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Humin as an Electron Mediator for Microbial Reductive Dehalogenation

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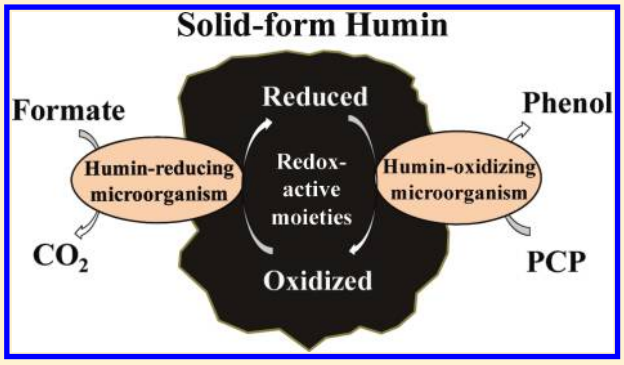
Supporting Information

ABSTRACT: We report that humins extracted as the solid fractions from paddy soils or sediment are involved in extracellular electron transfer, coupled with microbial reductive dehalogenation of pentachlorophenol (PCP), by serving as both electron acceptor and electron donor. In our system, humin is requisite for the dechlorination of PCP, and this activity cannot be maintained when humin is replaced with soluble humic substances or related compounds, including 0.1 M NaOH-extracted humic acid from soil, Aldrich humic acid, and anthraquinone-2,6-disulfonate. The function of humins is stable against treatments with H_2O_2 (30%, 30 min), HCl (0.1 M, 48 h), $NH_2OH{\cdot}HCl$ (0.1 M, 48 h), $NaBH_4$ (0.1 M, 15 h), and heat (121 °C, 30 min). Cyclic voltammograms indicated that humin harbors redox-active moieties, and electron spin resonance suggested that quinone moieties within humin are the redox-active centers. Fourier-transform infrared and nuclear magnetic resonance analyses verified the presence of the aryl carbonyl carbon group in humin. Although the proportion of redox-active carbon is very small, the potential electron-mediating ability is not negligible. The finding that humin, in solid form, is redox active has important implications for in situ bioremediation, given the wide distribution of humin and the diversity and ubiquity of humic substance-utilizing microorganisms.



INTRODUCTION

Humic substances (HSs) are redox-active natural organic macromolecules that are ubiquitous in the environment, including in soils, waters, sediments, peatlands, and bogs.[1−3] The role of HSs in promoting anaerobic bioremediation processes, by shuttling electrons, has been extensively studied during the past two decades because of their enormous application potential.[4,5] However, studies of the redox-mediating effects of HSs supporting the microbial reactions of organic/inorganic compounds under anaerobic conditions have focused on soluble HSs and their quinoid analogues,[5−7] and there are few reports on the HSs present in nature in solid form, namely humin.[1,8] In a recent report, which showed that the sediments in solid form have electron-mediating ability, the humic acids (HAs) were identified as the redox mediator.[9] However, the redox-mediating ability of humin has been neglected.

Pentachlorophenol (PCP) is the most highly chlorinated phenol manufactured. Formerly, it was widely used in wood preservation (fungicide) and other pesticide applications, but has been banned because of the high toxicity to liver and kidney with cancer risks.[10] PCP is still classified as a priority pollutant[11] and listed in the drinking water standards of the United States and the World Health Organization.[12] In Japan, PCP had been widely applied as a pesticide in paddy fields. In anoxic conditions, the microbial reductive dechlorination of PCP has been detected in sediments, soils, and sewage sludges, from which PCP-dechlorinating anaerobic strains have been isolated: *Desulfitobacterium* spp. and *Desulfomonile* spp.[13] However, no isolate has been obtained from paddy soil, despite the well-documented PCP-dechlorinating activity in paddy soil.[14] This was probably because the PCP-dechlorinating activity required the presence of paddy soil, as observed in our anaerobic PCP-to-phenol dechlorinating culture.[15] The role of soil in the dechlorinating culture remained uncharacterized. In this paper, we report the successful replacement of soil with humin and evaluate the role of humin as redox mediator in PCP dechlorination.

Humin is defined classically as the fraction of HSs that is not water-soluble under any pH conditions.[16] The humin fraction comprises a relatively small proportion of organic carbon and a larger proportion of inorganic materials, and the organic carbon is strongly bonded with inorganic materials.[16] Although the humin fraction is considered to contain both humic and nonhumic substances comprising biological molecules of plant and microbial origins, there is no consensus on the fundamental nature of humin[17] because of the lack of an efficient extracting

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solvent for humin.[18] Studies on the function of humin in the environment have been limited to the sorption of various chemicals to humin,[19,20] and its other functions have not been explored.

Here, we demonstrate the function of solid-form humin as an electron mediator in microbial reductive dechlorination of PCP, with chemical, electrochemical, and spectrophotometric characterizations of humin.

