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# Abiotic Formation of Humic-Like Substances through Freezing-Accelerated Reaction of Phenolic Compounds and Nitrite

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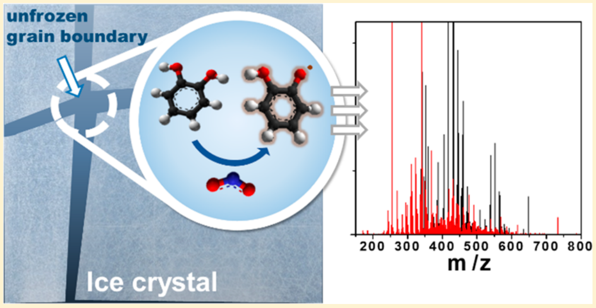
*Supporting Information*

**ABSTRACT:** A previously unknown abiotic humification pathway which is highly accelerated in frozen solution containing phenolic compounds and nitrite was investigated and proposed. The production of humic-like acids (HLA) and fulvic-like acids (FLA) was observed in the frozen solution (−20 °C) whereas it was negligible in aqueous solution (20 °C). Inorganic nitrogen was transformed into organic nitrogen during the humification process. Mass spectrometry (MS) and elemental analyses, including pyrolysis-GC/MS and FT-ion cyclotron resonance/MS, showed that humification products (HLA and FLA) have chemical structures and compositions similar to nature humic substances. The enhanced humification reaction could be attributed to the freeze-concentration effect, whereby nitrite ions in the unfrozen grain boundary region are transformed into nitrosonium ions which oxidize phenols to phenolic radicals. Confocal Raman microscopy confirmed that catechol and nitrite ions are preferentially concentrated at the ice grain boundary and electron paramagnetic resonance spectroscopic analysis of catechol/nitrite solution detected the phenolic radicals only in frozen solution, not in aqueous solution. The freezing-induced generation of phenolic radicals should lead to the formation of humic-like substances through polymerization. This study identifies and proposes a new humic formation pathway that might work as a model abiotic "bottom-up" mechanism in frozen environmental conditions.



## INTRODUCTION

Humic substances (HS) are ubiquitous components of natural organic matters (NOM), which play an important role in the environment. NOMs are one of the largest carbon storages on Earth and contribute significantly to carbon cycle.[1] These organic materials contain nitrogen, phosphorus, and nutrients, which are important for soil fertility and microorganism growth in water.[2,3] In geochemistry, the role of HS can be considered as an electron donor/acceptor and previous studies have shown that several biochemical reactions in geospheres could be mediated by HS.[4] In addition, HS are important chromophores that serve as a photosensitizer in the environment.[5] Under solar irradiation, HS can generate reactive species or solvated electrons that induce redox reactions in nature.[6]

HS can be formed via biochemical transformation, resynthesis, decomposition, and polymerization, all of which are generally defined as humification process.[7] The production pathways can affect the ultimate chemical properties of HS.[8] However, the humification mechanisms are poorly understood and based on many uncertain speculation. A major humification process is the decomposition of macromolecules derived from dead bodies of living organisms.[9] Another important pathway is polymerization of low-molecular-weight materials (e.g., phenolic compounds, glucose, and amino acids) and their conversion to larger sized HS.[10,11] The Maillard reaction[12] is typically regarded as an important humification process; the effects of phenolic compounds, minerals, and metal oxides toward the reaction products have been studied in previous research.[13−18]

However, the Maillard reaction is the only humification pathway yet to be studied and no other possible reactions have been suggested in the literature. In addition, previous

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studies[13−18] investigated humification with high concentration of organic precursors (e.g., dissolved organic carbon concentration (DOC) over 500 mg $L^{-1}$), but this concentration range is not comparable to DOC in natural waters.[19,20] Moreover, the above-mentioned studies focused only on organic nitrogen (e.g., glycine) as a source of nitrogen in HS instead of inorganic nitrogen species that are commonly present in natural aquatic systems.[21] In this study, the humification was studied with DOC concentration under 10 mg $L^{-1}$ and inorganic nitrogen species.

The aim of this study is to propose and investigate a previously unrecognized and purely abiotic humification process that takes place in the frozen solution containing phenolic compounds and nitrite. It is suggested that nitrite ions initiate the polymerization reaction of phenolic compounds (catechol or phenol), leading to the production of humic-like acids (HLA) and fulvic-like acids (FLA). The chemical characteristics of the produced HS were analyzed and compared with those of natural HS. According to mass spectrometric (MS) and elemental analyses, the humification products (HLA, FLA) have chemical structures and compositions similar to natural humic substances. The reaction is markedly accelerated in frozen conditions, which is attributed to the freeze-concentration effect. The humification rate measured in the frozen phase is much higher than those reported in the previous humification studies. The unique chemical transformation reactions taking place in frozen solutions could have important roles in organic carbon transformation in nature.

