

Using Humic Fractions to Understand Natural Organic Matter Processes in Soil and Water: Selected Studies and Applications

D. C. Olk,* P. R. Bloom, M. De Nobili, Y. Chen, D. M. McKnight, M. J. M. Wells, and J. Weber

Abstract

Natural organic matter (NOM) plays key environmental roles in both aquatic and soil systems. A long-standing approach for evaluating NOM composition and activity is to extract soils with alkali solutions to obtain humic substances, namely humic acids (HA), and fulvic acids (FA), or to briefly expose isolated fractions of dissolved organic matter to alkali. Critics have claimed these methods create laboratory artifacts and are thus unsuitable for studying NOM behavior in field conditions. In response, we describe case studies in which humic fractions were analyzed to identify significant processes in environmental or agricultural issues. Specifically, humic fractions played a key role in maintaining toxic levels of arsenic (As) in drinking water supplies in South and Southeast Asia. Elsewhere, binding reactions of FA and HA with prions were shown to provide a plausible mechanism for variable persistence of prion infectivity across soil types. Humic substances were also shown to enhance iron (Fe) uptake by plants in solution culture and field conditions. Their specific binding sites for mercury (Hg) as determined in laboratory conditions enabled accurate modeling of soil Hg binding under varying conditions. A young HA fraction reproduced in controlled conditions the capacity of animal manure to maintain potassium (K) availability in strongly K-fixing field soils, leading to development of a commercially successful humic-K fertilizer. Humic fractions accurately represented NOM across multiple settings and research objectives while providing novel opportunities for advanced analyses. The study of humic fractions has helped resolve scientific and practical issues in aquatic and soil systems.

Core Ideas

- Humic substances (HS) affect As, Hg, and Fe cycling in water, sediment, and soil matrices.
- Humic binding of prions may control the persistence of their infectivity across soil types.
- A humic fraction simulated the organic component of manure in improving soil K cycling.
- Soil and aquatic HS responded in quantity and composition to location and land use.

SOIL SCIENTISTS have used humic acid (HA) and fulvic acid (FA) fractions, prepared using alkali extraction methods, for >200 yr to study the structure and function of soil organic matter (SOM). Over the last few decades, aquatic scientists have used similar fractions of dissolved organic matter (DOM), extracted by resin adsorption followed by brief alkali desorption. Critics have claimed that alkali-extractable and alkali-exposed fractions are laboratory artifacts and are thus unsuitable for studying natural organic matter (NOM) structure and function in field conditions (Kleber and Johnson, 2010; Schmidt et al., 2011; Lehmann and Kleber, 2015; Kleber and Lehmann, 2019). Moreover, it has also been alleged that, because of the incomplete extraction of SOM, these fractions cannot offer a suitable approach to investigate environmental and agricultural functions of soils.

In a recent review, we addressed major issues raised by critics about the suitability of HA and FA, collectively named humic substances (HS), for use in environmental and agricultural research (Olk et al., 2019). Specifically, these issues included (i) the potential for chemical alteration during alkali extraction, (ii) a presumed necessary linkage of alkali extractions to belief in a macromolecular structure of HS, and (iii) an alleged lack of evidence for the occurrence of “humification,” which would bring about unique chemical structures in soil and water not inherited directly from organisms.

Responding to these allegations, Olk et al. (2019) drew on literature reviews to first conclude that HS are composed of a vast array of components. They contain both biological molecules and the products of humification reactions, and the majority of their components have molecular weights much lower than thought several decades ago. These traits are inconsistent with a single macromolecular structure of HS, as presented in variants by Stevenson (1994). As discussed by Olk et al. (2019),

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Abbreviations: DOM, dissolved organic matter; EXAFS, extended X-ray absorption fine structure; FA, fulvic acid; HA, humic acid; HS, humic substances; IHSS, International Humic Substances Society; MHA, mobile humic acid; NMR, nuclear magnetic resonance; NOM, natural organic matter; SOM, soil organic matter; TSE, transmissible spongiform encephalopathies; XANES, X-ray absorption near edge structure.

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practitioners of alkali extractions have proposed multiple chemical structures of NOM, in contrast with the belief by Lehmann and Kleber (2015) that the use of alkali extractions is necessarily linked to belief in a single macromolecular structure. A second conclusion by Olk et al. (2019) was that documented chemical alterations to HA and FA that were caused by exposure to sodium hydroxide (NaOH) appear to be so minor that they do not diminish the validity of humic fractions in representing NOM. Third, multiple lines of evidence in natural conditions indicate preferential decomposition over time of carbohydrates and other labile compounds together with the occurrence of abiotic reactions involving phenols and lignin residues that create new structures unlike biological molecules, consistent with the occurrence of humification. Olk et al. (2019) concluded overall that HS are not laboratory artifacts formed through alkali-induced reactions, and they are suitable for the study of NOM processes in field conditions.

Olk et al. (2019) also presented several case studies in which HA and FA were extracted from soils, waters, and organic materials to address meaningful problems across diverse research settings. They demonstrated that an understanding of HS properties can be key to understanding issues including environmental pollution due to toxic metals, pesticides, or persistent organic pollutants, and the effects on soil performance of organic amendments and other land management practices. Also, a modified alkali procedure for extracting a labile HA fraction not bound to soil Ca was proven suitable for understanding N cycling in intensively cropped paddy rice (*Oryza sativa* L.) soils, which led to improved crop management options for reversing long-term grain yield declines.

In the current review, we continue the report of Olk et al. (2019) by describing additional case studies in environmental research using humic fractions to understand important processes involving NOM. These selected studies include both soil and aquatic systems and involve NOM interactions with toxic metals, plant-essential nutrients, and prions. They also demonstrate the responsiveness of HS in composition and mass to environmental factors, including location, land use, and source material.

Humic Electron Shuttling Contributes to Groundwater Arsenic Toxicity in South and Southeast Asia

The exposure of >50 million people in communities in South and Southeast Asia, especially Bangladesh, to toxic levels of dissolved geogenic arsenic (As) in groundwater water supplies is considered to be the greatest mass poisoning in human history. This exposure was the inadvertent consequence of the introduction of shallow tube wells to access groundwater supplies in extensive wetland regions underlain with sediment from the Himalayas. The intent was to provide an alternative drinking water supply to surface waters that had become compromised by water-borne pathogens. Extensive research to address this problem has shown that the primary source of the As is geogenic and derived from weathering of eroded sediments from the Himalayas deposited over millennia (Polizzotto et al., 2005). In the sediments, the As is associated with iron (Fe) oxides and is released into the groundwater through reductive dissolution of the oxide surfaces driven by microbial respiration of labile organic substrates in

the groundwater (Zheng et al., 2004; Polizzotto et al., 2008). In addition to labile components of DOM, dissolved HS in these groundwaters can influence the release of As from sediments by both forming complexes with As and by acting as an electron shuttle, whereby bacteria that oxidize labile DOM transfer electrons to HS, which in turn reduce Fe oxides (Mladenov et al., 2010, 2015). Overall, the microbial degradation of the labile components of DOM is promoted by this cascade of electron transfer reactions. Further, the reductive dissolution at Fe oxide surfaces can also release HS that were adsorbed during sedimentary deposition in wetlands, as was demonstrated in earlier studies in freshwater wetlands (Chin et al., 1998). This redox cascade is illustrated in Fig. 1 (Kulkarni et al., 2018).

