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Prospects for nitrogen incorporation into humic acid as evidenced by alkaline extraction method

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Nitrogen-fixation is an important process in nature, transforming atmospheric N₂ into biological forms either via enzymatic pathways by nitrogen fixing microorganisms^{1,2} or by chemical methods using the Haber–Bosch process in industry^{3,4}. However, other pathways contributing to the global nitrogen cycle may exist. We made an attempt to show that the nitrogen content in humic acid extracted under air and pure N₂ gas increased compared with extraction under argon gas. ¹⁵N abundance in humic acid was increased to 1.7‰ by ¹⁵N₂ gas tracer measurement. Further evidence for the actual bond formation between nitrogen and humic acid was determined by FTIR spectroscopy. In addition, structural analyses using ¹³C nuclear magnetic resonance with cross polarization, magic angle spinning (CPMAS ¹³C NMR) and UV absorbance revealed structural changes in aromatic and aliphatic properties of the humic acid structure. Hence we envisage that alkaline humic acid might be undergoing a novel non-enzymatic N₂-fixation when extracted with air and N₂ through an unknown mechanism that consumes free radicals in the humic acid structure.

HUMIC substances are complex polymers originated from chemical and biological degradation of plant, animal residues and microorganisms and may contain 2–6% of elemental nitrogen^{5,6}. Humic acid is considered as a high quality growth substance for agricultural purposes⁷. Approaches towards characterizing N-content in humic substances utilize acid/base hydrolysis to separate nitrogen into acid insoluble-N, NH₃-N, amino acid-N, amino sugar-N, and hydrolysable

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unknown-N⁸. Variable amounts of polyamines, putrescine, spermidine and spermine were also identified in the hydrolysates of humic acid that are attributed to the hormone-like activity of humic substances⁹. Evidence for the formation of heteroaromatic nitrogen after prolonged humification of vascular remains has been recently documented¹⁰.

We found that the gas volume in plastic bottles when filled with alkaline humic acid solution sealed under air, was reduced by 39% (Figure 1 *a*, left) and 100% (Figure 1 *a*, right) after two years. Either nitrogenase activity or free-living, N₂-fixing bacteria were detected in the alkaline humic acid solution¹¹. These visible observations signify that N₂ gas might have been absorbed by the alkaline humic solution in the sealed bottle, since air contains over 70% N₂. Hence to understand the salient disappearance of N₂ in the alkaline humic acid, we proposed to study the composition and structural changes of peat humic acid extracted by the alkaline method under air, N₂ gas and argon (Ar) atmosphere in a unique reaction chamber (Figure 1 *b*). In addition, the alteration of free radicals during this process was also assessed.

Extraction and purification of humic acid under air, N₂ and Ar atmosphere were carried out as follows. A peat soil sample (pH 4.2; peat:H₂O = 1:10, w/v) was collected from Indonesia. This contained 52.3% C, 6.0% H, 36.7% O, 1.8% N and 3.2% ash. Humic acid was extracted from the peat samples and purified according to the main guidelines of the International Humic Substances Society (IHSS)¹². Three equilibrium suspensions of 2 g in 200 ml 0.1 M NaOH solution were prepared and each bubbled either

with N₂, Ar or air for 6 min with flow rate of 200 ml min⁻¹. The suspension in a centrifuge bottle (Hitachi, 500PA) was tightly capped and shaken for 168 h with 120 rpm. The dark coloured supernatant solutions were collected after shaking by centrifugation at 13,700 *g* for 40 min. Subsequently, the collected supernatants were acidified with 6 M HCl to precipitate humic acid under air, N₂ gas or Ar gas atmosphere for each corresponding treatment. Humic acid was collected by centrifugation at 4000 *g* for 10 min. To remove suspended materials, humic acid was redissolved in a minimum volume of 0.1 M KOH separately under air, N₂ gas or Ar gas atmosphere and the ionic strength adjusted to 0.3 M K⁺ by adding KCl followed by centrifugation at 13,700 *g* for 40 min to separate the dissolved humic acid with suspended materials from clay. After acidification of the supernatant with 6 M HCl and subsequent centrifugation, the precipitated humic acid was shaken with 200 ml HCl/HF solution (0.1 M HCl and 0.3 M HF) for 16 h separately under the three different atmosphere conditions to reduce the ash content, followed by washing the precipitate with distilled water, transfer to dialysis tube [Spectra/Por CE (cellulose ester) membrane MWCO: 1000] and dialyses against distilled water for 72 h. The dialysed humic acid was then freeze-dried, weighed and stored in a vacuum-dried chamber for further analyses.