## ■ METHODS

**Materials.** Chemicals and reagents used in this study were as follows: catechol ($C_6H_6O_2$, Sigma-Aldrich), phenol ($C_6H_6O$, Sigma-Aldrich), sodium nitrite ($NaNO_2$, Aldrich), sodium nitrate ($NaNO_3$, Acros Organics), sulfuric acid ($H_2SO_4$, Sigma-Aldrich), sodium phosphate diabasic ($Na_2HPO_4$, Sigma-Aldrich), sodium phosphate monobasic ($NaH_2PO_4$, Sigma-Aldrich), sodium sulfate anhydrous ($Na_2SO_4$, Shinyo Pure Chemicals Co., Ltd.), hydrochloric acid (HCl, Sigma-Aldrich), DAX-8 resin (Sigma-Aldrich), sodium hydroxide (NaOH, Sigma-Aldrich), potassium persulfate ($K_2S_2O_8$, Sigma-Aldrich), humic acid (Aldrich), ethyl acetate (J.T. Baker), and methanol (J.T. Baker). All chemicals were of analytical grade unless specified otherwise. Suwannee River humic acid (SRHA) and fulvic acid (SRFA) were purchased from the International Humic Substances Society (https://ihss.humicsubstances.org/). The deionized water used in the experiments was of ultrapure (18 MΩ·cm) grade and was prepared using a Barnstead purification system.

**Humification of Catechol and Phenol with Nitrite Ion.** Chemical reagent and catechol (or phenol) stock solutions were freshly prepared daily. Ten mL of sample solution with an appropriate substrate concentration was transferred to a 15 mL conical tube. The solution pH was adjusted by sulfuric acid solution (1.0 and 0.1 M). The conical tube was then transferred to a precooled ethanol bath (−20 °C) for the reaction in frozen solutions. The reaction time was measured from the moment when the sample tube was immersed into the ethanol bath (time zero, $t = 0$). After the reaction was completed (6 h), the frozen sample was thawed in a water bath (40 °C) prior to sample analysis. All the above procedures were the same for the control experiments using aqueous phase samples except that the reaction temperature was 20 °C.

**Humic/Fulvic-Like Acid Extraction.** For HLA/FLA extraction, liquid−liquid extraction was used to eliminate the remaining nitrite that might induce further reaction of HLA/FLA products. The sample solution was mixed with ethyl acetate (50 mL) in a separating funnel to extract organic compounds. The mixture was shaken for ∼5 min and settled for 10 min. The aqueous layer was discarded, and the organic layer was preserved for further analysis. The above procedure was repeated in triplicate. The organic solution was then treated by anhydrous sodium sulfate ($Na_2SO_4$) to remove excess water in the solution. The solution was then transferred to a round-bottom flask and the organic solvent was evaporated by a rotary evaporator (40 °C). The remaining organic substances in the flask were dissolved in deionized water (10 mL) for further separation of HLA and FLA. Over 90% of nitrite was eliminated and ∼80% of dissolved organic carbon (DOC) was recovered through this preparation procedure.

The extraction of humic-like acids (HLA) and fulvic-like acids (FLA) was conducted according to the International Humic Substances Society (IHSS) standard protocol. After nitrite elimination, the redissolved aqueous solution of humification products was acidified to pH 1.0 and maintained at room temperature for 24 h. The HLA fraction was precipitated and separated by centrifugation (10 000 rpm for 20 min). The HLA precipitates were redissolved in NaOH (0.1 M) solution for analysis. The FLA fraction in the supernatant was extracted using DAX-8 resin. One mL of the resin was used for each sample. After the absorption of FLA fraction, the DAX-8 resin was washed with deionized water until no monomer (catechol or phenol) was detected (3−5 times). The FLA was redissolved in NaOH (0.1M) solution before analysis. The organic substance fraction remaining after extraction HLA and FLA was classified as "residual".

**Quantitative Measurement.** The amount of HLA and FLA formation was quantitatively estimated by measuring the concentration of dissolved organic carbon (DOC). The DOC of extracted HLA and FLA was measured by a TOC analyzer (TOC-V CSH, Shimadzu) (detection limit: 0.10 mg $L^{-1}$).

The humification process can generate dark organic compounds with an elevated background absorption in the visible light region. As an indicating parameter for the humification process,[13,15−18] the $E_{600}$ value based on the absorbance at 600 nm can be calculated by the following equation.

$$E_{600}\ (\mathrm{Lg^{-1}cm^{-1}}) = \frac{\{\text{absorbance at 600 nm}\}}{\mathrm{DOC}\ (\mathrm{gL^{-1}}) \times \{\text{light path length (1 cm)}\}} \qquad (1)$$

The pH of the sample solution was adjusted to 7 by phosphate buffer before the absorbance measurements which were done using a UV/Visible spectrophotometer (Agilent 8453).

**Dissolved Organic Nitrogen (DON) Measurement.** DON production from the reaction was calculated from total dissolved nitrogen (TDN), which was measured by the persulfate oxidation method,[22] and the following equation.

$$\mathrm{DON} = \mathrm{TDN} - ([\mathrm{NO_2}^-] + [\mathrm{NO_3}^-] + [\mathrm{NH_4}^+]) \qquad (2)$$

The concentrations of nitrite, nitrate, and ammonium ions were detected with an ion chromatograph (IC, Dionex DX-120) having a column for anion (Dionex IonPac AS23; 4 × 250 $mm^2$) or cation (CS12A; 4 × 250 $mm^2$) analysis, together with a conductivity detector (detection limits for nitrite, nitrate, and ammonium: 0.3∼0.6 $\mu$M).