Consideration of the role of humic DOM in the mobilization of As can help to interpret variations in the relationships between DOM and As concentrations, with significant positive correlations (Anawar et al., 2003), significant negative correlations (Bhattacharya et al., 2009), or no correlations at all (Hoang et al., 2010) having been reported in different studies. The potential importance of the release of sedimentary humic DOM into groundwater in the Araihasar subdistrict in Bangladesh was indicated by the co-occurrence in groundwater of high As concentrations ($400 \mu\text{g L}^{-1}$) with increased dissolved organic carbon (C) concentrations having spectroscopic properties, such as relatively high specific ultraviolet absorbance at 254 nm (SUVA) and low fluorescence index, indicative of humic DOM derived from a plant–soil source that was released into the groundwater from a sedimentary source (Mladenov et al., 2010, 2015). Further, recent laboratory experiments of the electron shuttling capacity showed that humic DOM isolated from groundwater zones with high As concentrations in an aquifer in Bangladesh had comparable or much higher electron shuttling capacity than

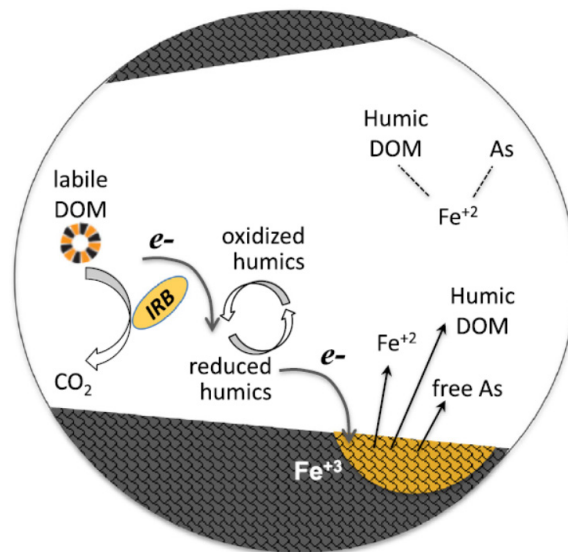


Fig. 1. Conceptual model of humics-enhanced microbial reductive dissolution by Fe-reducing bacteria (IRB), in which dissolved humics (humic dissolved organic matter, DOM) serve as electron shuttles to accelerate Fe(II) release and mobilization of As into solution from soil mineral matrix (gray patterned areas). The resulting increase in concentrations of free Fe(II) allows for greater complexation of Fe with other molecules (e.g., DOM and As ternary complex with Fe), which lowers the activity of free Fe(II) and diminishes surface complexation by Fe(II). Precipitation and competitive sorption reactions are not shown. Reprinted with permission from Kulkarni et al. (2018).

DOM in other aquatic humic studies (Fig. 2). The Bangladeshi groundwater FA sample (isolated using XAD-8 resin) with the highest electron shuttling capacity matched the capacities of HS extracted from solid phases (e.g., a FA extracted from the sediments of a subalpine lake and HAs extracted from soils).

These results demonstrate the importance of humic DOM in the redox cascade that drives the release of toxic As to the groundwater. In addition, these results indicate that an additional approach to assessing water quality, and the risk of As exposure in these regions could be simple spectroscopic measurements of DOM quality that can be made quickly and locally. In particular, values of specific ultraviolet absorbance and fluorescence index that are indicative of a plant–soil humic DOM source would suggest a risk of toxic As concentrations, which could be further evaluated with direct As measurements.

Additional evidence for the importance of the fractionation by “XAD” resin in aquatic research has been demonstrated by studies of membrane fouling in water purification. Separating DOM into HA, FA, and hydrophilic fractions has been key to understanding which components of DOM are important in the fouling of membrane filters of differing chemical compositions (see the supplemental material).

Reduction in Infectivity of Soil Prions

Enzyme encapsulation by NOM and HS in particular has been recognized for decades (Skujiņš and Burns, 1976). Proposed mechanisms depend on both amino acid composition and structural arrangement of the protein that allow interaction with hydrophobic HS moieties causing its physical entrapment within the HS (Zang et al., 2000; Hsu and Hatcher, 2006). The process is favored when the solution pH is sufficiently low to induce aggregation of humic molecules into supramolecular arrangements. Water-protected hydrophobic interior voids may thus form through hydrogen (H)-bonding among small molecules, generating an expanded three-dimensional arrangement (Ferreira et al., 2001). Molecular-level evidence for encapsulation of net positively charged proteins by HS in nanostructured films, driven by electrostatic attraction between the positively charged protein surface and the negatively charged HS molecules, was provided by combining optical waveguide lightmode spectroscopy and quartz crystal microbalance measurements (Tomaszewski et al., 2011). Encapsulation of trypsin and ribonuclease A, which to the contrary bear negatively charged surfaces, suggested that hydrophobic effects can overcome localized charge repulsion when the pH is sufficiently low to reduce surface charge density, fostering entrapment within the HA matrix (Tomaszewski et al., 2011). Encapsulation enhances resistance against proteases (Zang et al., 2000) and preserves activity of soil enzymes, albeit at lower levels, by inducing conformational changes (Ding et al., 2011) and/or hampering access of substrates to the active site (Chen et al., 2004b).

Prions are the well-known proteinaceous infective agents of transmissible spongiform encephalopathies (TSE), a class of fatal neurodegenerative diseases that includes bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep, and chronic wasting disease in cervids. Their pathogenic activity may persist in soil for years (Brown and Gajdusek, 1991; Seidel et al., 2007). However, within the large spread of potentially infected lands, prion diseases have become endemic only in geographically limited regions.

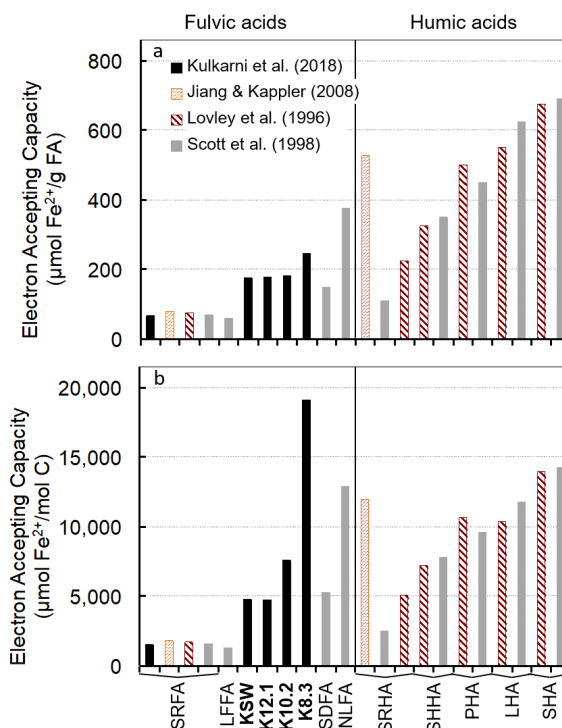


Fig. 2. Electron accepting capacities (a) per gram of fulvic (FA) or humic acid (HA), and (b) per mole of C for Bangladeshi FAs (black) and other FAs and HAs. Key: SRFA, Suwannee River fulvic acid; LFFA, Lake Fryxell fulvic acid; KSW, K12.1, K10.2, and K8.3 are Bangladeshi FAs described in Mladenov et al. (2015); S DFA, San Diego Bay sediment fulvic acid; NLFA, Nymph Lake sediment fulvic acid; SRHA, Suwannee River humic acid; SHHA, Summit Hill humic acid; PHA, peat humic acid; LHA, leonardite humic acid; SHA, soil humic acid. Reprinted with permission from Kulkarni et al. (2018).

In studies of the role of soil minerals in the spread of prion diseases, Johnson et al. (2006) and Kuznetsova et al. (2018) showed that prions remain infective when adsorbed to soil and source clay minerals in the laboratory. In a field investigation, large clay contents seemed to promote infectiveness (Walter et al., 2011), but other recent findings showed (i) the probability of horizontal transmission is greater where percent clay is <18% (Dorak et al., 2017), and (ii) there was a weaker negative correlation of chronic wasting disease endemicity with SOM than with clay. On a large scale, however, TSE endemic areas appear to be confined to soils of low SOM contents (Fig. 3). Indeed, the soil used by Seidel et al. (2007) to show long-term persistence in soil contained only 0.9% organic C.

