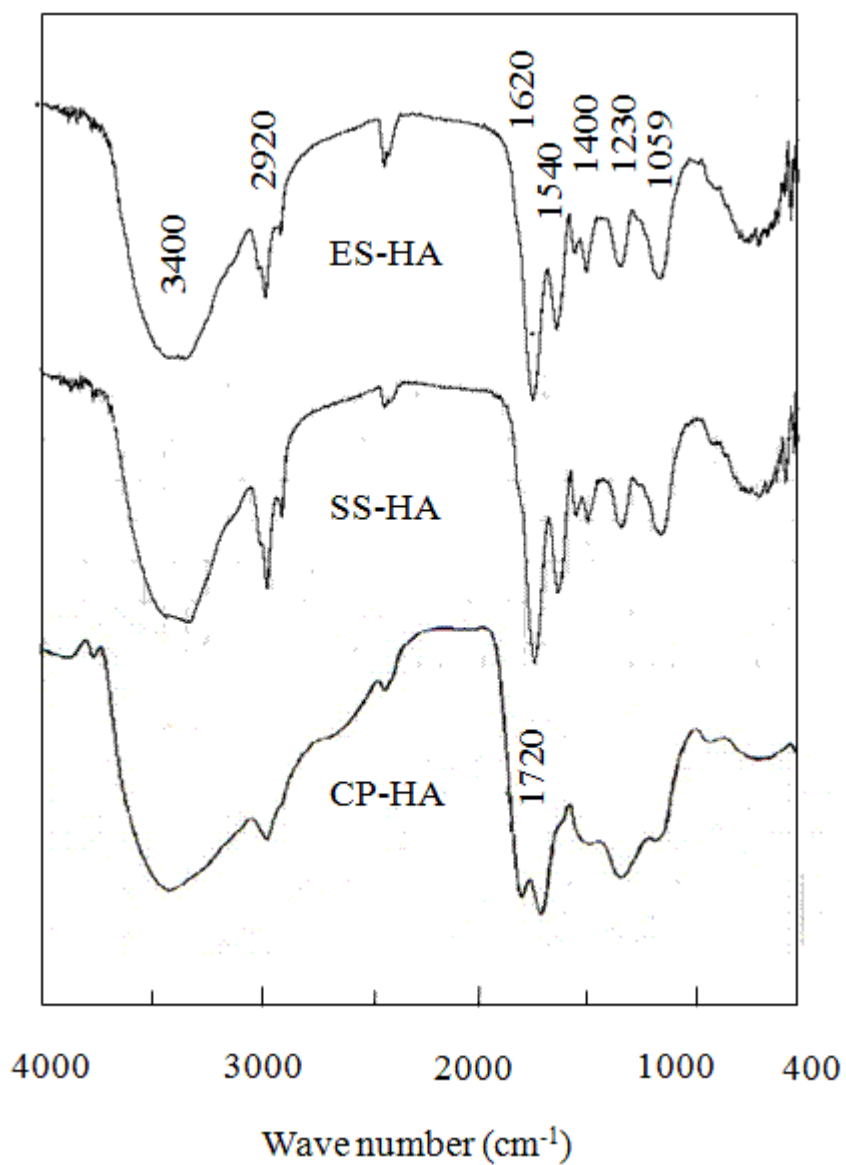


Fig 3

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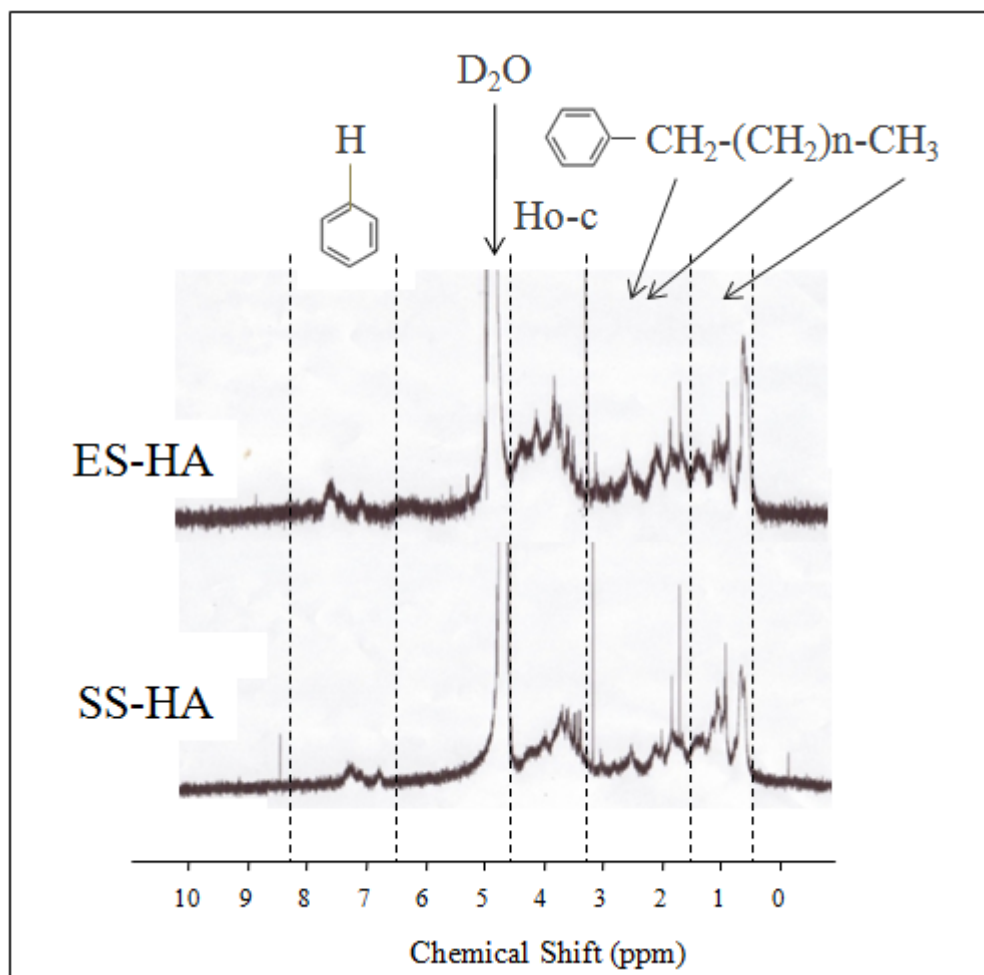