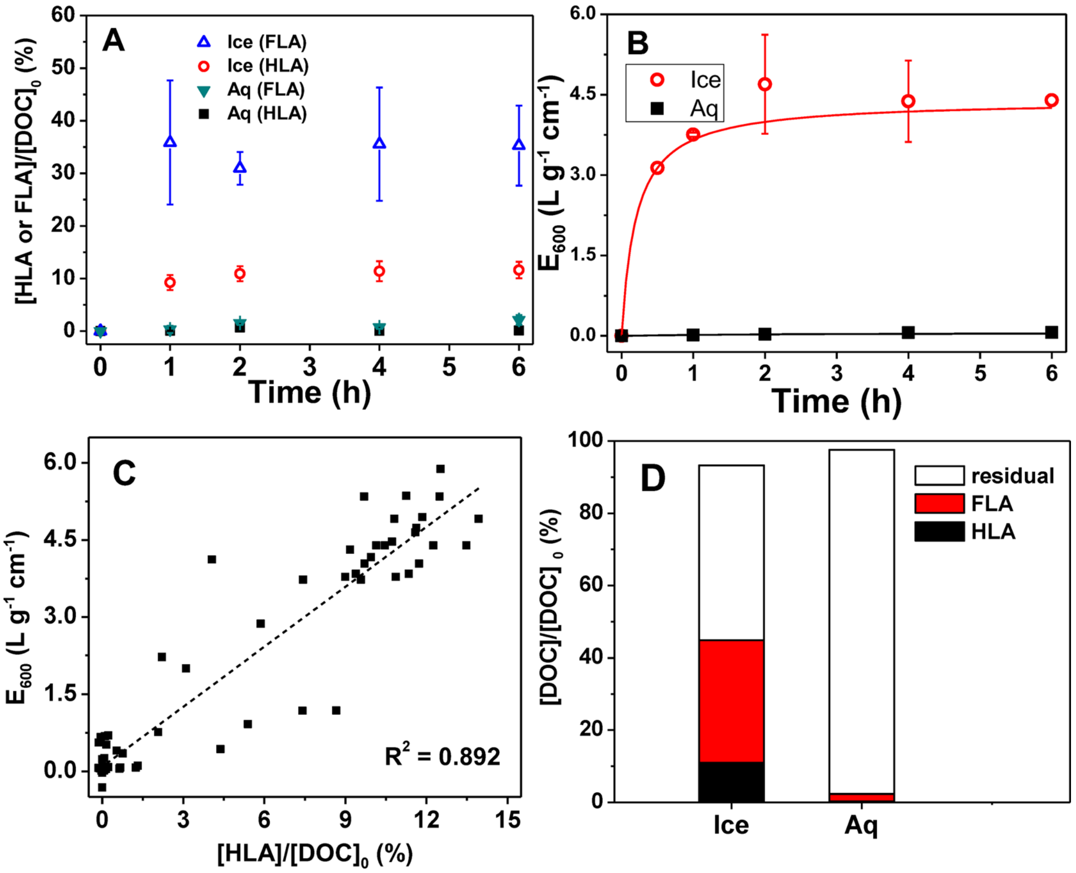

**Figure 1.** Time profiles of (A) catechol transformation to HLA and FLA(evaluated in terms of DOC) and (B) $E_{600}$ under aqueous (20 °C) and frozen solution (−20 °C) solutions. (C) Correlation between $[HLA]/[DOC]_0$ (%) and $E_{600}$ of this study. (D) Relative composition of HLA, FLA, and residual organics (residual) fractions in frozen (−20 °C) and aqueous (20 °C) solution after 6 h reaction. Experimental conditions: $pH_i = 4$, $[Catechol]_0 = 100\ \mu M$, $[NO_2^-]_0 = 5\ \mu M$, $pH_i = 4.0$.

The elemental analysis for FLA, HLA, and humic/fulvic acids from Suwannee River and Aldrich was carried out using an elemental analyzer (Vario-Micro Cube, Germany). FLA and HLA samples were freeze-dried prior to the elemental analysis.

**Fluorescence Measurement.** The excitation/emission matrix (EEM) of FLA, HLA, and humic/fulvic acids from Suwannee River and Aldrich was measured by using a spectrofluorometer (FluoroMAx-4, HORIBA).

**Pyrolysis-Gas Chromatography/Mass Spectrometry (py-GC/MS).** Prior to py-GC/MS measurement, HLA and FLA samples were freeze-dried. Py-GC/MS was performed by a GC (Agilent 7890A) with a quadrupole MS (5975C). Approximately 0.5−1.0 mg of powder sample was covered with pyrofoil and placed in a quartz tube. The sample was then heated to the Curie temperature (590 °C) using a Curie point injector (JCI-22; Japan Analytical Industries, Japan). After pyrolysis, organic fragments were went through GC (DB-5MS; Agilent Technologies, U.S.A.) equipped with a column (30 m × 0.25 mm × 0.25 μm) for separation. After separation, the organic molecules were identified with a mass spectrometer. For MS measurement, electron energy was 70 eV, scanning arrange was 40−500 amu, and ion source temperature was 220 °C.

**Fourier Transform Ion Cyclotron Resonance/Mass Spectrometry (FT-ICR/MS).** Inorganic ions in the humification product samples ($Na^+$, $Cl^-$, $SO_4^-$, $NO_3^-$, and $NO_2^-$) were removed by solid-phase extraction prior to FT-ICR/MS measurement. SUPELCLEAN ENVI-8 6 mL tubes (SUPELCO) were used for the extraction following a standard procedure detailed elsewhere.[23] The absorbent tubes were rinsed with methanol before use. The humic sample solution was acidified to pH 2 and passed through the absorbent tube, which was subsequently rinsed again with HCl solution (0.01M) to redissolve the ions in the absorbent. The absorbent was dried by purging air for 5 min, and the absorbed organic and humic products in the tube were redissolved by eluting methanol through the tube.