Research performed on the interactions of HS with prion proteins has shown that HS actively reduce the risk of horizontal transmission via soil ingestion by animals. Complexation of prion proteins by HS was first suggested by Polano et al. (2008). Corsaro et al. (2010) analyzed the interaction of human prion protein fragment 90-231 (HuPrP) with HA and FA. They observed an interaction driven by nonspecific electrostatic attraction involving regions situated within α -helix A and β -sheet S1 of human PrP. Fulvic acid binding altered the ability of HuPrP to acquire some PrPSc (PrP-scrapie)-like characteristics. In particular, in the presence of FA, HuPrP showed a reduced amount of β -sheet content, an increased sensitivity to protease K, a broad-spectrum serine protease, and an inhibition of the fibrillogenic pathway for infecting neural cells. Additional indications of decreased infectivity are indicated by the fact that FA-HuPrP interaction caused

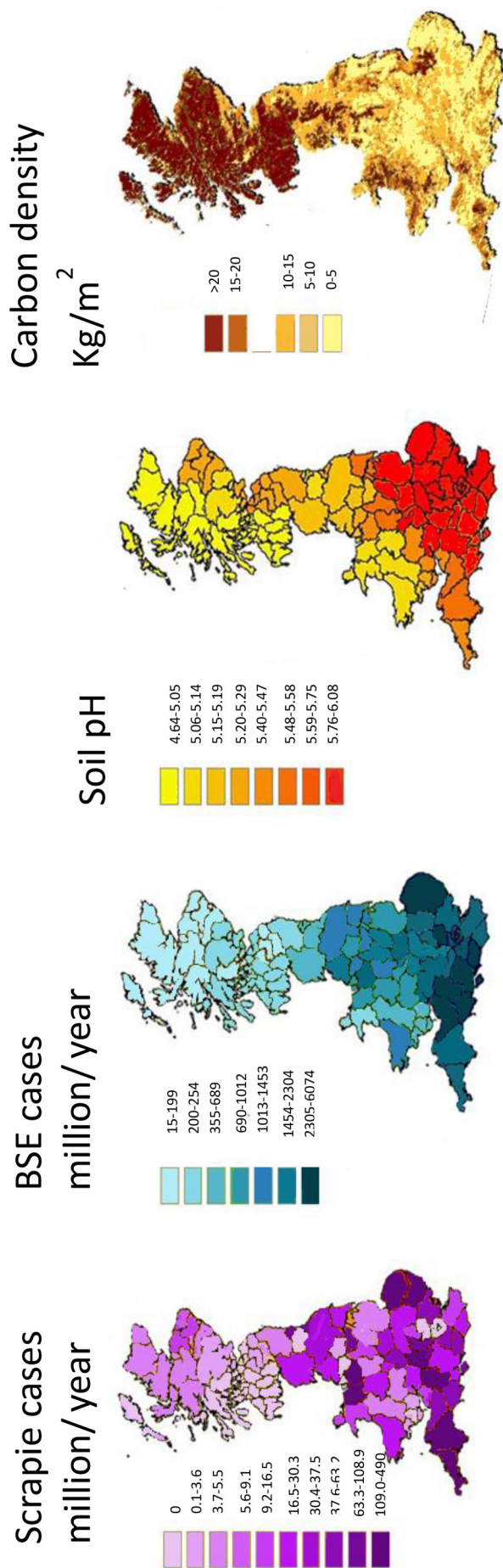


Fig. 3. Distribution of endemic areas of chronic wasting diseases, including bovine spongiform encephalopathy (BSE), in Great Britain compared with the pH and C content of soils. Modified from Imrie et al. (2009) and Ostle et al. (2009).

aggregation of the peptide into unstructured macrocomplexes, as demonstrated by the altered electrophoretic migration in the semi-denaturing detergent-agarose gel. The presence of FA reduced the number of fibrils and produced some macroaggregates (Fig. 4). Particle length distribution analysis also showed a reduction in length: in the absence of HS, proto-fibrillar structures grouped from 1.2 to 2.1 μm . Conversely, in the presence of FA, aggregate length was limited to 0.75 to 0.87 μm (Fig. 4).

Similarly, Kuznetsova et al. (2018) reported that HA fractions extracted from six soils and incubated with chronic wasting disease prions negatively affected prion molecular weight and extractability at higher HA concentrations. Animal bioassay of the samples showed a concomitant decline in prion infectivity with increasing concentration of HA.

Neurotoxic effects of the prion protein are related to its migration into the interior of cells, which depends on the acquisition of oligomeric β -rich conformation. In the presence of FA, the rate of internalization of HuPrP into human neuroblastoma cells was significantly reduced compared with that of the β -structured peptide (Corsaro et al., 2010). This showed that HS inhibited the acquisition of PrP(Sc)-like structural properties that otherwise are accountable for intracellular accumulation and lead to neuronal death. HuPrP-HS complexes, having limited capability for internalization into living cells, may hamper prion transmission from HS-rich soil, even in the presence of infected feces and carcasses. Giachin et al. (2014) confirmed that HS act as potent anti-prion agents in prion-infected neuronal cells and in the amyloid seeding assays: HS adsorbed both recPrP (recombinant-PrP) and prions, thus sequestering them from the prion replication process.

Protein-HS electrostatic attraction favors encapsulation of positively charged proteins by soil HA within the pH range that is common in soil. However, Dorak et al. (2017) found that when soil pH was above 6.6, exposure of white-tailed deer (*Odocoileus virginianus*) to prions was significantly greater. Hence, soil pH is another factor that may contribute to the persistence of infectivity. Potentially compounding the low amount of organic matter in soils of endemic areas, HS in neutral or alkaline soils can be strongly bound to soil minerals by Ca^{2+} ions (Olk, 2006). Therefore, their reactive functional groups may not be free to interact with prions. In some endemic areas of TSE for free-ranging animals, prion transmission in soils of low SOM content may depend on Ca^{2+} -induced impairment of HS stabilization of prions and soil pH values near or above neutrality (Fig. 3). This hypothesis merits further investigation.

In summary, controlled incubations and advanced laboratory analyses of HS interactions with prions led to novel insights into a potential mechanism for the deactivation of the self-replication capability of prions. The linkage of laboratory analyses to geographic distributions of soil properties and prion infectivity illustrates how studies with extracted HS can lead to important insights into NOM binding with proteins that have full significance to field conditions.

The Role of Humic Substances in Supplying Iron to Plants

Iron is frequently deficient for plants grown in calcareous soils, but a secured supply in a complexed form can distinctively and positively affect plant growth. The capacity of Fe-deficient

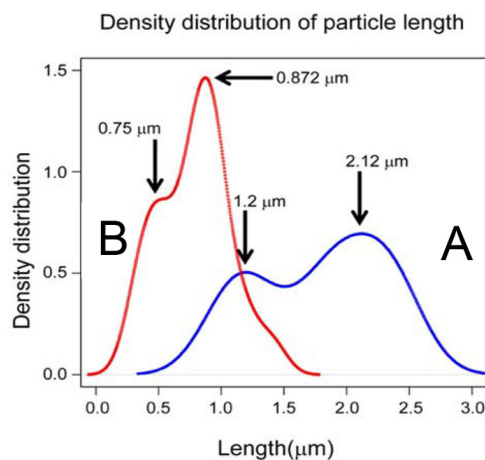
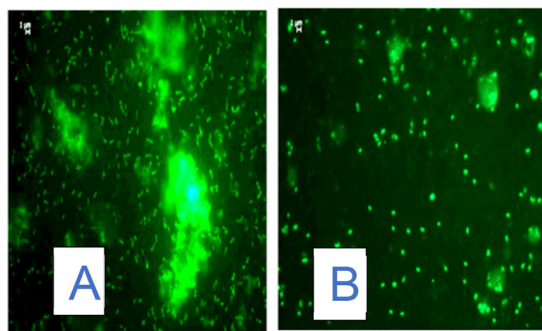
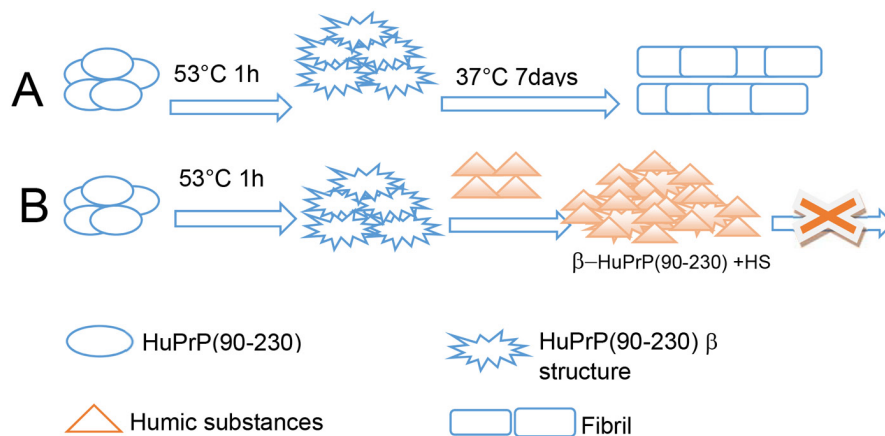


Fig. 4. Inhibition of the fibrillogenesis pathway by humic substances (HS), specifically humic acid (HA), and fulvic acid (FA) fractions. To produce fibrils in vitro, the HuPrP(90-230) fragment of the human prion protein was first heated at 53°C to induce acquisition of the β structure, then incubated for 7 d (37°C). When the β form was incubated at 37°C without HS (top figure), fibrils are produced (A), but in the presence of HS, noninfective aggregates are produced (B). Particle length distribution analysis of the micrographic images for both sequences (middle figure) indicates a reduction in fibril length in the presence of HS, as shown in the line graphs (bottom figure). In the absence of HS, proto-fibrillar structures grouped from 1.2 to 2.1 μm (A, blue line). Conversely, in the presence of HS, aggregate length was limited to 0.75 to 0.87 μm (B, red line). Modified from Corsaro et al. (2010).

plants to use Fe–HA and FA complexes as sources of Fe has been demonstrated in many nutrient solution studies (Vaughan and Malcolm, 1985; Chen and Aviad, 1990; Pinton et al., 1997; Chen et al., 2001, 2004a). This was also demonstrated in a field study of gladiolus (*Gladiolus grandiflorus*) grown on a calcareous soil, where the addition of peat containing 5% Fe as ferrous sulfate to the planting furrow was as effective in promoting Fe uptake as was FeEDDHA, a standard soil treatment for Fe deficiency (Chen et al., 1982).