MATERIALS AND METHODS

Extraction and Physicochemical Characterization of Humic Substances. Humin and HA were extracted from a paddy soil in Kamajima (KM), Aichi Prefecture, Japan, designated as KMHM and KMHA, respectively, and were used for most of the experiments in this study. Humins extracted from paddy soils in Kuridashi and Yatomi in Aichi Prefecture and from Arako River sediment, designated as KRHM, YAHM, and ARHM, were also prepared. The physicochemical properties of these soils and the sediment have been described previously.[14,21]

To extract HSs, soil was first treated with hydrofluoric acid (HF) to remove the clay minerals and silicate.[22] An air-dried soil sample of 100 g was mixed with 150 mL of 2% HF solution, and shaken at 150−180 rpm at room temperature for 16 h. The mixture was centrifuged, and the supernatant was discarded. This procedure was repeated with shaking durations of 24 h (three times), 48 h (twice), and 64 h (once). The residue was then treated with 0.1 M NaOH according to Schnitzer and Khan.[23] Briefly, the HF-treated residue was shaken with 150 mL of 0.1 M NaOH at 150−180 rpm for 24 h, then centrifuged, and the supernatant was collected as the extract. This extraction process was repeated at least 10 times until the dark color in the extract was not obvious. The collected alkaline extract was acidified to pH < 2 with 2 M HCl, and the formed flocculent precipitate, namely HA, was allowed to coagulate in the dark for 24 h, and was collected through centrifugation. The HA was redissolved in 0.1 M NaOH solution for 1 h, and centrifuged to remove any residual inorganic particulate matter. The HA solution was again acidified overnight to precipitate the HA and remove any residual fulvic acid, and then was again collected by centrifugation. The HA thus obtained was repeatedly rinsed with distilled water until pH was neutralized, and then freeze-dried. The soil residue remaining after exhaustive alkaline extractions, namely humin, was further treated with 200 mL of 2% HF by shaking for 24 h (three times) to concentrate the organic matter. The sample was thoroughly rinsed with distilled water and freeze-dried. All the solutions and distilled water used during the extraction were purged with N_2, and all the centrifugation was carried out at 6230*g* for 10 min.

The contents of carbon, hydrogen, and nitrogen for humin and HA were determined on a Yanaco MT-5 CHN-corder (Yanaco New Science Inc., Kyoto, Japan) by using antipyrine as the standard sample. The ash content was determined by weight. Metal species in the humin were analyzed by inductively coupled plasma atomic emission spectroscopy (Optima 3300DV, PerkinElmer, Yokohama, Japan), after digesting with perchloric acid and nitric acid.

Pretreatment of Humin. To examine if an organic fraction was involved in the dechlorination process, 1 g of KMHM was treated by drying in an oven at 105 °C for 24 h, followed by heating in a muffle furnace at 650 °C for 30 min to obtain organic-burnt KMHM. Hydrogen peroxide treatment was carried out by adding 50 mL of 30% H_2O_2 to 1 g of KMHM; this was then reacted for either 30 min or 15 h with stirring, followed by heat treatment at 90 °C for 45 min, and then washed with distilled water and freeze-dried. The pretreated humins thus obtained were designated as H_2O_2-treated KMHM (30 min) and H_2O_2-treated KMHM (15 h). HCl-treated KMHM was provided as a humin from which HCl-extractable labile metals had been removed. This treatment was carried out by mixing 1 g of KMHM with 100 mL of 0.1 M HCl. The mixture was shaken at 150 rpm for 24 h and centrifuged at 6230*g* for 10 min, and the precipitate was collected. This extraction process was repeated twice. The amorphous Fe(III) oxide was removed from KMHM by using hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$-treated KMHM), where 1 g of KMHM was shaken with 100 mL of 0.1 M $NH_2OH{\cdot}HCl$ at 150 rpm for 24 h, and then collected by centrifugation (6230*g*, 10 min). This extraction process was repeated twice. The chemical reduction of KMHM was carried out by mixing 5 g of KMHM with 100 mL of 0.1 M $NaBH_4$ in *N*,*N*-dimethylacetamide using a magnetic stirrer in a 600-mL bottle at room temperature for 15 h. Prior to the reduction procedure, the solution was thoroughly flushed with nitrogen. After the reduction, acetic acid was added to bring the pH to 7 and eliminate excess $NaBH_4$. $NaBH_4$-reduced KMHM was collected by centrifugation (6230*g*, 10 min) and rinsed with distilled water.

PCP-to-Phenol Dechlorinating Humin Culture. An anaerobic PCP-to-phenol dechlorinating humin culture was enriched from a soil-dependent PCP-to-phenol dechlorinating culture. The soil culture had been transferred using serial 10-fold dilutions for ten generations before transferring to fresh medium using KMHM as a substitute for soil. This humin culture is referred to hereafter as KMHM culture, which was maintained as described below.

For preparation of KMHM culture, 60-mL serum bottles containing 20 mL of mineral medium and 5 g L^{-1} of freeze-dried KMHM were flushed with nitrogen gas, sealed using Teflon-coated butyl rubber stoppers and aluminum seals, and steam-sterilized at 121 °C for 30 min. The bottle headspaces were then flushed with nitrogen gas, while vitamin solution[24] and 0.25 mM of Ti(III)-NTA were added to the medium through a 0.2-μm membrane filter. PCP and formate were added to give the final concentrations of 20 μM and 4 mM, respectively. The PCP-spiked humin medium was inoculated with 1 mL of the culture of the previous generation (about 10^8 cells mL^{-1}) using nitrogen-flushed syringes. The cultures were incubated statically at 30 °C in the dark in an incubator. The mineral medium consisted of (per liter): 1.0 g of NH_4Cl; 0.05 g of $CaCl_2{\cdot}2H_2O$; 0.1 g of $MgCl_2{\cdot}6H_2O$; 0.4 g of K_2HPO_4; 1 mL of trace element SL-10 solution;[25] 1 mL of Se/W solution;[25] and 15 mM MOPS buffer (pH 7.2). After the appropriate intervals, 1 mL of the culture was sampled, and PCP and its metabolites were extracted with acetonitrile and ethyl acetate, and analyzed using a QP5050 gas chromatography−mass spectrometry system (Shimadzu, Kyoto, Japan) equipped with a DB-5MS column (J&W Scientific, Folsom, CA), as described by Yoshida et al.[26]