The samples were analyzed on a 7 T FT-ICR/MS (Solarix 2XR, Bruker Daltonics, GmbH, Bremen, Germany) which was equipped with a quadrupole phase detection (2ω mode) system. The electrospray ionization (ESI) technique was used for the ionization of humic/fulvic substances in the samples. The sample solutions were prepared in 1:1 methanol−water mixture and directly injected into the syringe pump (flow rate: 180 μL/h) using a gastight syringe (Hamilton Co., Reno, NV). The mass spectra were recorded in negative ionization mode within a mass-to-charge ($m/z$) range of 150.5−3000.0. All recorded spectra were externally calibrated with an ESI-L low concentration tuning mixture (Agilent technologies). For nebulizing and drying, high purity nitrogen (N2) was used. For ESI source, pressure of nebulizer gas was 0.8 bar and temperature of drying gas was 200 °C with 4.0 L/min flow rate. For the FT-ICR measurement, a capillary voltage of 3.5 kV was used. The ion accumulation time was 10 ms for a single spectrum and nearly 200 scans were summed for the final mass spectra. Voltage of the skimmer was −45.0 V. For the ICR

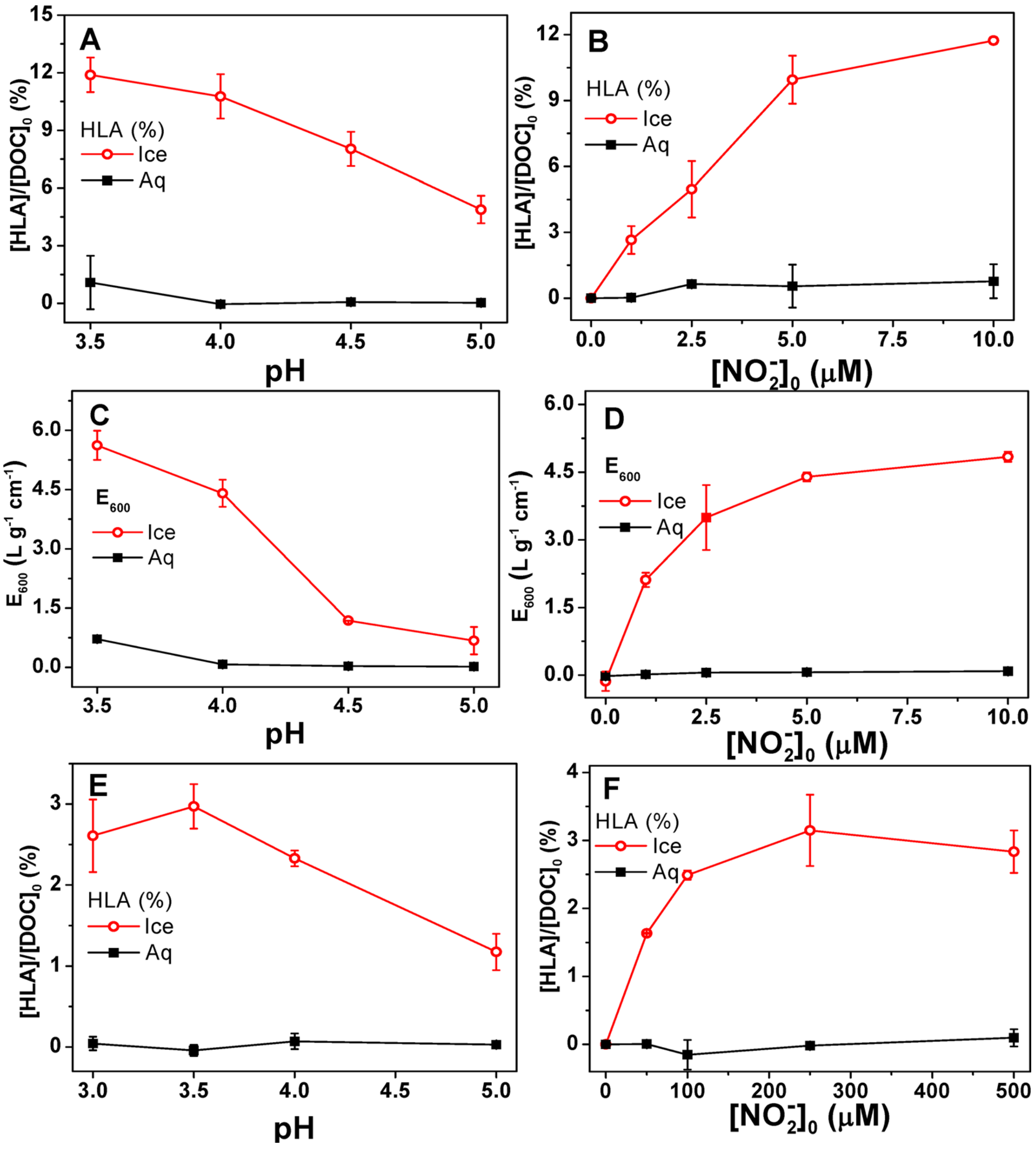

**Figure 2.** (A, B) HLA production (measured in DOC) and (C, D) $E_{600}$ generation from catechol conversion in frozen (−20 °C) and aqueous (20 °C) solutions as a function of (A, C) pH and (B, D) $[NO_2^-]_0$. (E, F) HLA production from phenol conversion in frozen (−20 °C) and aqueous (20 °C) solutions as a function of pH and $[NO_2^-]_0$. Experimental conditions: $[\text{Catechol}]_0$ = 100 μM or $[\text{Phenol}]_0$ = 100 μM (for E, F), $pH_i$ = 4.0 (for B, D, F), $[NO_2^-]_0$= 5 μM (for A, C), or 100 μM (for E), reaction time of 6 h.

trap, the voltages of both the front and back trap plates were −1.6 V. The ions were excited with a sweep excitation power of 25%.