The elemental composition of the three humic acid samples was determined on a C, H, O, N elemental analyser (Heraeus, CHN-O-Rapid) with four replications for each sample. Duncan's multiple-range test was used to find the significance of differences among the three gas treatments.

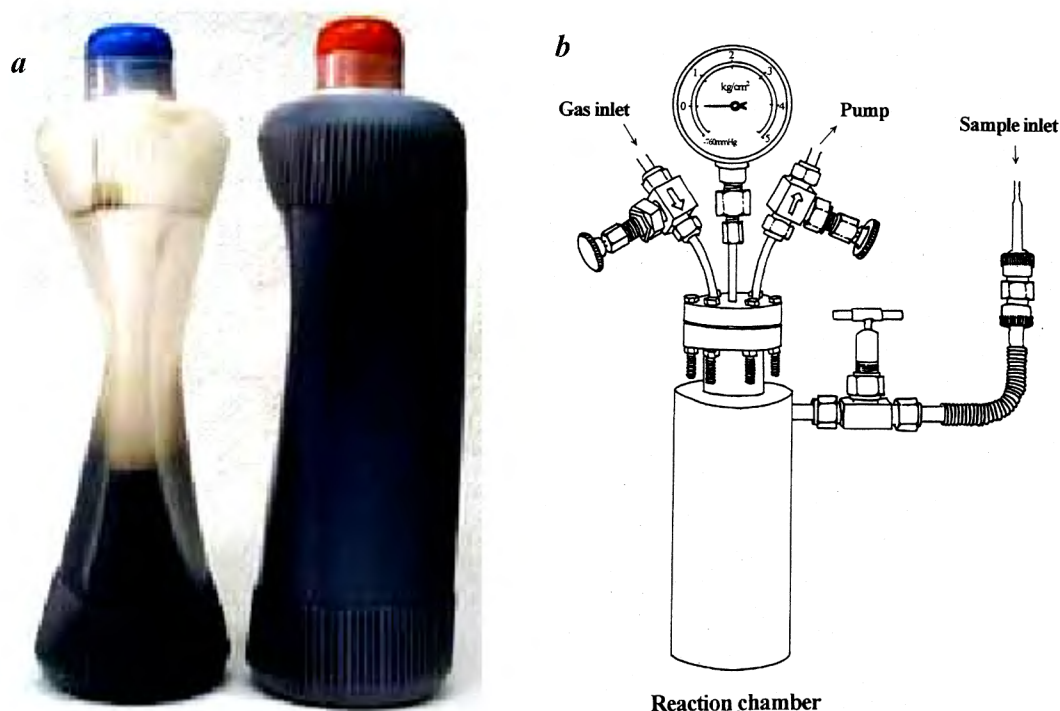


Figure 1. *a*, (Left) Contraction of sealed containers filled with alkaline humic acid solution after two years. (Right) N₂ gas disappeared from the bottle. *b*, Design of stainless reaction chamber with pressure gauge, individual gases and sample inlet.

EPR spectra of freeze-dried humic acid were recorded on an EPR spectrometer (Bruker, EMX10), employing a modulation of 1.997 mW, operation frequency of 9.78 GHz and a central magnetic field of 3486.4 G. Thoroughly freeze-dried samples were transferred to standard EPR sample tubes (3 mm inner diameter), filled to a height of 5 cm and tightly stopped. The magnetic field at the sample was calibrated by diphenylpicryl hydrazyl.