KMHM culture was used as the inoculum source throughout the entire study unless otherwise stated. The stability of the microbial community structure of KMHM culture as the inoculum was confirmed at regular maintenance intervals by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis targeting the partial 16S rRNA gene of the domain bacteria. The microbial community

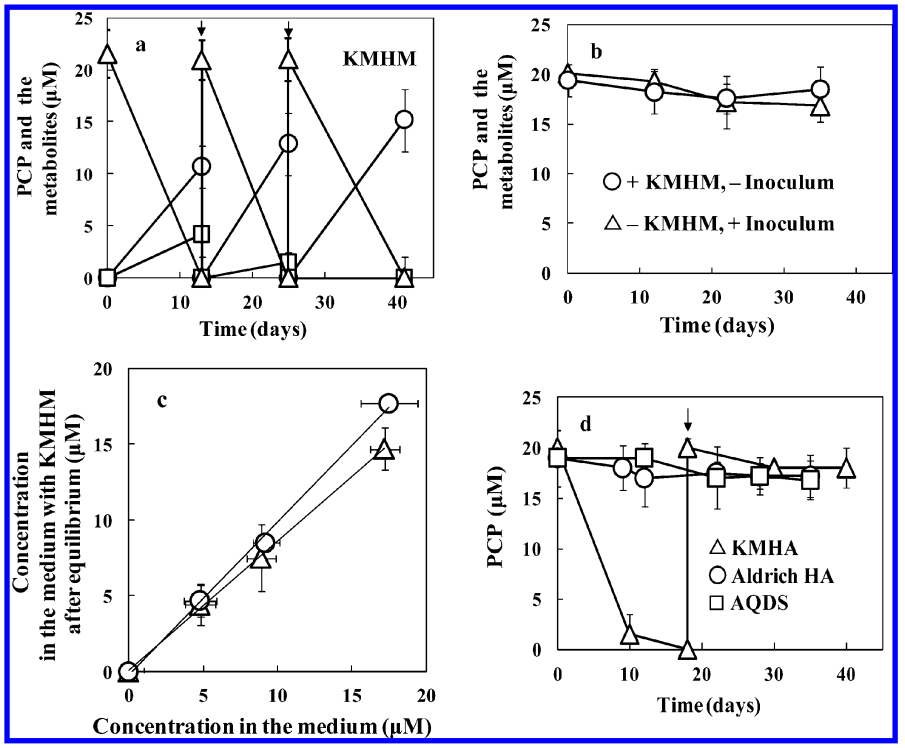

Figure 1. Influence of humic substances on dechlorination of PCP in the enrichment culture and on the concentration of PCP and phenol in solution: (a) reductive dechlorination of PCP (Δ) with metabolites production of 3-chlorophenol (□) and phenol (○) in the culture with 5 g L^{-1} Kamajima humin (KMHM) and 5% inoculum; (b) no dechlorination of PCP under the control conditions with 5 g L^{-1} KMHM and without inoculum (○) and with inoculum but without KMHM (Δ); (c) sorption of PCP and phenol: comparison of the concentrations of PCP (Δ) and phenol (○) between the medium solutions with and without KMHM (5 g L^{-1}) ; (d) no dechlorination of PCP in the cultures with soluble Kamajima humic acid (KMHA) (Δ), Aldrich HA (○), and anthraquinone-2,6-disulfonate (AQDS) (□). Arrows indicate the transfer of 5% of the microcosm to fresh medium spiked with PCP. The experiments were carried out at least three times and the representative results are shown. Data show mean values of duplicate parallel cultures, and vertical bars show the difference in duplicate samples.

structure of KMHM culture was determined by constructing the full-length 16S rRNA gene clone library. All the nucleotide sequence data obtained have been deposited in the DDBJ nucleotide sequence database (http://ddbj.sakura.ne.jp) under accession numbers AB713999–AB714004. The detailed procedure of microbial community analysis is described in Supporting Information.

Influence of HSs on Microbial Reductive PCP Dechlorination. The influence of KMHM, pretreated KMHMs, KRHM, YAHM, ARHM, soluble HSs, and HS-related compounds on the microbial PCP-to-phenol dechlorination activity was examined by incubation experiment, which was carried out in the same way as that of KMHM culture maintenance. All the humins were added as freeze-dried powder and autoclaved with the medium. Soluble HSs and HS-related compounds were sterilized by filtering of stock solutions through 0.2-μm membrane filters and added to the autoclave-sterilized medium. Anthraquinone-2,6-disulfonate (AQDS), 1,4-naphthoquinone, 1,2-naphthoquinone, hydroquinone, vitamin B12, hematin, or hemin was added at a concentration of 250 μM, riboflavin was added at 20 μM, and KMHA or Aldrich humic acid was added at 125 mg/L. Preparation procedures of stock solutions of HSs and related compounds are described in Supporting Information. The influence on the dechlorination was judged after at least two generations of incubation. All the experiments were conducted in duplicate sets, and each set was repeated at least three times to confirm the results.

Sorption Experiment. PCP or phenol was added at concentrations of 5 μM, 10 μM, or 20 μM to 60-mL serum bottles containing 20 mL of mineral medium amended with/without KMHM (5 g L^{-1}) and incubated for 5 d under the same conditions as those used for the humin cultures. KMHM was removed by centrifugation, and the concentration of PCP or phenol in the supernatant was analyzed.