**Confocal Raman Analysis.** To analyze the reagents (catechol and nitrite) in the grain boundary region in the frozen solution, a confocal Raman microscope (inVia Qontor confocal Raman microscope, Renishaw) equipped with a 785 nm edge laser was used. A Linkam stage (THMS 600, Linkam) was used for freezing the sample solution.

**Electron Paramagnetic Resonance (EPR).** Catechol radicals produced by reaction with nitrite in the frozen solution were detected by electron paramagnetic resonance (EPR) analysis with an EMX plus −9.5/2.7 (Bruker). The measurement conditions were as follows: microwave power of 0.6 mW, microwave frequency of 9.4 GHz, and modulation frequency of 100 kHz.

## RESULTS AND DISCUSSION

**Freezing-Induced Humification of Phenolic Compounds.** Figure 1A shows the time profiles of humic-like acids (HLA) formation in the presence of catechol and nitrite ions in aqueous and frozen solutions. The concentration of HLA produced from the initial monomer (catechol) was expressed in terms of DOC concentration. The increase of HLA concentration is a result of the humification of catechol. The reaction was markedly enhanced under the frozen condition compared to that in the aqueous phase. The HLA concentration rapidly increased during the initial 2 h of reaction, which leveled off afterward. Figure 1B compares the time profiles of $E_{600}$ values (eq 1) under aqueous and frozen conditions. In the frozen phase, there was a rapid increase in $E_{600}$ value over the initial 2 h, which subsequently reached a plateau, whereas $E_{600}$ in the aqueous phase was negligible. This is consistent with the behavior of HLA formation (Figure 1A).

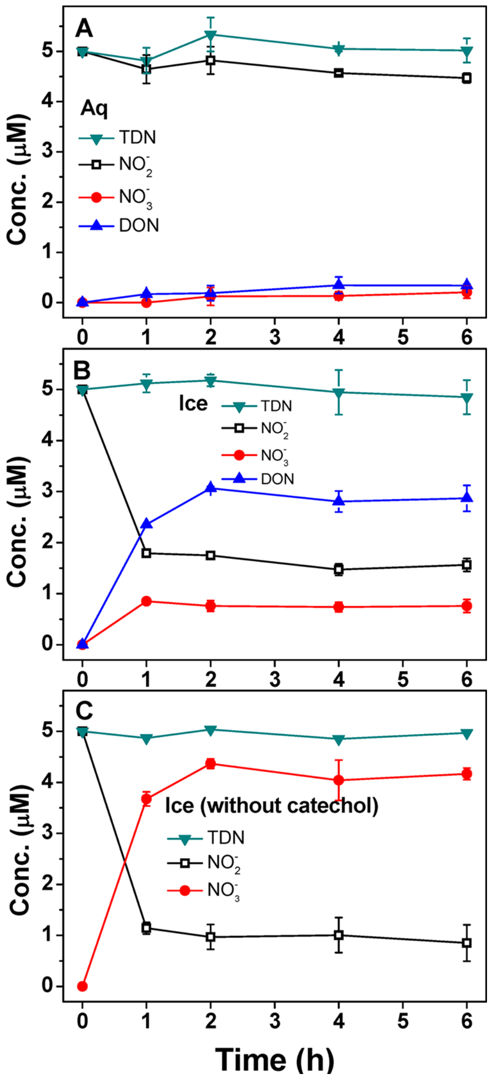

**Figure 3.** Time profiles of total dissolved nitrogen (TDN), nitrite ($NO_2^-$), nitrate ($NO_3^-$), and DON concentration under (A) aqueous (20 °C) and (B) frozen (−20 °C) conditions in the presence of catechol. (C) Conversion of nitrite ($NO_2^-$) to nitrate ($NO_3^-$) in the frozen phase in the absence of catechol. Experimental conditions: $[\text{Catechol}]_0 = 100\ \mu M$ (for A, B), $[NO_2^-]_0 = 5\ \mu M$, $pH_i = 4$.

The HLA and $E_{600}$ values from this study exhibited a reasonably good linear correlation (Figure 1C), indicating that the $E_{600}$ value could be used to estimate the amount of HLA formation. Figure 1D shows the relative composition (%) of three organic fractions (HLA, FLA, and organics residual) after 6 h reaction of catechol and nitrite. The concentration of HLA and FLA fraction generated in the frozen solution was 11% and 34%, respectively, which was much higher than those obtained in aqueous solution (0.3% and 2.1%). The fraction of FLA was higher than HLA in both conditions.

Table S1 of the Supporting Information (SI) compares the production rate of HLA and $E_{600}$ values among this and other studies.[13−18] The calculated HLA and $E_{600}$ formation rates in the ice phase were orders of magnitude higher than those in the aqueous phase. The production rates of HLA and $E_{600}$ observed in the frozen condition are significantly higher than any of the reported values measured in the aqueous solution. The production of HLA and $E_{600}$ was favored at lower pH (Figure 2A and C) and increased with increasing $[NO_2^-]$ up to 10 μM (Figure 2B and D). In the absence of nitrite ions, no HLA and $E_{600}$ were generated at all. The humification of catechol essentially requires the presence of nitrite and is highly accelerated at lower pH. Instead of catechol, phenol was also tested as a humification precursor (Figure 2E and F). The behavior of HLA production in the frozen solution of phenol was very similar to that of catechol although the HLA concentration level was much lower compared with the case of catechol.