Chen and colleagues used HA and FA in a series of studies on Fe deficiencies in plants. In a study with melon plants (*Cucumis melo* L.) grown in nutrient solutions that were either devoid of Fe or had received FeEDDHA, or ferric nitrate [$\text{Fe}(\text{NO}_3)_3$] plus Elliott soil HA (International Humic Substances Society [IHSS]), Chen et al. (2001) found that the chlorophyll concentration in the leaves after 35 d was highest in the HA + Fe treatment. In a study of the effect of added Fe, zinc (Zn), FA, and HA in nutrient solution on ryegrass (*Lolium* sp.) growth, Chen

et al. (2001, 2004a) found that plants grown in nutrient solution devoid of Fe and Zn and without FA or HA exhibited very low chlorophyll levels and retarded growth, resulting from limited photosynthesis. Addition of HA and FA separately did not result in a significant improvement in chlorophyll concentration. Addition of Fe and Zn sulfates to the nutrient solution resulted in a significant, yet insufficient, improvement in plant chlorophyll levels: plant growth did not improve significantly. Finally, addition of Fe and Zn together with either a chelator, HA, or FA resulted in healthy plant growth, with a near doubling of plant biomass and chlorophyll content.

Studies on the role of HS in Fe supply to plants provide another illustration of the importance of NOM in metal ion retention and transport. In our previous review, we discussed how studies of binding of metals to carboxyl and phenol sites in HS enabled numeric modeling of metal retention and mobility in soils (Olk et al., 2019). The supplemental material discusses an application of the metal ion binding potential of HS in phytoremediation or soil washing remediation of metal-contaminated soils.

The Role of Humic Substances in Retention and Transport of Mercury in Soils, Water, and Sediments

Mercury (Hg) is a global pollutant that affects human and ecosystem health. Its fate and transport in soil, water, and sediments is to a great degree a function of complexation with SOM and DOM (Skylberg, 2012; Driscoll et al., 2013). Elemental mercury [Hg(0)] from natural and anthropogenic sources is transported long distances in the atmosphere while being oxidized to Hg(II) before being deposited onto the earth's surface. Under anoxic conditions, as in organic-rich subaqueous sediments, Hg(II) can be methylated to CH_3Hg^+ , a very powerful neurotoxin that is bioaccumulated up the food chain.

The association of Hg with NOM has been known for many decades, based on the correlation of Hg in stream water with dissolved organic C (Mierle and Ingram, 1991), yet the nature of its bonding to NOM and the resulting significance for environmental fate have only more recently been elucidated (Skylberg, 2012). Mercury is a soft Lewis acid and therefore has a much higher affinity for thiol groups in NOM than for the abundant phenolic and carboxyl groups that bind most other metals. However, the early studies of complexation of Hg^{2+} in soil HA and aquatic HS produced formation constants that were similar to those expected for carboxylic ligands (Cheam and Gamble, 1974; Kerndorff and Schnitzer, 1980; Lovgren and Sjoberg, 1989). The formation constant reported by Lovgren and Sjoberg (1989) was $10^{10.37}$, similar that of Hg^{2+} binding to oxalate and much too low for complexation of Hg^{2+} by thiol ligands. All of these studies were conducted at levels of added Hg^{2+} that more than saturated the strong binding thiol sites.

To quantify thiol groups in HS that might be the bonding sites for Hg^{2+} , Xia et al. (1998) used X-ray absorption near edge structure (XANES) spectroscopy to differentiate the oxidation states of sulfur (S) in Suwanee River HA and FA and two soil HAs. They were able to separate six oxidation states ranging from reduced (RSH, RSR, and RSSR, where "R" represents any organic group with a terminal C or H) to sulfate ester S. Xia et

al. (1999) determined the bonding environment of Hg^{2+} in a soil HA at a molar ratio of Hg/reduced S of 3.1:1 using extended X-ray absorption fine structure (EXAFS) spectroscopy. They found that the Hg was bound to one oxygen (O) and one S. Skylberg et al. (2006) added Hg^{2+} separately to both an organic soil and an HA at ratios varying from 8:1 to 0.01:1. Their EXAFS spectra showed that at the highest Hg/reduced S ratios, when the reduced S sites are more than saturated with Hg^{2+} , the bonding is predominantly at nitrogen (N) or O ligand sites, whereas at the lowest ratio, Hg^{2+} is complexed in linear coordination with two S atoms. The lowest ratio in an organic soil, corresponded to 1 mg Hg kg^{-1} , is nevertheless still higher than for all but highly polluted soils. At the same Hg/reduced S ratios, the EXAFS spectra for HA were equivalent to the results for the organic soils; however, the spectra were better resolved for the HA. Skylberg et al. (2006) estimated that high-affinity two-coordinate sites represent 20 to 30% of the reduced S. In a similar study of CH_3Hg^+ in an organic soil and aquatic NOM, Qian et al. (2002) found that CH_3Hg^+ is bound to reduced S with a 1:1 coordination. They also concluded that 20 to 30% of reduced S is involved in high-affinity sites.

The bonding of Hg^{2+} to thiol ligands is very strong, and the determination of formation constants for Hg^{2+} complexation requires the use of a competing ligand with a similar affinity for Hg^{2+} as the thiol sites in NOM. This is needed to have a measurable concentration of Hg^{2+} in solution while maintaining free aquo Hg^{2+} at extremely low activities. Skylberg et al. (2000) determined formation constants in organic-rich forest soil horizons using bromide (Br^-) as a competing ligand. Khwaja et al. (2006) used penicillamine, a thiol amino acid, as the competing ligand in a study of Hg^{2+} in IHSS Pahokee peat (euic, hyperthermic Lithic Haplosaprists), five organic soils, and HA extracted from one of the organic soils. The studies of Skylberg et al. (2000) and Khwaja et al. (2006) yielded similar complexation constants. Skylberg (2008) recalculated the constants from these two studies assuming two independent RS^- sites react with Hg^{2+} to form $(\text{RS})_2\text{Hg}$. The $\log(K)$ values ranged for 43.3 to 47.7 at pH 3.15 to 4.82 with a trend of higher $\log(K)$ at higher pH, suggesting a possible dependency on pH. This compares with $\log(K) = 44.4$ for Hg^{2+} binding to penicillamine (Casas and Jones, 1980). It is interesting to note that when one organic soil was compared with the HA extracted from that soil, the results for determination of relative abundance of S species by XANES and for determination of the binding constants were very similar. Therefore, the extraction of HA with NaOH did not change the S chemistry.

The complexation of CH_3Hg^+ with HS and NOM is strong but not nearly as strong as for Hg^{2+} . Khwaja et al. (2010) determined formation constants for CH_3Hg^+ with Suwanee River FA using competitive complexation with Br^- . Karlsson and Skylberg (2003) determined formation constants for organic soils and DOM that desorbed from the soils, using a competitive complexation method with both chloride (Cl^-) and Br^- . The results were similar in both studies: at pH 3.0, both reported $\log(K) = 15.7$ for the formation of a monodentate RSHCH_3 complex with RS^- . Both research groups observed a strong pH dependence for the formation constant. Khwaja et al. (2010) postulated the existence of thiol sites with pK_a of 4.0, much lower than the value of 10.0 for mercaptoacetic acid, which is typically

used to estimate the acidity of NOM thiols (Skylberg, 2008). With the assumption of a sufficient content of low-pKa thiols to complex with the low quantity of added CH_3Hg^+ (Hg/reduced S < 0.001), they were able to eliminate the pH dependence.