Microscopic Observation of Microorganisms. To achieve better visualization, the amount of KMHM in the culture was decreased to 0.5 g L^{-1}. An aliquot of the culture suspension (1 mL) was transferred to a 1.5-mL tube, and the microbial cells were collected by centrifugation at 21 880*g* for 1 min, stained with 0.01% ethidium bromide for 15 min, and then rinsed three times with 0.1 M phosphate-buffered saline.[27] The microbial cells were resuspended in 1 mL of 0.01% ethidium bromide, and placed on a 0.22-μm polycarbonate black membrane (Millipore, Bedford, MA, USA). The samples were observed with an epifluorescence microscope (BX50W1, Olympus, Tokyo, Japan) equipped with a WIG cube (excitation 520–550 nm, emission >580 nm) and a DP7 digital camera (Olympus).

Cyclic Voltammetry. Cyclic voltammetry measurements were carried out on an HZ-5000 automatic polarization system equipped with an HX-111 electrolytic cell (Hokuto Denko Inc., Osaka, Japan), consisting of a platinum working electrode, an Ag/AgCl (saturated KCl) reference electrode, and a platinum auxiliary electrode at room temperature. Cyclic voltammograms (CVs) of humin samples were obtained using dimethyl sulfoxide (DMSO) as solvent, referring to Nurmi and

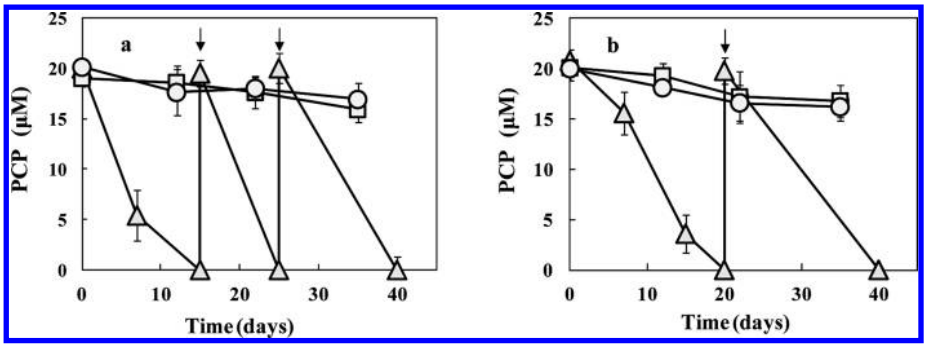

Figure 2. Influence of the pretreatments in KMHM, the inoculum and electron donor on the reductive dechlorination of PCP. Formate and inocula were provided in all the incubations unless otherwise indicated. (a) Reactions in the enrichment cultures with H_2O_2-treated KMHM (30 min) (Δ), with organic-burnt KMHM (□), and with H_2O_2-treated KMHM (15 h) (○). (b) Reactions in the culture with $NaBH_4$-reduced KMHM and without formate (Δ), in the culture with KMHM and without formate (□), and in the medium with $NaBH_4$-reduced KMHM but without inoculum (○). Arrows indicate the transfer of 5% of the microcosm to fresh medium spiked with PCP. The experiments were carried out at least three times and the representative results are shown. Data show mean values of duplicate parallel cultures, and vertical bars show the difference in duplicate samples.

Tratnyek.[28] The experimental conditions were as follows: 0.2 M Na_2SO_4 electrolyte, 10 mL of DMSO solvent, 5 g L^{-1} humin sample, 25 mV s^{-1} scan rate, and potential range from −0.25 to −2.0 V (vs Ag/AgCl). All test solutions were purged with high-purity N_2 for 15 min before each set of scans to remove oxygen. Background scans were performed prior to every measurement, and proper performance of the overall system was periodically verified by reproducing CVs for the ferrocyanide/ferricyanide redox couple.

Spectroscopic Characterization of Humin. Electron spin resonance (ESR) spectra were recorded at room temperature with a JES-FA200 ESR spectrometer (JEOL Co. Ltd., Tokyo, Japan), operating at 9.4 GHz with a 100 kHz modulation of the steady magnetic field. The measurement was carried out using 15 mg of humin in quartz glass tubes with an inner diameter of 4 mm. Other spectrometric conditions for the measurements were as follows: magnetic field centered at 336.4 mT, 0.998 mW microwave power, 30 s sweep time, and 0.03 s time constant. To examine the effect of pH on the signal intensity of the ESR radicals produced, the pH of humin was adjusted using either 0.1 M HCl or 0.1 M NaOH to pH 3 or pH 11, and freeze-dried.

Fourier-transform infrared (FTIR) spectra were determined using a KBr pellet technique in the 400−4000 cm^{-1} range using a JASCO FT/IR-6100 spectrometer (JASCO, Tokyo, Japan), with a resolution of 4 cm^{-1} and eight accumulations. The spectra were corrected against pure KBr and ambient air.

Solid-state ^{13}C CP/MAS nuclear magnetic resonance (NMR) spectra were acquired using an ECA-700 spectrometer (JEOL Co. Ltd.) with resonance frequency of 176 MHz, sweep speed of 70.4 kHz, ramp-CP contact time of 2 ms, 1 s recycle delay, and 80 000 scans per sample.