$^{15}\text{N}_2$ as a tracer for humic acid- N_2 reaction was carried out as follows. Three grams of sterilized peat was shaken with 300 ml 0.1 M NaOH under $^{15}\text{N}_2$ gas (MESA International, Canada, purity 99.9%) at 1 atm pressure for 240 h with four replications, in a stainless reaction chamber (Figure 1b) equipped with a pressure gauge. The humic acid samples were extracted and purified using the IHSS procedures described above and analysed using the automatic nitrogen and carbon analyser with GC-MS (ANCA-NT, Europe Scientific, Crewe, UK). Urea (Merck, USA) was the standard sample with $^{15}\text{N}/^{14}\text{N} = 0.0036765$. ^{15}N abundance and $\delta^{15}\text{N}$ were calculated^{13,14}.

The three humic acid samples were subjected to analyses using FTIR spectrophotometer (Bio-Rad, FTS-7). KBr pellets were prepared by compressing under vacuum (mixture of 1.00 mg of freeze-dried humic acid with 250.00 mg KBr (spectrometry grade) at 70–100 MPa pressure for 2 min). To compare the differential spectra of the three humic acid samples, the absorbance of the bands at 400–4000 cm^{-1} was computed by the differences between each spectrum using the software supplied with the equipment. The coefficients of variation between replicate FTIR analyses were $\pm 2.5\%$.

^{13}C CPMAS NMR analysis was carried out with three freeze-dried humic acid specimens and subjected to analysis of solid state ^{13}C NMR using a Bruker DSX-400 MHz spectrometer (Bruker G.M.B.H., Kasslruhe, Germany) at 100.47 MHz. Single cross polarization contacts of 1 ms were used with relaxation delays of 1 s between pulses. A sweep width of 30.12 kHz was employed to collect 7601-point decay curves. The magic angle-spinning rate was 7.0 kHz in a 7 mm (diameter) rotor using a Bruker probe. The chemical shift is expressed in parts per million (ppm) on a scale relative to the aromatic signal from hexamethylbenzene at 132.3 ppm as an external reference. Percentages of aliphatic C (0–110 ppm), aromatic C (110–160 ppm) and

carboxyl C (160–190 ppm) were estimated by integration of the spectrum accordingly^{15–17}.

The UV absorbance at 272 nm was used to measure the aromatic C content of humic acid. The humic acid solutions (100 mg l^{-1}) were shaken in bottles filled with air, N_2 gas or Ar gas. The UV absorbance at 272 nm was measured after incubations at 0, 0.5, 1, 1.5, 2, 3, 4, 6 and 8 h with four replications for each gas treatment.

Elemental analysis of humic acid showed that the N and H contents of humic acid extracted under air and N_2 gas were higher than those under Ar gas (Table 1). The O content of humic acid extracted under air showed higher values than that under Ar gas. The C content, on the other hand, showed a comparatively lesser value for humic acid extracted under air and N_2 gas than that under Ar gas. Collectively, the N/C ratio in humic acid increased under air and N_2 atmosphere extraction. The higher H/C ratio obtained under air and N_2 atmosphere extraction (Table 1), indicates a low degree of chemical condensation (i.e. less aromatic structure)¹⁸. Not surprisingly, the O/C ratio increased from 0.500 to 0.524 (Table 1), which specifies auto-oxidation of humic acid extracted under air that contains O_2 , which is in corroboration with earlier reports¹⁹. The alkaline extraction method used was recommended by IHSS^{12,20}. Previous investigations using this protocol indicated that no differences exist in C, H, O, N and S content of humic acid and fulvic acid²¹. Moreover, extraction of humic acid under noble gas (Ar or He) as control was not considered in those studies.

Based on elemental analysis, a further assessment using $^{15}\text{N}_2$ as a tracer to measure the N incorporation into humic acid during extraction indicates that ^{15}N abundance in humic acid increased from 0.36676 atom % (as the control set) to 0.36738 atom %, and $\delta^{15}\text{N}$ value was also increased by 1.7‰ as measured by GC-MS (data not shown). The increase was not a consequence of the adsorption of N_2 molecules on the humic acid structure, since the extraction protocol utilized a high vacuum system during lyophilization. The results indicated that N_2 gas might have reacted with and/or incorporated into the humic acid solution. The physico-chemical properties of isotopes of an element are different to some degrees. This difference in physico-chemical properties of an isotope is a property of the mass. The difference in atomic mass for ^{15}N and ^{14}N is 7.14%.