The studies described above have had a large impact on the understanding of the role of NOM in the fate and transport of Hg^{2+} and CH_3Hg^+ in soils, sediments, and natural waters. They made possible the use of numerical chemical modeling to partition Hg between solid-phase organic matter and the various species in solution and to obtain species distributions that better match measured data (Skylberg, 2008, 2012; Liem-Nguyen et al., 2017). Field measurements confirm that under oxic conditions, both Hg^{2+} and MeHg^+ are found only as NOM complexes with no significant free aquo ions or complexed with hydroxide (OH^-) or Cl^- (Skylberg et al., 2003). In previous chemical modeling (e.g., Morel et al., 1998), there was no consideration of the NOM in the numerical calculations. Model calculations by Liem-Nguyen et al. (2017) accurately predicted porewater concentrations of MeHg^+ in the soils of a boreal wetland forest, and model calculations for Hg^{2+} successfully predicted the mean concentration in the porewaters but over predicted the variability among samples.

The inclusion of bonding to NOM has implications for quantification of methyl mercury in soils and sediments and for studies on the production and fate of CH_3Hg^+ in the environment. The humic-based studies discussed above investigated Hg(II) binding at addition rates that did not exceed the capacity of reduced S binding sites, resulting in mechanistic understanding of the very strong bonding of Hg and MeHg in NOM. They brought more focus to the importance of specific sites for binding of metals to NOM and for considering the possibility of saturation of strong binding sites when studying the bonding of metals in NOM. Also, the similarity of stability constants gained from HS versus NOM not subjected to alkali extraction show that the S chemistry in extracted HS is not significantly affected by the alkali separation procedures.

Humic Fractions and Potassium Fixation in Vermiculitic Soils for California Cotton

A long-term decline in cotton (*Gossypium hirsutum* L.) lint yield in the San Joaquin Valley (California) has been attributed to strong potassium (K) fixation by vermiculitic soil minerals (Cassman et al., 1989). Two years of steer or poultry manure application better maintained cotton lint yield and residual soil K availability against mineral fixation than did amendment with fertilizer K equivalent to the K content of the manure (Cassman et al., 1992), indicating that the organic component of the manure helped maintain available soil K against mineral fixation.

To further examine the possible role of young organic matter in this field benefit of manure application to K availability, humic fractions were extracted from a local vermiculitic soil using a modification of the standard alkali extraction. The mobile HA (MHA) fraction is distinguished from less labile material by its immediate extraction from the soil with NaOH (Olk et al., 1995). Only subsequently is the soil washed by hydrochloric acid (HCl) solutions, a conventional step for removing calcium (Ca), which can bind and stabilize SOM. Hence, a second alkali extraction recovers the Ca humate (CaHA) fraction. The cycling

rates and chemical compositions of the two fractions have been responsive to field treatments in multiple cropping systems, especially for the MHA (Olk, 2006, Olk et al., 2019).

The MHA and CaHA were added separately at prescribed rates as freeze-dried powders in laboratory studies to another high K-fixing vermiculitic soil, together with soluble KCl added at rates comparable with fertilizer K applications in production fields. Addition of the MHA fraction reduced soil fixation of the amended solubilized K in (i) soil suspensions, (ii) during wetting–drying cycles that simulated soil surface moisture conditions, and (iii) during 80-d aerobic incubations at constant moisture that simulated subsoil moisture conditions (Olk and Cassman, 1995). Corresponding increases in K availability with addition of the more humified CaHA were much smaller or nonexistent. Soil amendment with MHA enabled 42% greater uptake of amended K by ryegrass (*Sorghum vulgare sudansense* L.) after 21 d of growth in a growth chamber compared with the control lacking MHA amendment. This increase was not due to the minimal K content of the MHA. Model compound studies suggested that MHA enhancement of K availability might arise from interactions between the abundant amino groups of the young MHA with the K-fixing interlayers of the vermiculitic soil minerals. Accordingly, addition of the relatively amino-depleted and less soluble CaHA fraction provided less notable boosts in ammonium (NH_4)–extractable soil K than did the MHA.

Addition of the labile MHA fraction in controlled incubations accurately reproduced the field benefit of manure application to available soil K and crop K uptake. These results became known among agricultural industry staff, especially those who work with K-fixing soils, including researchers at Actagro, a biostimulant company based in Fresno, CA. They used this knowledge in their development of Katalyst, a specialty K fertilizer product that contains a proprietary form of organic matter, derived in part from a humic substance. Today this product is widely applied to high value tree crops and vineyards on vermiculitic soils in the San Joaquin Valley (California) for the purpose of economically maintaining K availability against mineral fixation.

Further Studies on Humic Fraction Responses to Land Management and Location

Additional studies of humic fractions in diverse soil and aquatic systems also describe the responsiveness of humic properties to location or land management practices. These studies were not always designed to identify the mechanisms of significant environmental processes, yet they merit brief mention because logical humic responses in composition and mass to environmental factors refute claims that humic fractions are laboratory artifacts and thus unsuitable proxies for NOM (Lehmann and Kleber, 2015; Kleber and Lehmann, 2019).

Soil Humic Response to Land Use Patterns

Wingeyer et al. (2012) studied soil C sequestration after deep incorporation of crop residues to 20-cm depth, which was intended to slow their decomposition. In a long-term no-tilled field in Nebraska (USA), deep incorporation during 3 yr resulted in mixing of accumulated surface C (0–5 cm) into the deeper soil

depths (5–15 and 15–30 cm). Total soil C stocks in the surface 0- to 5-cm layer decreased by 17% during the first year of deep incorporation and by 23% after the third year. Corresponding decreases in the MHA fraction extracted from this depth were greater, by 44 and 52%, respectively. The more recalcitrant CaHA and humin fractions decreased by 13 to 15%, or slightly less than did SOM. At the greatest depth, total C stocks were unchanged in the first year of deep incorporation and increased by 3% by the third year, whereas the MHA content increased by 42% in the third year. The authors hypothesized that these increases in the third year signaled the beginning of continued C accumulation at depth that would continue into future years. Vertical mixing of SOM within the soil profile during deep incorporation was also evident in humic properties: by the third year, the MHA and CaHA showed narrower variation across the soil depths in their C/N ratios and light absorption at 465 nm than before deep incorporation.

De Nobili et al. (2008) collected soil samples from long-term field experiments at Rothamsted Research (UK) for alkali extraction before and after a 215-d laboratory incubation. The alkali-extracted HS were then passed through cross-linked polyvinylpyrrolidone columns, which adsorb phenolic-enriched materials. Arable soils, unless fertilized with farmyard manure, had a C ratio for non-phenol-enriched to phenol-enriched HS about twice that of the other soils. During incubation, the C ratio of MHA to CaHA, which was positively related to soil C inputs, decreased, showing that stabilization by humification plus Ca binding is yet more effective than by humification alone. Phenolic enrichment was associated with a more refractory nature: In the arable soils with low C inputs, the C ratio of non-phenol-enriched to phenol-enriched HS decreased during incubation. This trend supports the concept of lignin persistence (Olk et al., 2019). The ^{13}C nuclear magnetic resonance (NMR) spectra of the CaHA fraction showed an increase in aromatic C during incubation. In contrast, the spectra of the MHA were mostly unchanged. In summary, these results indicate the potential responsiveness of HS to both land use and incubation time.

Abdelrahman et al. (2017) studied changes in SOM pools during the transition from conventional farming to organic farming with different types of organic fertilizers. For both phases of a wheat (*Triticum aestivum* L.)–lentil (*Lens esculenta* Medik.) rotation at two sites in Italy, the MHA mass was statistically more responsive to crop phase and fertilizer type than was mass of the particulate organic matter in three of four cases. Both the MHA and the particulate organic matter were poorly responsive in the fourth case.

Bongiovanni and Lobartini (2006) compared an undisturbed forest soil in central Córdoba, Argentina, with a nearby long-term tilled soil. Tillage was shown to lead to decreases in HA and FA contents of whole soil and aggregate size classes, and their decreases were of the same proportions as decreases in other SOM binding agents. These results indicate the responsiveness of HS to tillage.

Mao et al. (2008) extracted the MHA and CaHA fractions from fertilizer treatments in a Nebraska maize (*Zea mays* L.)–soybean [*Glycine max* (L.) Merr.] field. Animal manure and mineral N fertilizer treatments had received the same input rate of total N. Analysis by ^{13}C NMR spectroscopy found that the CaHA from the manure treatment had a smaller aromatic signal

and enhanced peptide and fatty acid signals compared with the CaHA from both the mineral N equivalent treatment and an unamended control. The manure treatment increased maize grain yield in poorer-yielding portions of the field, and stepwise regression analysis best correlated this yield boost with improved soil availability of N and P. Hence, the greater peptide signal of the CaHA was consistent with the increased soil N supply provided by manure application.