RESULTS

Physicochemical Characterization of HSs Extracted from Soil. The carbon content of KMHM was 17.8%, which is equivalent to 1.43 mg-C g $soil^{-1}$, given the recovery of 8 mg-KMHM g $soil^{-1}$. KMHA had a carbon content of 53.9%, which is equivalent to 6.13 mg-C g $soil^{-1}$. The carbon content of KM soil was 10.1 mg-C g $soil^{-1}$. This carbon distribution was consistent with the nature of HSs. KMHM also contained 2.71% hydrogen, 1.28% nitrogen, and 60.28% ash, and KMHA contained 4.93% hydrogen, 4.15% nitrogen, and 0.05% ash. KMHM contained a much higher concentration of ash than did KMHA. KMHM contained considerably higher concentrations of iron (2.39 mg-Fe g $KMHM^{-1}$) and cobalt (3.19 mg-Co g $KMHM^{-1}$), while low concentrations of Cr, As, Mn, and Cu were detected, and Ni, As, Se, and Mo were below the detection limit. The high concentrations of Fe and Co in KMHM may have formed during the extraction of KMHM in alkaline conditions using large amounts of soil.[29,30]

Characterization of KMHM Culture. Stable PCP-to-phenol dechlorination activity was maintained in KMHM culture for more than 30 transfers using 5% by volume of inoculation at intervals of 10−25 d. The representative results are shown in Figure 1a and b. The activity was not observed without inoculation, suggesting that PCP dechlorination was microbial activity. The dechlorination pathway was determined as being from PCP to 2,3,4,5-tetrachlorophenol, 3,4,5-trichlorophenol, 3,5-dichlorophenol, 3-chlorophenol, and then to phenol. Humin was requisite for the PCP-dechlorinating activity in KMHM culture, as no dechlorination activity was observed in the absence of humin. The minimum amount needed for dechlorinating 20 μM PCP was 10 mg KMHM 20 mL^{-1} culture. Although the PCP concentration was decreased by 20% by the sorption to humin, metabolite production was not observed in the system where phenol was not sorbed to humin at all (Figure 1c), suggesting only a slight sorption of PCP to humin and no chemical reaction between PCP and KMHM.

The stability of the microbial community structure in KMHM culture was confirmed prior to the inoculation for further study by PCR-DGGE analysis targeting the domain bacteria. A full-length 16S rRNA gene clone library revealed that KMHM culture, based on operational taxonomic units at a level of sequence similarity ≥97%, was dominated by *Sulfurospirillum* spp. (32 out of 82 clones; see Supporting Information Figure S1), *Macellibacteroides* spp. (26 clones), and *Clostridium* spp. (16 clones). Two clones of *Dehalobacter* spp. were also detected, which are known obligate dehalogenators. The microbial community composition was different from that of the original soil culture, which showed a broader bacterial diversity and was predominantly *Clostridium* species.[26]

Influence of HSs on Microbial Reductive PCP Dechlorination. KMHM was able to sustain dechlorination activity stably, whereas KMHA was unstable in mediating the activity in the dissolved form, as indicated by the loss of PCP-dechlorinating activity after subculture (Figure 1d). We

reincubated the KMHA-amended cultures several times; in the best case, the activity was preserved for two generations, but in most cases, the activity only showed in the first generation, or no dechlorination was observed. Humins obtained from the other paddy soils and sediment, namely KRHM, YAHM, and ARHM, also stably mediated the PCP-dechlorinating activity (Supporting Information Figure S2). It is therefore suggested that the solid-form humin played a role in maintaining the PCP dechlorination in various environments.

We also examined the mediating activity of various soluble HSs and related compounds (Figure 1d and Supporting Information Figure S3). Hematin, hemin, and riboflavin showed mediating activity in the first incubation, but the activity was not maintained after subculture. Other compounds, including Aldrich humic acid, AQDS, 1,4-naphthoquinone, 1,2-naphthoquinone, hydroquinone, and vitamin B12, showed no dechlorination activity. The stable mediating function of solid-form humin in the PCP dechlorination was attributable neither to sorption, which had only a slight effect (Figure 1c), nor to the PCP toxicity, which, at a concentration of 20 μM, was below the minimum inhibitory concentration to most microorganisms.[31]

Electron-Mediating Fraction in Humin. Humin comprises both organic and inorganic fractions, and removal of or change in the fractions of humin during pretreatments suggested the role of humin in the microbial reductive PCP dechlorination. Partial oxidation of the organic fraction in H_2O_2-treated KMHM (30 min) did not result in the loss of mediating activity in PCP dechlorination, but the activity was lost when the organic fraction was substantially destroyed as in the cases of organic-burnt KMHM and H_2O_2-treated KMHM (15 h) (Figure 2a and Supporting Information Figure S4a). The carbon content of humin (17.8%) decreased to 7.5% in H_2O_2-treated KMHM (30 min) and then to 2.6% in H_2O_2-treated KMHM (15 h). The NH_2OH·HCl and HCl treatments did not influence the PCP-dechlorinating activity (Supporting Information Figure S4bc). When $NaBH_4$-reduced KMHM was used without formate as an electron donor, the PCP-dechlorinating activity was stably maintained although at a lower activity level, while intact KMHM without formate did not result in PCP dechlorination (Figure 2b). This activity of $NaBH_4$-reduced KMHM was observed only with the inoculation (Supporting Information S4d), which was the same as that of intact KMHM.

Microscopic Observation of Microorganisms Attached to Humin. Fluorescence microscopic observation showed that most of the microbial cells were attached to the solid-form KMHM, and were observed especially in the organic fraction (Figure 3). Ethidium bromide in the fluorescent stain binds not only with nucleic acids of microorganisms, but also with organic matter nonspecifically, although only weakly. Therefore, a dimly lit background is obtained in the organic matter fraction of humin. The total number of microbial cells attached to or embedded in humin was about 8×10^7 cell mL^{-1}, and the number of microbial cells in the free form on the filter was about 1×10^4 cell mL^{-1}.