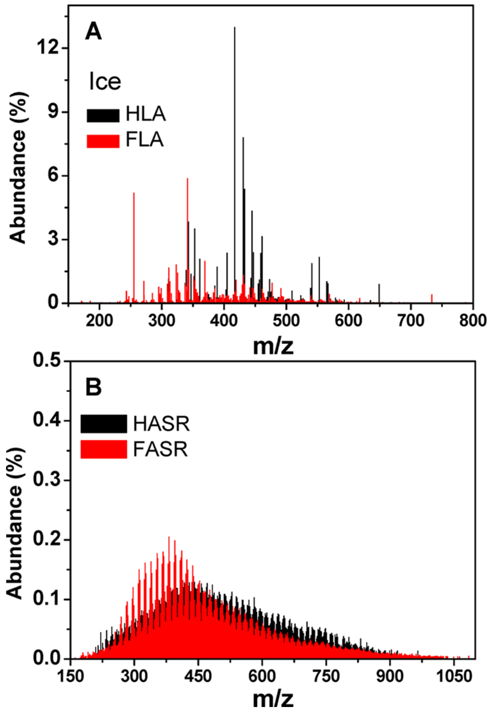

**Figure 4.** (A) Mass spectra (HLA and FLA) of humification products generated from the catechol transformation in frozen; (B) reference mass spectra of humic and fulvic acids from Suwannee River (SRHA and SRFA). Experimental condition: (A) $[\text{Catechol}]_0 = 100\ \mu M$, $[NO_2^-]_0 = 10\ \mu M$, $pH_i = 4$, reaction time of 6 h.

**Dissolved Organic Nitrogen (DON) Formation.** Dissolved organic nitrogen (DON) production from the humification process of catechol was measured in aqueous and frozen conditions (Figure 3). The formation of DON and nitrate was significant only in the frozen condition, which was accompanied by a rapid removal of nitrite during the first hour. The nitrate ion should be produced from the oxidation of nitrite ion by dissolved oxygen, which is highly enhanced under frozen condition (eq 3).[24]

$$2HNO_2 + O_2 \rightarrow 2NO_3^- + 2H^+ \qquad (3)$$

Although nitrite ions were quantitatively converted to nitrate ions in the absence of the organic precursor (Figure 3C), a majority of reacted nitrite ions (~84%) were transformed to DON after frozen reaction in the presence of catechol. This indicates that nitrite-N elements were incorporated into the organic substances during the catechol humification process in the frozen condition whereas such process was negligible in the aqueous solution.

The elemental analysis showed that both HLA and FLA contained the nitrogen content and FLA has higher N content than HLA (see Table S2). The nitrogen composition of HLA was approximately the same as that in natural humic

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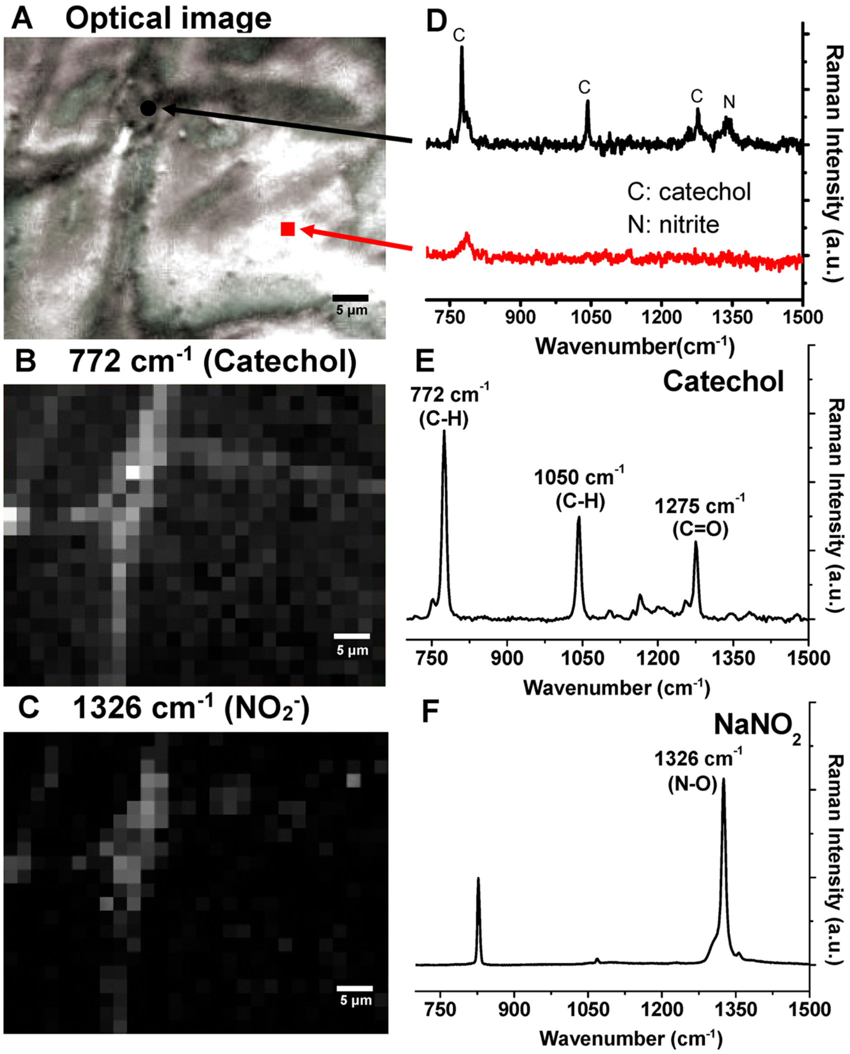