Fig 4

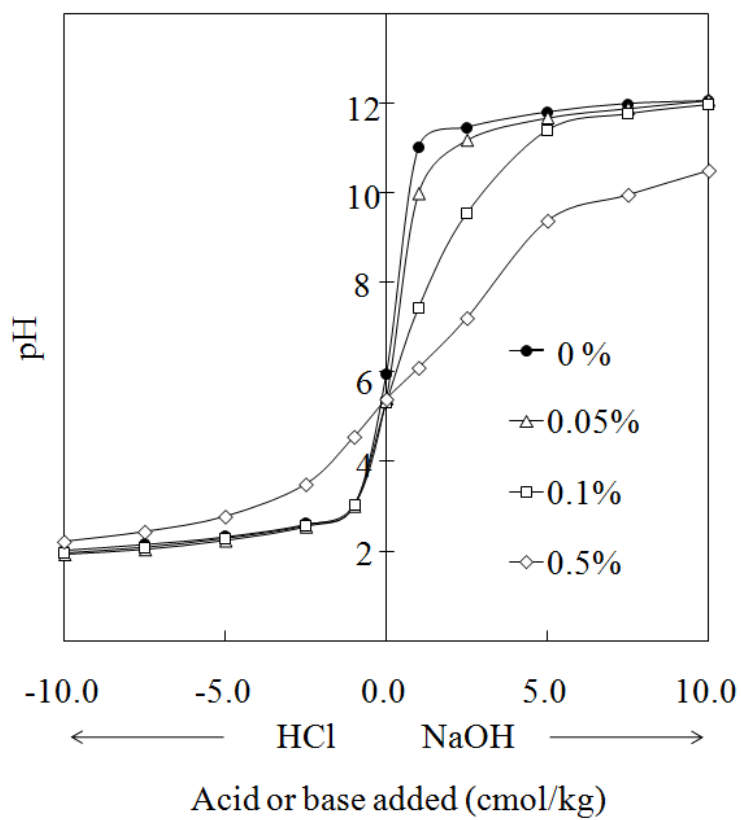


Fig 5