Electrochemical Properties of Humin. The presence of redox-active moieties in the humin samples was shown by CVs (Figure 4). Much more current was produced in the presence of KMHM compared with H_2O_2-treated KMHM (15 h), resulting in a CV with a wider shape. The same results were obtained for KRHM, YAHM, and ARHM (Supporting Information Figure S5). As HSs generally produce CVs with

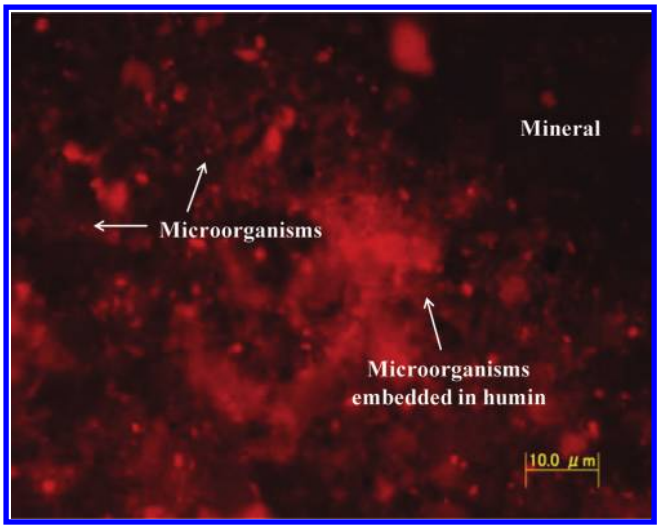

Figure 3. Fluorescence microscopy of KMHM culture. Fluorescence microscopy showed that most of the microorganisms (bright red) were embedded in the humin (weakly stained) observed in the PCP-dechlorinating humin culture stained with ethidium bromide in phosphate buffer.

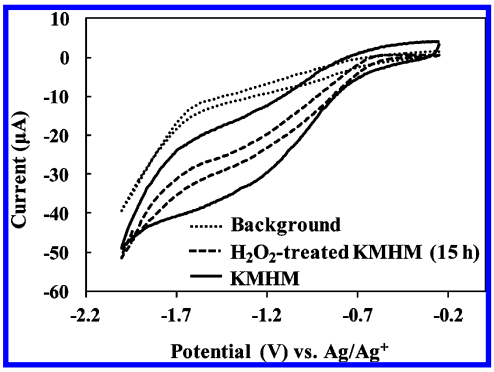

Figure 4. Cyclic voltammograms of intact and H_2O_2-treated KMHM (15 h) using DMSO as solvent. "Background" refers to the condition without humin sample.

little or no useful structure because of the lack of electrode activity,[32] we assessed the redox potential of humin by applying both aqueous solution (mineral medium) and nonaqueous solvent (DMSO). The redox potential of humin was estimated from CVs in DMSO by the correction of Nurmi and Tratnyek,[28] namely that the potential in DMSO vs Ag/AgCl equals the potential in H_2O vs SHE plus 0.45 V. Based on this correction to peak potentials, the redox potential of humin was estimated at −0.08 V, which agreed well with the results obtained using medium/humin under aqueous conditions (Supporting Information Figure S6). Although the mechanism of how DMSO influences the interaction of humin with Pt electrodes requires further investigation, it is known that DMSO can improve the response of quinone moieties on Pt electrodes because of the strong chemisorption of DMSO to Pt, which surpasses the formation of comparatively unreactive surface complexes between the quinone moieties and Pt.[33] We therefore attribute the improved response of humin observed in DMSO to the activated quinones as redox-active moieties in humin.

Spectroscopic Properties of Humin. KMHM showed an ESR signal at g = 2.0042, around which the organic radicals typically appear, whereas the H_2O_2-treated KMHM (15 h)

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showed little ESR signal. KMHM adjusted at pH 11 produced a remarkable increase in radical signal compared with that of KMHM at pH 3 (Figure 5). The same tendency was observed in KRHM, YAHM, and ARHM (Supporting Information Figure S7). The increase in ESR signal at high pH is typical for the semiquinone-type radicals, which supports the interpretation that quinone moieties were responsible for the observed signal increase.[34,35] However, we cannot exclude the contribution of ESR signals from other origins such as thiols, nitrogen functional groups, or metal−organic complexes.