**Figure 5.** (A) Optical image of the grain boundary in the frozen solution and the corresponding spacial distribution of (B) catechol and (C) nitrite ions detected by a confocal Raman microscope within the frozen solution. (D) Raman spectra of the grain boundary region (black circle) and ice crystal region (red square); Reference Raman spectra of (E) catechol and (F) sodium nitrite. Freezing condition: a solution of catechol (1 mM) and nitrite (0.1 mM) was frozen at 253.2 K (−20 °C).

substances. The inorganic nitrogen species is transformed into organic nitrogen species typically through a biotic process in nature.[25,26] However, this study demonstrated that inorganic nitrogen can be incorporated into the organic substances via a purely abiotic pathway occurring in frozen solution.

**Fluorescence Spectra of FLA and HLA.** Spectrofluorometric analyses are widely used for chemical characterization of organic matters in nature. Figure S1 shows the excitation/emission matrix (EEM) of catechol, FLA, and HLA. Catechol showed a fluorescence peak at 275/316 nm (= excitation/emission). This peak was shifted to 300/350 nm (= ex/em) for FLA (Figure S1B and C). Such a red shift of the fluorescence peak was also observed in a humification study.[27] The fluorescence spectra of FLA obtained from aqueous and frozen solution exhibited similar shapes. However, HLA produced from the frozen solution demonstrated fluorescence peaks at 295/416, 345/424, and 435/480 nm (= ex/em) and the spectra ranged from 360−550 nm emission and 260−450 nm excitation (Figure S1D). Fluorescence spectra in this excitation/emission region are the typical sign of humic-like substances.[28] HLA was not obtained in the aqueous condition.

**Mass Spectrometric Analysis of Humification Products.** Pyrolysis-Gas Chromatography/Mass Spectrometry (Py-GC/MS) was used to analyze the chemical structures of HLA and FLA generated from the humification process. The pyrolysis products of HLA and FLA are composed of various chemical structures formed during humification (Table S3). Alcohol, ketone, aldehyde, carboxylic acid, ester, and methoxy compounds were detected by GC/MS. The formation of such products with functional groups could be induced by the cleavage of aromatic rings during the oxidation of phenolic compounds.[29−31] Several nitrogen-containing compounds (nitrile, amide, indole, and pyrrolidine) were also detected from the humification products, which reconfirmed that organic nitrogen compounds were produced from the reaction of catechol and nitrite. Although the transformation of inorganic nitrogen into organic nitrogen is commonly mediated via plants and microorganisms in ecosystem,[25,26] the present result demonstrated the possibility of abiotic mechanism that converts inorganic nitrogen to organic nitrogen in the frozen condition.

FLA, HLA, and standard HS (humic and fulvic acids obtained from Suwannee River) were further analyzed by

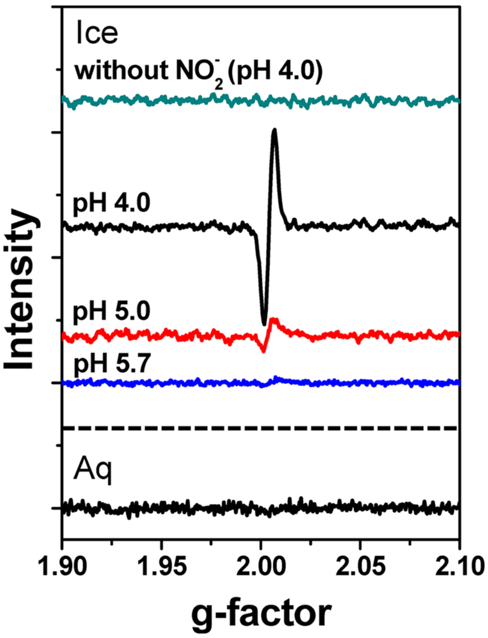

**Figure 6.** Electron paramagnetic resonance (EPR) spectra of catechol/nitrite solution in frozen (−20 °C) and aqueous (20 °C) solution. The EPR spectra of frozen solution with catechol only (no $NO_2^-$), with catechol (10 mM)/nitrite (1 mM) at different pH, and aqueous solution with catechol (10 mM)/nitrite (1 mM) at pH 4.0.

Fourier Transform-Ion Cyclotron resonance/MS (FT-ICR/MS). A high sample concentration (DOC > 500 ppm) was required for the analysis. Table S4 compares the number of $m/z$ peaks with assigned chemical formula, the average molecular weight ($M_n$), and percentage of the same $m/z$ peaks among HLA, FLA, and the standard HS (SRFA and SRHA). The number of $m/z$ peaks in SRFA and SRHA was much higher than in HLA and FLA, indicating that natural HS have more diverse chemical compositions than the laboratory-prepared HLA and FLA in frozen conditions. The mass spectra of SRFA and SRHA exhibited broad and evenly distributed peaks in the range of 150−1100 $m/z$ (Figure 4) whereas HLA and FLA samples showed mass peaks in a narrower range of 200−800 $m/z$. However, in general, HLA and FLA exhibited a high level of similarity (70−80%) to SRHA and SRFA in terms of % peaks identical with SRHA (or SRFA). This implies that HLA and FLA generated in the frozen solution of catechol and nitrite are structurally similar to natural humic substances.