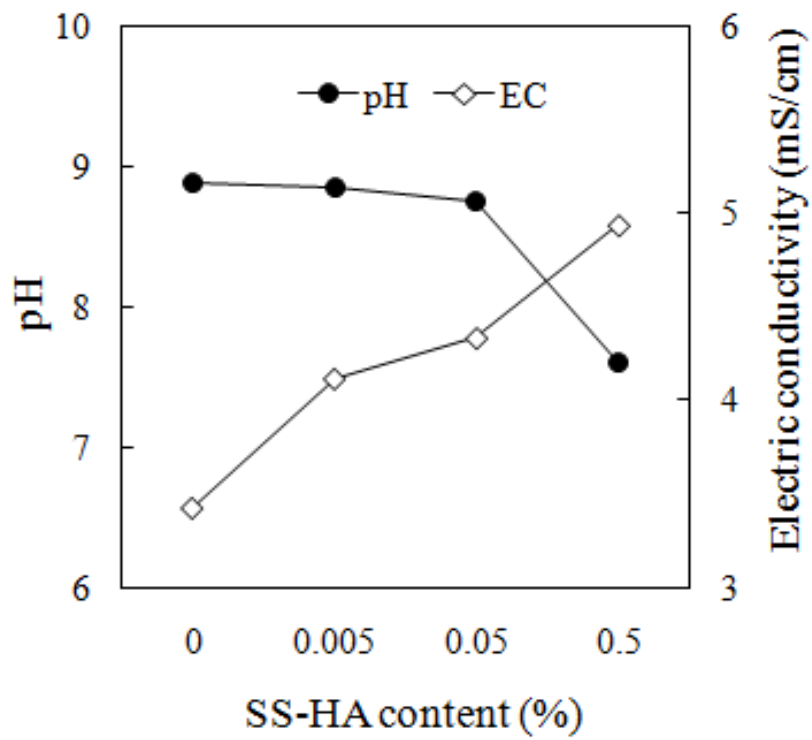
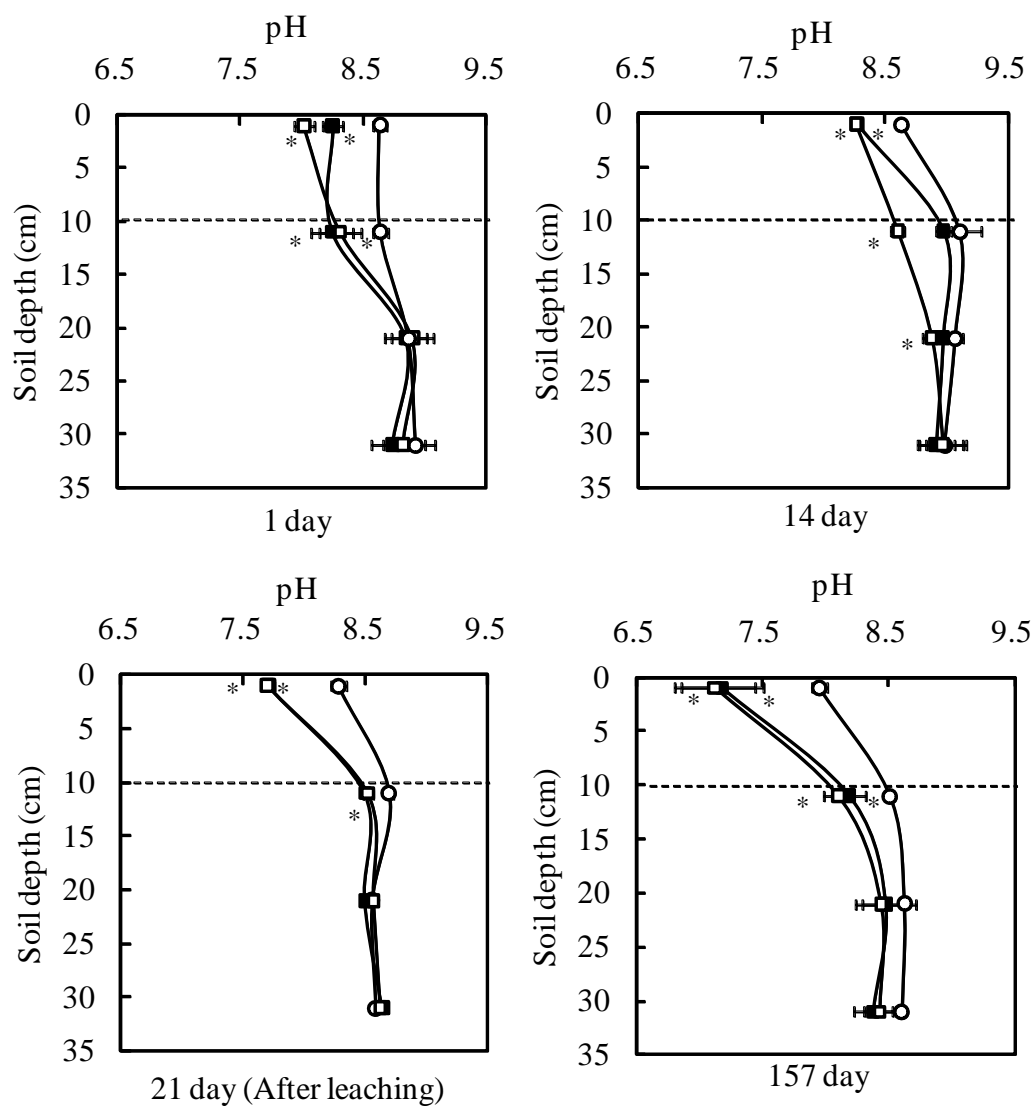