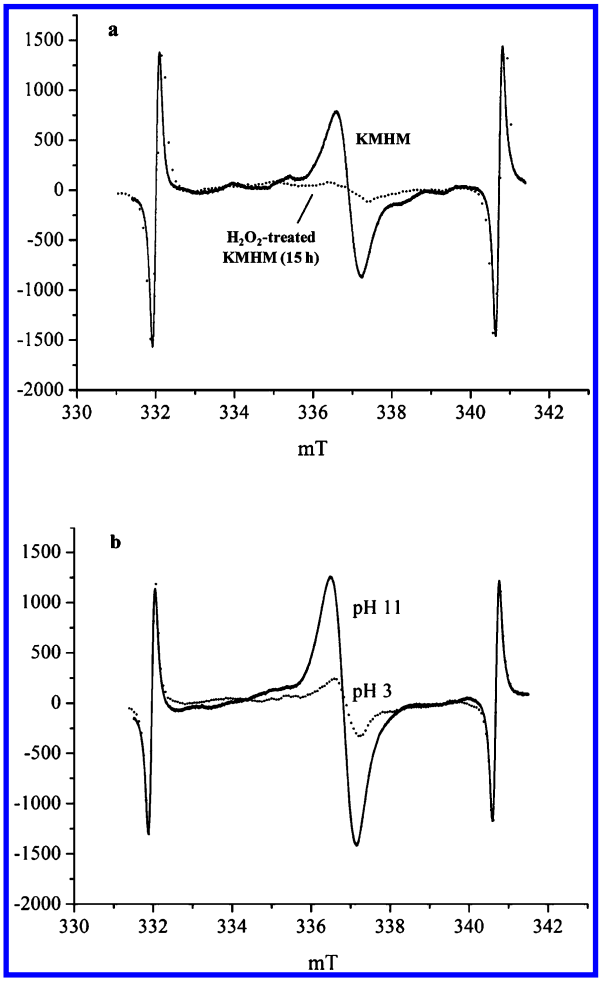

Figure 5. ESR spectra of humin samples. (a) Intact KMHM and H_2O_2-treated KMHM (15 h) at pH 7. (b) KMHM at pH 3 (dotted line) and pH 11 (solid line). For each ESR analysis, 15 mg of dry humin sample was used. The two peaks to the right and left of the organic radical peak are from a manganese calibration standard.

The FTIR spectrum of KMHM suggested the presence of the aryl carbonyl carbon group at 1650 cm^{-1}, 1550 (shoulder) cm^{-1}, and 1500 cm^{-1}, according to Tatzber et al.,[36] while the H_2O_2-treated KMHM (15 h) lost all these bands (Figure 6a). The NMR spectrum showed that the relative abundance of the aryl carbonyl carbon group in KMHM was estimated at about 1%, by integration based on the magnetic range for aryl carbon as reported previously (Figure 6b).[37,38] Given the carbon content of 17.8% in KMHM and the requisite minimum amount of 10 mg-KMHM 20 mL^{-1} culture for dechlorinating 20 μM PCP, the aryl carbonyl carbon content was calculated as

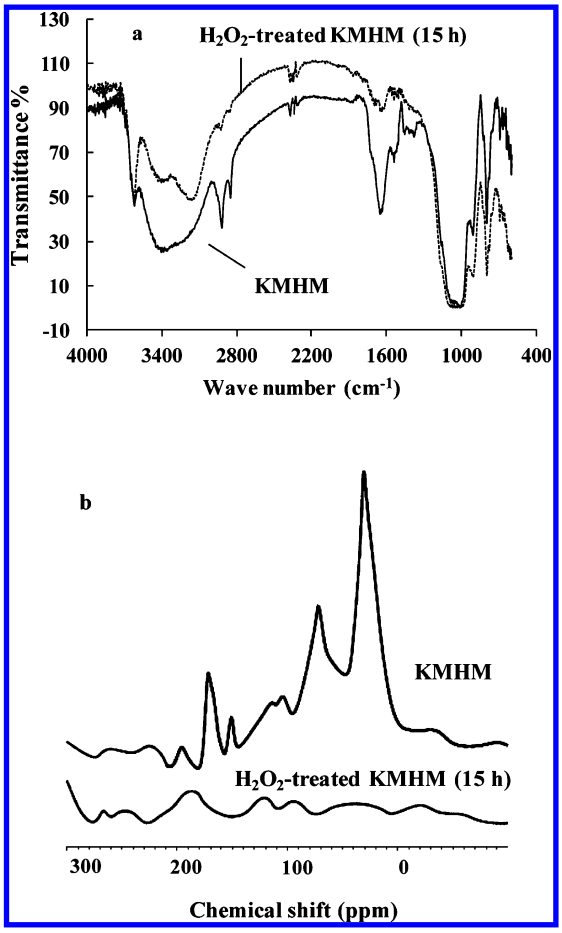

Figure 6. FTIR and solid-state ^{13}C CPMAS NMR spectra of KMHM and H_2O_2-treated KMHM (15 h). For FTIR spectra, transmittance values of the H_2O_2-treated KMHM (15 h) were increased by adding 15% for the purpose of comparison. For NMR spectra, the relative abundance of C was calculated according to the following chemical shift regions: aliphatic carbon (0−45 ppm), 64%; carbohydrate carbon (45−100 ppm), 19%; aromatic carbon (100−160 ppm), 1%; and carboxylic carbon (160−215 ppm), 16%.

17.8 μg, indicating that 1.48 μM of redox-active carbon, at most, was involved in the dechlorination of 20 μM PCP.

DISCUSSION

In this study, we have demonstrated that humin functions as a chemically stable solid-form redox mediator in anaerobic PCP-to-phenol dechlorinating cultures. Humin from different origins—KMHM, KRHM, YAHM, and ARHM—showed stable mediating activity in the PCP-to-phenol dechlorination. By contrast, KMHA and Aldrich humic acid could not maintain the dechlorination activity. We reported previously that the soil was an essential factor for maintaining the PCP-dechlorination activity.[15] The results in this study indicated that humin was the main functional fraction for mediating the PCP-dechlorinating activity in soil and sediment. The chemical stability of the humin function was also shown: against acids (exposure to 2% HF, and to 0.1 M HCl for 48 h), alkaline (0.1 M NaOH), heat (autoclaving 121 °C, 30 min), chemical oxidation (30% H_2O_2 for 30 min), and chemical reduction (0.1 M $NH_2OH{\cdot}HCl$ for 48 h, and 0.1 M $NaHBH_4$ for 15 h). These findings on ubiquity and chemical stability suggest that humin plays an extensive role as the solid-form mediator of the anaerobic microbial

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reaction in various environments. To date, this role has been neglected.