Olk et al. (1998) compared the quantities of the MHA and CaHA fractions under optimal N–phosphorus (P)–K fertilization versus a minus N–P–K control for paddy rice at three locations. The optimal fertilizer treatment had greater crop biomass production and return of crop residues into soil than did the controls. Soil organic C content increased with optimal fertilization, consistent with the hypothesis that greater biomass of incorporated crop residues generally leads to greater accumulation of SOM (Liu et al., 2014). Extracted masses of the MHA and CaHA fractions increased proportionately yet more so with optimal fertilization than did SOC, demonstrating their sensitivity to crop management.

Humic Substances in Natural Waters Vary with Environmental Factors

Esteves et al. (2009) found that humic fractions in an open ocean water sample showed less phenolic and lignin residue signals and more branched aliphatic and olefin molecules than did humic fractions of stream and estuarine aquatic samples as measured through ultraviolet–visible, molecular fluorescence, Fourier-transform infrared (FTIR), and cross-polarization magic angle spinning (CP-MAS) ^{13}C NMR spectroscopies. These results are consistent with the expected greater microbial input to open ocean C, versus greater terrestrial source influence on the stream and estuarine samples.

Kalmykov et al. (2005) found that HA binding dominated the migration behavior of aquatic radionuclides, especially actinides, and in manners that depended on the actinide. In bottom sediments of the Yenisey River (Russia), for example, plutonium was mostly associated with the humin and mineral components of the sediments, whereas americium was bound to mobile FA or other low-molecular-weight organic substances. This difference could explain why sediment plutonium concentrations decreased much faster downstream with increasing distance from a chemical plant nearby the river than did americium concentrations.

Linnik and Vasilchuk (2005) found that aqueous forms of Fe, manganese (Mn), copper (Cu), Zn, lead (Pb), chromium (Cr), and cadmium (Cd) in three reservoirs along the Dnieper River (Ukraine) were mostly (at least >70%) complexed with DOM, primarily FA. Humic and fulvic acids accounted for 40 to 74% of the metals bound to DOM. Seasonal and spatial fluxes in the capacity of DOM for metal binding might have been linked to simultaneous shifts in its relative abundances of (i) lower- versus higher-molecular-weight HS, and (ii) HS versus nonhumic compounds such as carbohydrates. In a controlled study, the HA fraction formed relatively strong bonds with Cu, which reduced its toxicity to the large water flea (*Daphia magna* Straus.).

Lu et al. (2000) extracted HA and FA from swamp water and also from a nearby surface soil. Analysis by ^{13}C NMR

spectroscopy and pyrolysis–gas chromatograph–mass spectrometry concluded that vascular plants were the main contributor to HS and that both the soil and swamp water had similar humic markers, suggesting the swamp HS were derived from the nearby surface soil.

Discussion and Conclusions

Natural organic matter is of unquestioned importance to multiple environmental processes in both aquatic and soil systems. Its components can vary tremendously in chemical and biological reactivity and also in cycling rates, encouraging their separation to enable study of those components that possess specific reactivity traits or cycling rates befitting specific research objectives. Separation of relevant SOM fractions from the soil matrix also makes possible their chemical characterization by a wider array of advanced instruments and in cases at greater precision.

Multiple approaches exist for separating NOM fractions; all have their own strengths and deficiencies (Olk and Gregorich, 2006). The general suitability of any of these approaches is best evaluated based on the quality and breadth of accurate results that its use provides, not on theoretical arguments whether it is flawed. Therefore, we presented several studies that used HS toward a range of research objectives in both aquatic and soil settings. Collectively, these studies highlight tangible benefits of studying HS.

In specific case studies, both field measurements and laboratory incubations demonstrated that dissolved humic organic matter accelerates the mobilization of Fe oxide-bound As in groundwater in Bangladesh. Key to this is the role of FA as an electron shuttle, whereby bacteria that oxidize labile DOM transfer electrons to FA, which in turn reduces Fe oxides, leading to release of toxic levels of As to drinking water (Mladenov et al., 2010, 2015).

Mercury²⁺ and methyl mercury are very strongly bonded to thiol sites in FA and HA, in soils, sediments and water, illustrating the importance of these very high affinity sites in the modeling of Hg retention, transport, and bioavailability (Skylberg, 2008, 2012; Liem-Nguyen et al., 2017). Research results comparing Hg²⁺ binding in O-horizon soils to Hg²⁺ binding by HA extracted from one of the soils showed that the alkali extraction had no effect on the chemical nature of the S binding sites (Khwaja et al., 2006).

Similarly, prions, the proteinaceous infective agents of TSE, can be encapsulated by HS and made inactive. This can account for the reduced transmissibility in soils with high SOM content (Corsaro et al., 2010). The extent of process-level knowledge and the capabilities for landscape-scale assessment that have been gained through humic studies of prions might not have been attained through alternative fractionation procedures for NOM or through the absence of any NOM fractionation.

Addition of a young HA fraction to vermiculitic soils in controlled conditions reproduced the field benefits of animal manure application to increased soil K availability and plant K uptake (Olk and Cassman, 1995). These concepts led to the development of a commercially successful fertilizer product that contains, in part, HS.

Humic fractionation is an imperfect method. Studies elsewhere have identified chemical alterations that occur during alkali extraction. If such alteration were debilitating, as claimed by Lehmann

and Kleber (2015), then HS should prove unable to accurately describe NOM and its responses to environmental conditions. Studies described here and by Olk et al. (2019) rebut this claim. We acknowledge that properties of humic fractions are less suitable to some research objectives than alternative fractionation procedures, yet the opposite also applies: for multiple other research objectives, humic fractions are more suitable than alternative, similarly flawed fractionation procedures or the study solely of total NOM. Specifically, HS were shown here and by Olk et al. (2019) to be highly appropriate for studies of aquatic C and for environmental processes that involve NOM components such as aromatic C or oxygenated functional groups as preferential binding sites for metals and other contaminants. Successful application of HS to identify significant environmental processes and to develop successful solutions should be the primary evaluator of the suitability of an NOM approach. Study of HS has proven to be a robust approach in resolving environmental issues and developing meaningful paths for mitigation steps.

Supplemental Material

The supplemental material describes additional applications HS in studying membrane fouling by NOM and developing HS-based solutions for cleansing soils of heavy metal contaminants.

Conflict of Interest

The authors declare no conflict of interest.

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Reference to any specific commercial product is for the information of the public and does not constitute endorsement or recommendation by the US government or other sponsors of this report.

References

- Abdelrahman, H., C. Cocozza, D.C. Olk, D. Ventrela, and T. Miano. 2017. Carbohydrates and amino compounds as short-term indicators of soil management. *Clean: Soil, Air, Water* 45:1600076. doi:10.1002/clen.201600076
- Anawar, H.M., J. Akai, K. Komaki, H. Terao, T. Yoshioka, T. Ishizuka, et al. 2003. Geochemical occurrence of arsenic in groundwater of Bangladesh: Sources and mobilization processes. *J. Geochem. Explor.* 77:109–131. doi:10.1016/S0375-6742(02)00273-X
- Bhattacharya, P., M.A. Hasan, O. Sracek, E. Smith, K.M. Ahmed, M. Von Brömssen, et al. 2009. Groundwater chemistry and arsenic mobilization in the Holocene flood plains in south-central Bangladesh. *Environ. Geochem. Health* 31:23–43. doi:10.1007/s10653-008-9230-5
- Bongiovanni, M.D., and J.C. Lobartini. 2006. Particulate organic matter, carbohydrate, humic acid contents in soil macro- and microaggregates as affected by cultivation. *Geoderma* 136:660–665. doi:10.1016/j.geoderma.2006.05.002
- Brown, P., and D.C. Gajdusek. 1991. Survival of scrapie virus after 3 years' interment. *Lancet* 337:269–270. doi:10.1016/0140-6736(91)90873-N
- Casas, J.S., and M.M. Jones. 1980. Mercury(II) complexes with sulfhydryl containing chelating agents: Stability constant inconsistencies and their resolution. *J. Inorg. Nucl. Chem.* 42:99–102. doi:10.1016/0022-1902(80)80052-2
- Cassman, K.G., B.A. Roberts, and D.C. Bryant. 1992. Cotton response to residual fertilizer potassium on vermiculitic soil: Organic matter and sodium effects. *Soil Sci. Soc. Am. J.* 56:823–830. doi:10.2136/sssaj1992.03615995005600030025x
- Cassman, K.G., B.A. Roberts, T.A. Kerby, D.C. Bryant, and S.L. Higashi. 1989. Soil potassium balance and cumulative cotton response to annual potassium additions on a vermiculitic soil. *Soil Sci. Soc. Am. J.* 53:805–812. doi:10.2136/sssaj1989.03615995005300030030x
- Cheam, V., and D.S. Gamble. 1974. Metal-fulvic acid chelation equilibrium in aqueous NaNO₃ solution: Hg(II), Cd(II), and Cu(II) fulvate complexes. *Can. J. Soil Sci.* 54:413–417. doi:10.4141/cjss74-055
- Chen, Y., and T. Aviad. 1990. Effects of humic substances on plant growth. In: P. MacCarthy, et al., editors, *Humic substances in soil and crop sciences: Selected readings*. ASA and SSSA, Madison, WI. p. 161–186.