Fig 6

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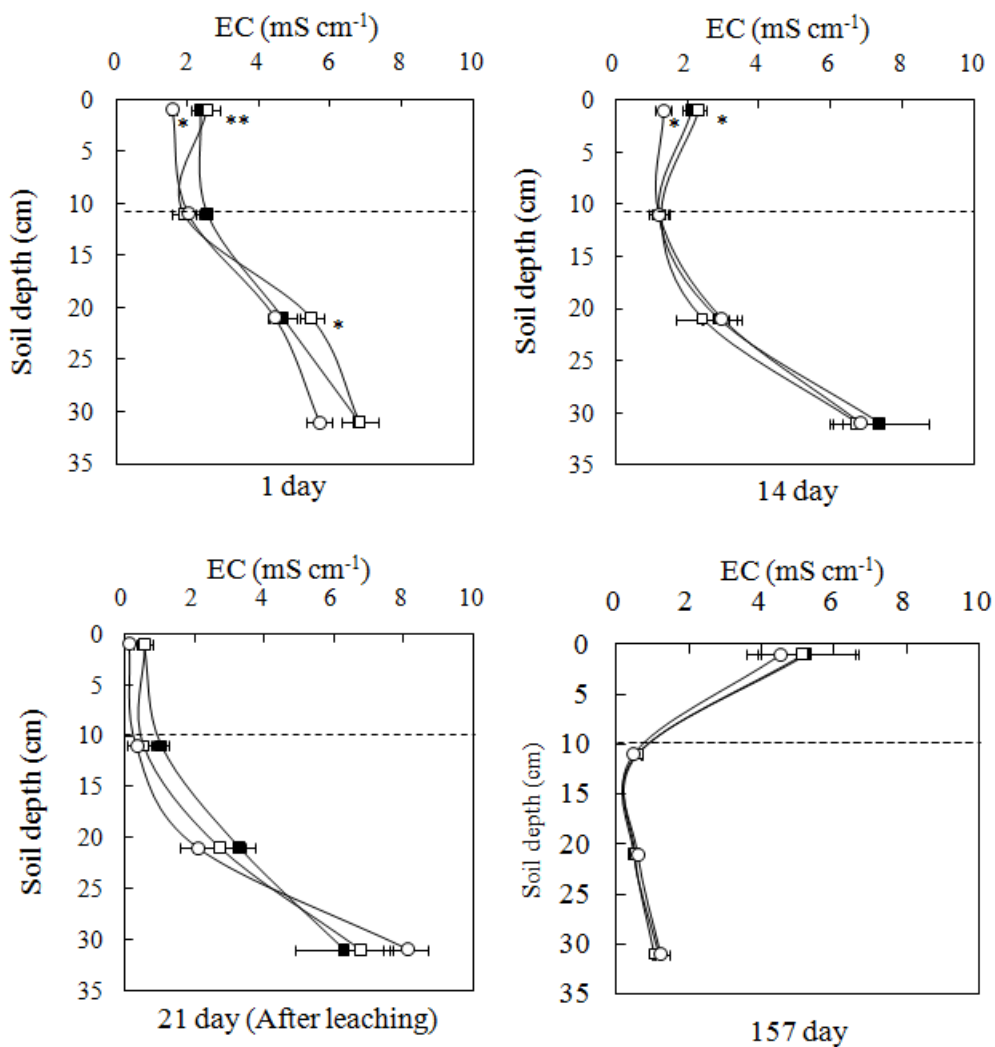
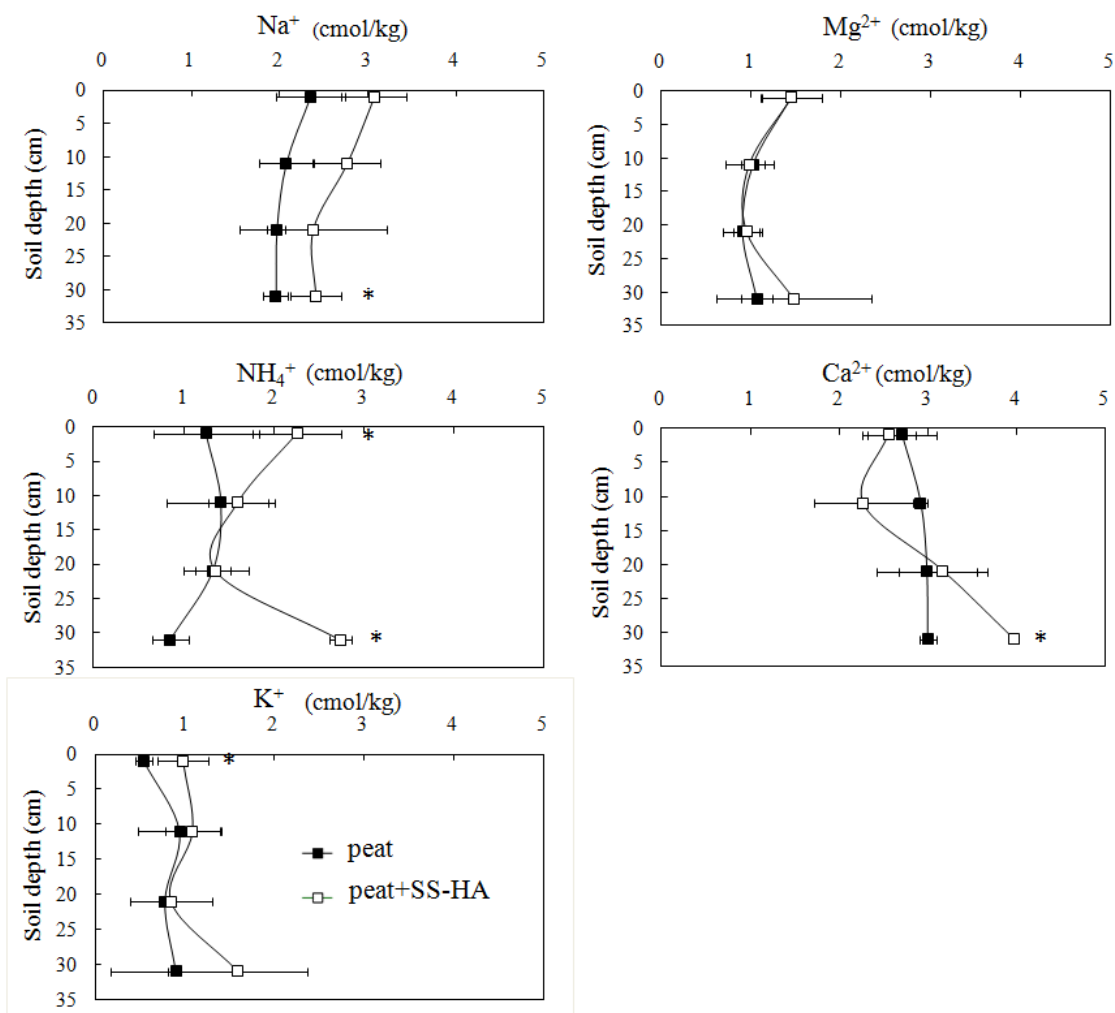


Fig 8

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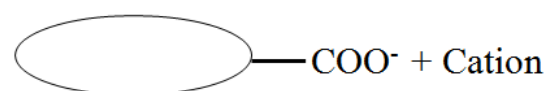
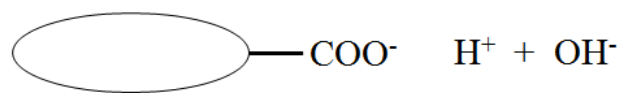
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8 Fig 9

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(A)



(B)

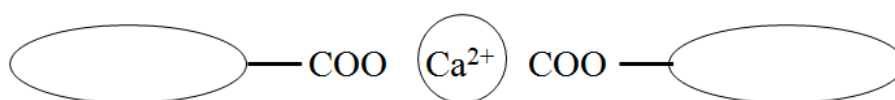


Fig 10