The presence of redox-active moieties in humin is shown by the wider CV of intact KMHM compared with that of H_2O_2-treated KMHM (15 h). When $NaBH_4$-reduced KMHM was used, PCP was dechlorinated without formate in the culture, whereas dechlorination was not observed in the absence of inoculum, demonstrating that the reduced form of KMHM did not reduce PCP by chemical reaction, but needed the involvement of microorganisms for the electron transfer (Figure 2b). When intact KMHM (not reduced) was used, PCP was not dechlorinated without formate in the culture. Furthermore, in the absence of humin, the culture did not dechlorinate PCP even with the presence of formate. These findings suggest that microorganisms in the culture used formate as the electron donor coupled with the reduction of KMHM. Formate is known to be used directly as an electron donor by halorespiring bacteria for reductive dehalogenation; however, this was not the case in this reductive dechlorination of PCP. Thus, it is considered that humin served as an electron acceptor for the microbial anaerobic oxidation of formate, and the reduced humin was used as an electron donor for the microbial reduction of PCP (electron acceptor). The standard redox potential, *Eo*′, of formate (to CO_2) is −0.430 V[39] and the *Eo*′ of PCP (to 2,3,4,5-tetrachlorophenol) is +0.399 V.[40] The redox potential of humin was estimated at −0.08 V. The intermediate value of redox potential between formate and PCP is consistent with the function of humin as electron mediator between the two compounds.

Soluble HSs and the related compounds did not maintain the dechlorinating activity of the culture. We carried out a supplementary experiment to examine the redox-mediating functions of KMHM, Aldrich humic acid, and AQDS in the reduction of amorphous Fe(III) oxide by *Shewanella putrefaciens* strain CN-32 (see Supporting Information for the detailed procedure). The results (Supporting Information Figure S8) demonstrate that KMHM, Aldrich humic acid, and AQDS enhanced the microbial reduction of amorphous Fe(III) oxide, suggesting positive redox-mediating functions of all these substances. These findings suggest the presence of multiple redox-mediating functions in humin; one type of function is common to both humin and the soluble HSs and related compounds, whereas the other type is specific to humin, which facilitates PCP dechlorination. The positive mediating function observed in the first generation of soluble HSs and the related compounds may be attributable to the presence of carryover of KMHM brought in through the inocula.

Study using pretreated KMHM suggests that the redox-mediating function of humin is attributable to the organic fraction, which proved labile to oxidization with 30% H_2O_2 during reaction times from 30 min to 15 h. It was unlikely that labile metals and amorphous Fe(III) oxide were involved in the functional component as suggested by the experiments using $NH_2OH{\cdot}HCl$- and HCl-treated KMHM. The involvement of the organic fraction in the electron mediation agrees with the observation that most of the microbial cells were attached to the organic fraction of humin (Figure 3). The ESR signals of active/inactive humins and humins at different pH suggested the presence of semiquinone-type radicals.[35] FTIR also suggested the presence of carbonyl carbon and carbon−carbon double bonds in the active humin, whereas all these bands were lost in H_2O_2-treated humin (15 h). The ESR signals of organic radicals and carbonyl carbon in FTIR did not disappear in H_2O_2-treated KMHM (30 min), $NH_2OH{\cdot}HCl$-treated KMHM, and HCl-treated KMHM, which were the active humins (Supporting Information Figures S9 and S10, respectively). The NMR spectra showed that carbonyl carbon was present as 1% of total carbon in the active humin but was absent in the inactive humin. These findings suggest that the quinone structure in the organic fraction of solid-form humin functioned as electron mediator. However, the quinone structure is known to be present in the soluble HSs. Contribution of other types of electron-mediating moieties such as metal−organic complexes cannot be excluded, given the multiple functions suggested by the enhanced reduction of the amorphous Fe(III) oxide. Humin also contained a large proportion of inorganic materials, especially Fe and Co in KMHM. The microbial attachment to the solid-form humin may also explain the difference between soluble and solid HSs, as various microbial species may be organized on the interface in the electron-mediating reactions. Further study is required to explore the electron-mediating nature of humin.

The finding that humin in its solid form is redox active has important implications for in situ bioremediation, given the wide distribution of humin as well as the diversity and ubiquity of HS-utilizing microorganisms.[41−43] It is also suggested that, in many environments, humin may exhibit a previously unrecognized stable electron-mediating capacity that supports the anaerobic microbial world. In addition, we have been maintaining another humin-mediated culture that debrominates tetrabromobisphenol A (TBBPA), in which *Dehalobacter* spp. and *Geobacter* spp., as well as *Sulfurospirillum* spp., predominate. The same humin works as an electron mediator for TBBPA debromination as well as for PCP dechlorination. This suggests that humin may be the naturally occurring solid electron acceptor/donor for the widespread application of halorespiring microorganisms.

■ ASSOCIATED CONTENT

Supporting Information

Detailed descriptions of the procedures for the preparation of soluble HSs; influence of HSs on microbial reduction of amorphous Fe(III) oxide; microbial community analysis of KMHM culture, and the results of microbial community composition of KMHM culture (Figure S1); dechlorination activity in humin samples extracted from paddy soils and river sediment (Figure S2); effect of soluble HS-related compounds on the dechlorination activity (Figure S3); effect of pretreatments of humin on the dechlorination activity (Figure S4); CVs of different humin samples using DMSO as solvent (Figure S5); CVs under aqueous conditions (Figure S6); ESR spectra of humin samples extracted from different soils/sediment at different pH (Figure S7); influence of HSs on microbial reduction of amorphous Fe(III) oxide (Figure S8); spectroscopic properties of chemically treated humin, including the ESR spectra (Figure S9) and FTIR spectra (Figure S10). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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