**Freeze Concentration Effect.** The increase in the humification rate in the frozen solution can be ascribed to the freeze concentration effect occurring in the unfrozen liquid-boundary layer within the ice sample. When a solution starts to freeze, ice crystals proliferate and expel the solutes into unfrozen areas.[32] The liquid-boundary layer with concentrated solutes remains as a thin interfacial layer between the ice crystals. This unfrozen area, the ice grain boundary, concentrates the solutes in it.[24,32−34] This grain boundary can be observed by various microscopic techniques.[35−37] A confocal Raman microscope was used to observe catechol and nitrite ions concentrated at the ice grain boundary. Figure 5 shows the optical image of the frozen sample and the spacial distribution of catechol and nitrite ions in the same frozen sample. Catechol and nitrite ions exhibit characteristic Raman peak at 772 and 1326 cm$^{-1}$, respectively.[38,39] The Raman signals can be used as a probe to locate the distribution of catechol and nitrite ions in the frozen samples. Dark crack observed in the optical image is the grain boundary layer whereas the bright area indicates the ice crystal region in the frozen solution. The spacial Raman image of the same frozen sample shows that both catechol and nitrite are concentrated in the grain boundary region (Figure 5B and C) as compared with the optical image (Figure 5A). The Raman signals of 772 cm$^{-1}$ (for catechol) and 1326 cm$^{-1}$ (nitrite) are preferentially detected around the grain boundary region of the optical image. This observation provides a clear evidence that the humification reactions of catechol and nitrite should take place in the ice grain boundary region.

**Mechanism of Humification in the Frozen Solution and Environmental Implication.** The humification of phenolic compounds should occur via oxidative polymerization since nitrite ions can serve as an oxidant to initiate the humification process. The humification reaction was enhanced under acidic conditions (Figure 2) where nitrite ions are present in the protonated forms. The nitroacidium ions ($H_2ONO^+$) can be dehydrated to generate nitrosonium ions ($NO^+$) (eqs 4 and 5).[40,41]

$$NO_2^- + H^+ \leftrightarrow HONO \ (pK_a = 3.4) \tag{4}$$

$$HONO + H^+ \leftrightarrow H_2ONO^+ \rightarrow NO^+ + H_2O \ (pK_a = 1.7) \tag{5}$$

Nitrosonium ion ($NO^+$) is a strong oxidant, which can abstract an electron from phenolic compounds to generate phenolic radicals (eq 6).[42]

$$PhOH + NO^+ \rightarrow PhO^\bullet + NO + H^+ \tag{6}$$

$$PhO^\bullet \rightarrow \text{polymerization} \rightarrow \text{FLA, HLA} \tag{7}$$

The produced phenolic radicals can then induce polymerization and lead to the formation of humic-like substances (eq 7).[13,14,16,29−31]

The formation of catechol radicals through reaction with nitrite ions was confirmed by electron paramagnetic resonance (EPR) under aqueous and frozen conditions (Figure 6). The EPR measurements were performed at relatively high catechol and nitrite concentration to obtain high signal intensity for phenolic radicals. No phenolic radicals were produced in the absence of nitrite ions, which implies that the humification reaction of phenols should be initiated by nitrite ions. In the presence of nitrite, more radicals were produced under more acidic condition, which confirms that proton concentration is an important factor in the humification process. Phenolic radical peak which is comparable to previous studies[43] were observed only in frozen solution, not in aqueous solution according to the EPR analysis. This is fully consistent with the observed fact that HLA and FLA are produced only under the frozen condition. In the frozen sample, solutes (protons, nitrites, and phenolic compounds) are migrated from the ice crystal lattice and aggregated in a narrow ice grain boundary region.[24,32−34] The increased proton concentration might accelerate the production of $NO^+$ from nitrite ions in the frozen solution. Under such condition, the enhanced concentration of catechol and $NO^+$ in the ice grain boundary should provide a unique environment where the humification reaction of phenols are highly accelerated.

Humification processes occurring via the decomposition of living organisms and macromolecular biomass substances ("top-down" mechanism) have been studied for a long time.[8,9] However, HS formation from low molecular weight compounds ("bottom-up" mechanism) has been studied only in limited cases. Furthermore, the bottom-up mechanisms induced very slow humification rates[13−18] which might not be significant as HS formation mechanism in nature. This study demonstrated for the first time that HS could be produced from the freezing-induced polycondensation reaction of phenolic compounds initiated by nitrite ions. The produced HS (HLA, FLA) exhibited the structures and compositions similar to those of standard HS (HASR, FASR). Considering that frozen solutions and ice phases are ubiquitous in natural environments (e.g., ice in polar region, seasonal sea ice, snow, aerosols in upper atmosphere, and frozen soils) and that phenolic compounds and nitrite are also common solutes in various aquatic environments, the present observation of freezing-induced humification process presents a new model of "bottom-up" humification mechanism working in cold environments, which was previously unrecognized.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.9b00950.

> Figure S1, Excitation/emission matrix (EEM) of catechol, FLA and HLA; Table S1, rates of production of HLA and $E_{600}$; Table S2, elemental composition of HLA, FLA, and standard humic substances; Table S3, pyrolysis fragments of HLA and FLA detected by GC/MS; and Table S4, information of $m/z$ peaks measured by FT-ICR/MS (PDF)

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### Notes

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