Table 1. Elemental analysis and electron paramagnetic resonance (EPR) analysis of humic acid extracted under air, N_2 gas and Ar gas atmosphere

Treatment	Element analysis							EPR analysis	
	Element (%)				Atomic ratio			For free radical concentration (10^{17} spins g^{-1})	
	C	H	O	N	N/C	H/C	O/C		
Air	54.51 ^{b*}	4.98 ^a	38.11 ^a	2.41 ^a	0.038 ^a	1.10 ^a	0.524 ^a	3.11 ^b	
N_2	54.79 ^b	5.01 ^a	37.85 ^{ab}	2.36 ^a	0.037 ^a	1.10 ^a	0.518 ^{ab}	3.48 ^b	
Ar	55.87 ^a	4.77 ^b	37.21 ^b	2.15 ^b	0.032 ^b	1.02 ^b	0.500 ^b	4.34 ^a	

*Means in columns followed by the same letter are not significantly different ($P < 0.05$) by Duncan's multiple-range test with four replications.

Thus, they have significantly different molecular velocity and molecular vibration frequency in a chemical reaction²². In our experiment, ¹⁴N molecules had higher molecular velocity and molecular vibration frequency than ¹⁵N molecules. Consequently, the amount of the entire fixed ²¹N in the sample in a 240 h reaction period under ¹⁵N atmosphere was less than that of fixed N (mostly as ¹⁴N) (Table 1) in a 168 h reaction period bubbled with ¹⁴N₂ for 6 min.

It has been well documented that free radicals in humic acid play a role in the synthesis and degradation of humic acid from organic compounds, in metal-complexing and in the physiological activity^{18,23–26}. Three electron paramagnetic resonance (EPR) spectra for humic acid extracted under air, N₂ and Ar gases were similar to those published previously^{18,27}. The characteristic parameters of line width and *g*-value of the EPR spectra of humic acid extracted under air, N₂ and Ar gases were also close to those reported^{18,27}. However, under air and N₂ gas treatments the free radical concentration in humic acid had decreased compared to Ar gas treatment (Table 1).

These data led us to probe into the structural changes and bonding relationships of N element in humic acid after three different extraction conditions using FTIR spectroscopy, according to a previous publication²⁸. The wavenumbers and their assignments for the subtractive spectra are presented in Table 2. The differential FTIR spectra of humic acid extracted under N₂ and Ar gases (i.e. N₂–Ar) show increased N-bonding in humic acid extracted under N₂ gas, i.e. the occurrence of N₂–humic acid reaction. Moreover, humic acid extracted under air and Ar gas (i.e. Air–Ar) in the differential intensities may be related to the increase in O-bonding. Those of 1610 cm^{–1}, 1542 cm^{–1} and 1263 cm^{–1} may be related to the increased N-bonding. It was reported earlier²⁷ that no difference was noticed in the IR spectra of humic acid attributable to air and N₂ extrac-

tion methods for 24 h. Data from differential FTIR spectroscopy thus indicate that O₂ in air might have affected structure/bonding of humic acid extracted for 168 h, which was different from that extracted under pure N₂ gas. This increased bonding ability of N and O with humic acid is consistent with the increase in N/C and O/C ratios from elemental analysis (Table 1).

It was reported that distinct ranges of chemical shift in ¹³C CPMAS NMR spectra were attributed to a specified set of chemical environments²⁹, such as aliphatic or aromatic^{15,30}. Using ¹³C CPMAS NMR spectroscopy, we found no apparent difference among the ¹³C CPMAS NMR spectra from all the three treatments. However, differences among the relative distributions of characteristic C in humic acid were noted when computed (Table 3). The results show that the difference in chemical shift of air and N₂ gas treatments were relatively decreased in aromatic (110–160 ppm) and increased in aliphatic (0–90 ppm) constituents. Air and N₂ treatments thus decreased in aromatic subunits with low degree of chemical condensation in the structure of humic acid, which may have concomitantly resulted in the H/C ratio in Table 1. The increased aromatic amines of humic acid also supports the data obtained from FTIR spectroscopy (Table 2) and the increasing N/C ratio (Table 1).