- Chen, Y., C.E. Clapp, and H. Magen. 2004a. Mechanisms of plant growth stimulation by humic substances: The role of organo-iron complexes. *Soil Sci. Plant Nutr.* 50:1089–1095. doi:10.1080/00380768.2004.10408579
- Chen, Y., M. De Nobili, and T. Aviad. 2004b. Stimulatory effects of humic substances on plant growth. In: F. Magdoff and R.R. Weil, editors, *Soil organic matter in sustainable agriculture*. CRC Press, Boca Raton, FL. p. 103–129. doi:10.1201/9780203496374.ch4
- Chen, Y., H. Magen, and C.E. Clapp. 2001. Plant growth stimulation by humic substances and their complexes with iron. In: *Proceedings of the Dalia Greidinger Symposium*, Lisbon, Portugal. 4–6 Mar. 2001. *Int. Fert. Soc., Colchester, UK*. p. 1–14.
- Chen, Y., B. Steinitz, A. Cohen, and Y. Elber. 1982. The effect of various iron-containing fertilizers on growth and propagation of *gladiolus-grandiflorus*. *Sci. Hortic.* 18:169–175. doi:10.1016/0304-4238(82)90132-7
- Chin, Y.P., S.J. Traina, C.R. Swank, and D. Backhus. 1998. Abundance and properties of dissolved organic matter in pore waters of a freshwater wetland. *Limnol. Oceanogr.* 43:1287–1296. doi:10.4319/lo.1998.43.6.1287
- Corsaro, A., C. Anselmi, M. Polano, A. Aceto, T. Florio, and M. De Nobili. 2010. The interaction of humic substances with the human prion protein fragment 90-231 affects its protease K resistance and cell internalization. *J. Biol. Regul. Homeostatic Agents* 24:27–39.
- De Nobili, M., M. Contin, N. Mahieu, E.W. Randall, and P.C. Brookes. 2008. Assessment of chemical and biochemical stabilization of organic C in soils from the long-term experiments at Rothamsted (UK). *Waste Manag.* 28:723–733. doi:10.1016/j.wasman.2007.09.025
- Ding, F., J.X. Diao, X.L. Yang, and Y. Sun. 2011. Structural analysis and binding domain of albumin complexes with natural dietary supplement humic acid. *J. Lumin.* 131:2244–2251. doi:10.1016/j.jlum.2011.06.005
- Dorak, S.J., M.L. Green, M.M. Wander, M.O. Ruiz, M.G. Buhnerkempe, T. Tian, et al. 2017. Clay content and pH: Soil characteristic associations with the persistent presence of chronic wasting disease in northern Illinois. *Sci. Rep.* 7:18062. doi:10.1038/s41598-017-18321-x
- Driscoll, C.T., R.P. Mason, H.M. Chan, D.J. Jacob, and N. Pirrone. 2013. Mercury as a global pollutant: Sources, pathways, and effects. *Environ. Sci. Technol.* 47:4967–4983. doi:10.1021/es305071v
- Esteves, V.I., M. Otero, and A.C. Duarte. 2009. Comparative characterization of humic substances from the open ocean, estuarine water and fresh water. *Org. Geochem.* 40:942–950. doi:10.1016/j.orggeochem.2009.06.006
- Ferreira, J.A., O.R. Nascimento, and L. Martin-Neto. 2001. Hydrophobic interactions between spin-label 5-SASL and humic acid as revealed by ESR spectroscopy. *Environ. Sci. Technol.* 35:761–765. doi:10.1021/es010251
- Giachin, G., J. Narkiewicz, D. Scaini, A.T. Ngoc, A. Margon, P. Sequi, and L. Leita. 2014. Prion protein interaction with soil humic substances: Environmental implications. *PLoS One* 9:e100016. doi:10.1371/journal.pone.0100016
- Hoang, T.H., S. Bang, K.W. Kim, M.H. Nguyen, and D.M. Dang. 2010. Arsenic in groundwater and sediment in the Mekong River delta, Vietnam. *Environ. Pollut.* 158:2648–2658. doi:10.1016/j.envpol.2010.05.001
- Hsu, P.H., and P.G. Hatcher. 2006. Covalent coupling of peptides to humic acids: Structural effects investigated using 2D NMR spectroscopy. *Org. Geochem.* 37:1694–1704. doi:10.1016/j.orggeochem.2006.07.026
- Imrie, C.E., A. Korre, and G. Munoz-Melendez. 2009. Spatial correlation between the prevalence of transmissible spongiform diseases and British soil geochemistry. *Environ. Geochem. Health* 31:133–145. doi:10.1007/s10653-008-9172-y
- Johnson, C.J., K.E. Phillips, P.T. Schramm, D. McKenzie, J.M. Aiken, and J.A. Pedersen. 2006. Prions adhere to soil minerals and remain infectious. *PLoS Pathog.* 2:e32. doi:10.1371/journal.ppat.0020032
- Kalmykov, S.N., A.P. Novikov, A.B. Khasanova, N.S. Scherbina, and Yu.A. Sapozhnikov. 2005. Humic acids as barriers in actinide migration in the environment. In: I.V. Perminova, et al., editors, *Use of humic substances to remediate polluted environments: From theory to practice*. Springer, Dordrecht, the Netherlands. p. 175–184. doi:10.1007/1-4020-3252-8_8
- Karlsson, T., and U. Skjellberg. 2003. Bonding of ppb levels of methyl mercury to reduced sulfur groups in soil organic matter. *Environ. Sci. Technol.* 37:4912–4918. doi:10.1021/es034302n
- Kerndorff, H., and M. Schnitzer. 1980. Sorption of metals on humic acid. *Geochim. Cosmochim. Acta* 44:1701–1708. doi:10.1016/0016-7037(80)90221-5
- Khawaja, A.R., P.R. Bloom, and P.L. Brezonik. 2006. Binding constants of divalent mercury (Hg²⁺) in soil humic acids and soil organic matter. *Environ. Sci. Technol.* 40:844–849. doi:10.1021/es051085c
- Khawaja, A.R., P.R. Bloom, and P.L. Brezonik. 2010. Binding strength of methylmercury to aquatic NOM. *Environ. Sci. Technol.* 44:6151–6156. doi:10.1021/es101088k
- Kleber, M., and M.G. Johnson. 2010. Advances in understanding the molecular structure of soil organic matter: Implications for interactions in the environment. *Adv. Agron.* 106:77–142. doi:10.1016/S0065-2113(10)06003-7
- Kleber, M., and J. Lehmann. 2019. Humic substances extracted by alkali are invalid proxies for the dynamics and functions of organic matter in terrestrial and aquatic ecosystems. *J. Environ. Qual.* 49:207–216. doi:10.2134/jeq2019.01.0036
- Kulkarni, H.V., N. Mladenov, D.M. McKnight, Y. Zheng, M.F. Kirk, and D.R. Nemerugut. 2018. Dissolved fulvic acids from a high arsenic aquifer shuttle electrons to enhance microbial iron reduction. *Sci. Total Environ.* 615:1390–1395. doi:10.1016/j.scitotenv.2017.09.164
- Kuznetsova A., C. Cullingham, D. McKenzie, and J.M. Aiken. 2018. Soil humic acids degrade CWD prions and reduce infectivity. *PLoS Pathog* 14:e1007414. doi:10.1371/journal.ppat.1007414
- Lehmann, J., and M. Kleber. 2015. The contentious nature of soil organic matter. *Nature* 528:60–68. doi:10.1038/nature16069
- Liem-Nguyen, V., U. Skjellberg, and E. Bjorn. 2017. Thermodynamic modeling of the solubility and chemical speciation of mercury and methylmercury driven by organic thiols and micromolar sulfide concentrations in boreal wetland soils. *Environ. Sci. Technol.* 51:3678–3686. doi:10.1021/acs.est.6b04622
- Linnik, P.N., and T.A. Vasilchuk. 2005. Role of humic substances in the complexation and detoxification of heavy metals: Case study of the Dnieper reservoirs. In: I.V. Perminova, et al., editors, *Use of humic substances to remediate polluted environments: From theory to practice*. Springer, Dordrecht, the Netherlands. p. 135–154. doi:10.1007/1-4020-3252-8_6
- Liu, C., M. Lu, J. Cui, B. Li, and C. Fang. 2014. Effects of straw carbon input on carbon dynamics in agricultural soils: A meta-analysis. *Glob. Change Biol.* 20:1366–1381. doi:10.1111/gcb.12517
- Lovgren, L., and S. Sjoberg. 1989. Equilibrium approaches to natural water systems: 7. Complexation reactions of copper(II), cadmium(II) and mercury(II) with dissolved organic matter in a concentrated bog-water. *Water Res.* 23:327–332. doi:10.1016/0043-1354(89)90098-5
- Lu, X.Q., J.V. Hanna, and W.D. Johnson. 2000. Source indicators of humic substances: An elemental composition, solid state ¹³C CP/MAS NMR and Py-GC/MS study. *Appl. Geochem.* 15:1019–1033. doi:10.1016/S0883-2927(99)00103-1
- Mao, J., D.C. Olk, X. Fang, Z. He, and K. Schmidt-Rohr. 2008. Influence of animal manure application on the chemical structures of soil organic matter as investigated by advanced solid-state NMR and FT-IR spectroscopy. *Geoderma* 146:353–362. doi:10.1016/j.geoderma.2008.06.003
- Mierle, G., and R. Ingram. 1991. The role of humic substances in the mobilization of mercury from watersheds. *Water Air Soil Pollut.* 56:349–357. doi:10.1007/BF00342282
- Mladenov, N., Y. Zheng, M.P. Miller, D.R. Nemerugut, T. Legg, B. Simone, et al. 2010. Dissolved organic matter sources and consequences for iron and arsenic mobilization in Bangladesh aquifers. *Environ. Sci. Technol.* 44:123–128. doi:10.1021/es901472g
- Mladenov, N., Y. Zheng, B. Simone, T.M. Bilinski, D.M. McKnight, D. Nemerugut, et al. 2015. Dissolved organic matter quality in a shallow aquifer of Bangladesh: Implications for arsenic mobility. *Environ. Sci. Technol.* 49:10815–10824. doi:10.1021/acs.est.5b01962
- Morel, F.M.M., A.M.L. Kraepiel, and M. Amyot. 1998. The chemical cycle and bioaccumulation of mercury. *Annu. Rev. Ecol. Syst.* 29:543–566. doi:10.1146/annurev.ecolsys.29.1.543
- Olk, D.C. 2006. A chemical fractionation for structure–function relations of soil organic matter in nutrient cycling. *Soil Sci. Soc. Am. J.* 70:1013–1022. doi:10.2136/sssaj2005.0108
- Olk, D.C., P.R. Bloom, E.M. Perdue, D.M. McKnight, Y. Chen, A. Farenhorst, et al. 2019. Environmental and agricultural relevance of humic fractions extracted by alkali from soils and natural waters. *J. Environ. Qual.* 48:217–232. doi:10.2134/jeq2019.02.0041
- Olk, D.C., and K.G. Cassman. 1995. Reduction of potassium fixation by two humic acid fractions in vermiculitic soils. *Soil Sci. Soc. Am. J.* 59:1250–1258. doi:10.2136/sssaj1995.03615995005900050007x
- Olk, D.C., K.G. Cassman, and T.W.M. Fan. 1995. Characterization of two humic acid fractions from a calcareous vermiculitic soil: Implications for the humification process. *Geoderma* 65:195–208. doi:10.1016/0016-7061(95)94048-9
- Olk, D.C., K.G. Cassman, N. Mahieu, and E.W. Randall. 1998. Conserved chemical properties of young humic acid fractions in tropical lowland soil under intensive irrigated rice cropping. *Eur. J. Soil Sci.* 49:337–349. doi:10.1046/j.1365-2389.1998.00163.x
- Olk, D.C., and E.G. Gregorich. 2006. Overview of the symposium proceedings, “Meaningful pools in determining soil carbon and nitrogen dynamics. *Soil Sci. Soc. Am. J.* 70:967–974. doi:10.2136/sssaj2005.0111
- Ostle, N.J., P.E. Levy, C.D. Evans, and P. Smith. 2009. UK land use and soil carbon sequestration. *Land Use Policy* 26:S274–S283. doi:10.1016/j.landusepol.2009.08.006