Previous work had showed that the per cent aromatic C determined by NMR was correlated (*r* = 0.94) with UV absorption measurements. It was suggested to determine the UV absorption at 272 nm, which can be used to provide a quantitative estimation of the aromatic C content of dissolved humic acid³¹. To further support the results from ¹³C CPMAS NMR, we measured the UV absorbance of humic acid shaken under air, N₂ and Ar atmosphere. The obtained UV absorbance at 272 nm for the dissolved humic acid shaken under air and N₂ atmosphere during 8 h de-

Table 2. Assignment of observed FTIR bands of subtractive spectra (N₂–Ar, air–Ar, and air–N₂) of humic acid extracted under air, N₂ gas and Ar gas atmosphere

Wavenumber (cm ^{–1})*			Proposed assignment†
N ₂ –Ar	Air–Ar	Air–N ₂	
2348			NH ₃ ⁺ of tertiary amine salts
		1771	C=O of cyclic anhydrides
		1734	C=O of ketones and esters
1726			C=O of 5-ring lactams
	1718		C=O of ketones or acid dimers
1710			C=O of 5-ring lactams
1639			C=O of tertiary amides or C=N of RCH=NR
1610	1610		N–H of primary aromatic amines or C=N of RCH=NR
	1542	1540	N–H of secondary amides
1343			C–N of secondary or tertiary aromatic amines
1264	1263		C–N of primary aromatic amines
	1224	1223	Cyclic anhydrides, ketones or esters
404			Aromatic amines

*Wavenumber of bands in differential spectra of humic acids extracted under N₂ and Ar gases (N₂–Ar), air and Ar gas (air–Ar), and air and N₂ gas (air–N₂).

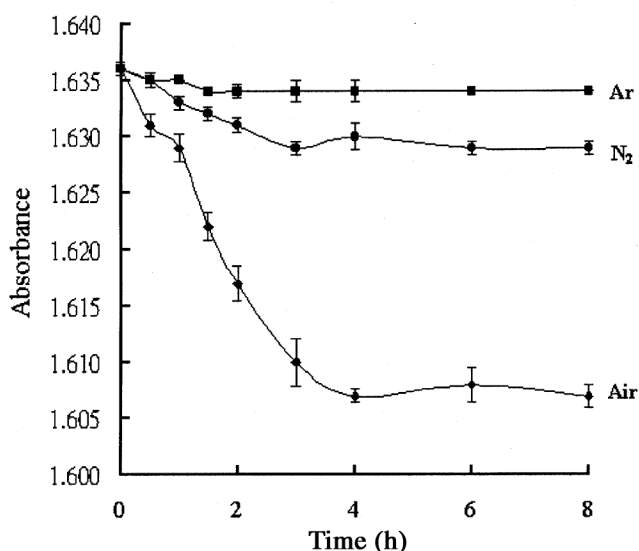
†According to Silverstein *et al.*²⁸.

Table 3. Relative contribution (%) of characteristic carbon in ^{13}C CPMAS NMR spectra of humic acid extracted under air, N_2 gas and Ar gas atmosphere

Chemical shift (ppm)	Relative contribution (%)			Assignment
	Air	N_2	Ar	
0–50	40.16 ^{a*}	39.08 ^{ab}	37.56 ^b	Alkyl carbon
50–90	25.42 ^a	24.73 ^b	24.64 ^b	C=O carbon
90–110	5.72 ^b	6.70 ^a	6.82 ^a	Acetals, ketals
110–140	15.97 ^b	16.41 ^{ab}	16.84 ^a	C, H aromatic
140–160	5.12 ^b	5.69 ^{ab}	6.24 ^a	O, H aromatic
160–190	7.60 ^a	7.39 ^a	7.91 ^a	Carboxyl, amides, esters
Aliphatic (0–110)	71.30	70.51	69.02	
Aromatic (110–160)	21.09	22.10	23.08	
Aromaticity [†]	22.83	23.86	25.06	

*Means in rows followed by the same letter are not significantly different ($P < 0.05$) by Duncan's multiple-range test with four replications.

[†]Aromaticity = [Aromatic/(Aliphatic + Aromatic)] \times 100.

**Figure 2.** Change in UV absorbance at 272 nm of humic acid solution extracted under air, N_2 gas and Ar gas atmosphere with different shaking times.

creased, particularly under air condition, but no apparent change was observed under Ar atmosphere (Figure 2). This indicated that humic acid shaken under air might contain less aromatic content than humic acid shaken under N_2 atmosphere. Supportive evidence was also shown in the distribution of characteristic carbon in ^{13}C CPMAS NMR spectra (Table 3) and H/C ratio (Table 1), but less categorically. The results revealed that the reaction of humic acid with air or N_2 gas might be related to the decrease in aromatic structure.

Humic acid is ubiquitous in the environment and has the potential to bind to certain transition metals that are harmful to biological organisms. The metal-loaded humic acid can also produce deleterious oxidizing species such as hydroxyl radical through the metal-driven Fenton reaction³². Mobility of humic acid in soil depends on its

charging behaviour and is controlled by the amount of functional groups³³. Recently, cation-binding ability of humic and fulvic acids was linked to the presence of reactive functional groups, their type and respective amount³⁴. We presume that alkaline humic acid might be undergoing N_2 -fixation by consumption of free radicals in the structure or by binding to certain strong transition metal oxides converting nitrogen to other intermediates and subsequently leading to the formation of ammonia. Once incorporated into humic acid, N element might have altered the overall bonding structure by changing the contents of aliphatic and aromatic constituents, which was evidenced in FTIR analysis (Table 2). We have recorded similar differences consistently in our recent experiments on humic acid from different sources. The differences obtained are insufficient to conclusively derive the mechanism of nitrogen fixation by alkaline humic acid. Detailed analysis is underway in our laboratory to ascertain the larger differences to conclusively derive the nitrogen fixation. Identification of a lesser known pathway in the global N-cycle has important implications for our understanding of N_2 -fixation by humic acid from natural humic substance-containing materials such as peat and compost. In particular, utilization of this process could greatly reduce N fertilizer cost by further modification studies that would ultimately be applicable in the industry.

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The status of the baobab (*Adansonia digitata* L.) in Mannar Island, Sri Lanka

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The baobab (*Adansonia digitata*) is not native to Sri Lanka, but was introduced centuries ago, perhaps by Arab traders. Today about 40 trees survive in Sri Lanka, of which 34 have been identified and measured in the island of Mannar. The most abundant size class was between 5 and 9.9 m (girth at breast height), which accounted for more than 50% of the trees. About 40% of the trees in Mannar are between 300 and 400 years old. The oldest tree is estimated to be about 723 years of age. Despite the baobab being an introduced species, it is protected in Sri Lanka given its rarity and antiquity. Mannar Island appears to be the last bastion for the baobab in Sri Lanka. That the baobab still survives in the island is largely due to its status as a ‘zero-cost species’. A potential threat for the future may come from the aspirations of the people for a better quality of life, and through ill-conceived and over-ambitious tourism development projects.

THE baobab (*Adansonia digitata* L.) belongs to the family Bombacaceae. It is unmistakable in the field, given its strange appearance and barrel-like enormous trunk, which tapers into branches. It is a deciduous tree whose rounded crown is bare during the dry season. When the leaves are shed, the tree gives the impression that it had been planted upside down. According to an Arabian legend, the baobab’s strange appearance is due to the devil plucking the tree up, thrusting its branches into the earth, and leaving its roots in the air. In mature trees, the extensive lateral roots rarely extend beyond 2 m, which is why they are often toppled in old age. The large, dark-green leaves are like the fingers of the human hand, with five (seldom seven) oblong blades; hence the specific name *digitata*¹. It was the dark-green foliage of the baobab that led the early mariners refer to Africa’s western bulge as the Cape Verde (French for green). The flowers which are pendulous, white, large and solitary are seen mostly when the leaves have been shed. They open at night and emit a scent attractive to bats which pollinate them². The sour scent also attracts certain flies and moths at night. The large, white, oval fruits are provided with a dense coat of velvety hairs, and are gourd-shaped, spongy, acidic and farinaceous³. The fruit is edible and contains a pleasant, cool-tasting mucilaginous pulp in which seeds are buried, and each fruit hangs from a thick stalk. The seed coat is hard and drought-resistant.

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