- Pinton, R., S. Cesco, M. De Nobili, S. Santi, and Z. Varanini. 1997. Water- and pyrophosphate-extractable humic substances fractions as a source of iron for Fe-deficient cucumber plants. *Biol. Fertil. Soils* 26:23–27. doi:10.1007/s003740050337
- Polano, M., C. Anselmi, L. Leita, A. Negro, and M. De Nobili. 2008. Organic polyanions act as complexants of prion protein in soil. *Biochem. Biophys. Res. Commun.* 367:323–329. doi:10.1016/j.bbrc.2007.12.143
- Polizzotto, M.L., C.F. Harvey, S.R. Sutton, and S. Fendorf. 2005. Processes conducive to the release and transport of arsenic into aquifers of Bangladesh. *Proc. Natl. Acad. Sci. USA* 102:18819–18823. doi:10.1073/pnas.0509539103
- Polizzotto, M.L., B.D. Kocar, S.G. Benner, M. Sampson, and S. Fendorf. 2008. Near-surface wetland sediments as a source of arsenic release to ground water in Asia. *Nature* 454:505–508. doi:10.1038/nature07093
- Qian, J., U. Skyllberg, W.G. Frech, W.F. Bleam, P.R. Bloom, and P.E. Petit. 2002. Bonding of methyl mercury to reduced sulfur groups in soil and stream organic matter as determined by X-ray absorption spectroscopy and binding affinity studies. *Geochim. Cosmochim. Acta* 66:3873–3885. doi:10.1016/S0016-7037(02)00974-2
- Schmidt, M.W.I., M.S. Torn, S. Abiven, T. Dittmar, G. Guggenberger, I.A. Janssens, et al. 2011. Persistence of soil organic matter as an ecosystem property. *Nature* 478:49–56. doi:10.1038/nature10386
- Seidel, B., A. Thomzig, A. Buschmann, M.H. Groschup, R. Peters, M. Beekes, and K. Tertytze. 2007. Scrapie agent (strain 263K) can transmit disease via the oral route after persistence in soil over years. *PLoS One* 2:e435. doi:10.1371/journal.pone.0000435
- Skujinš, J., and R.G. Burns. 1976. Extracellular enzymes in soil. *Crit. Rev. Microbiol.* 4:383–421. doi:10.3109/10408417609102304
- Skyllberg, U. 2008. Competition among thiols and inorganic sulfides and polysulfides for Hg and MeHg in wetland soils and sediments under suboxic conditions: Illumination of controversies and implications for MeHg net production. *J. Geophys. Res. Biogeosci.* 113:14. doi:10.1029/2008jg000745
- Skyllberg, U. 2012. Chemical speciation of mercury in soil and sediment. In: G. Liu, et al., editors, *Environmental chemistry and toxicology of mercury*. John Wiley & Sons, Hoboken, NJ, p. 219–250.
- Skyllberg, U., P.R. Bloom, J. Qian, C.M. Lin, and W.F. Bleam. 2006. Complexation of mercury(II) in soil organic matter: EXAFS evidence for linear two-coordination with reduced sulfur groups. *Environ. Sci. Technol.* 40:4174–4180. doi:10.1021/es0600577
- Skyllberg, U., J. Qian, W. Frech, K. Xia, and W.F. Bleam. 2003. Distribution of mercury, methyl mercury and organic sulphur species in soil, soil solution and stream of a boreal forest catchment. *Biogeochemistry* 64:53–76. doi:10.1023/A:1024904502633
- Skyllberg, U.L., K. Xia, P.R. Bloom, E.A. Nater, and W.F. Bleam. 2000. Binding of mercury(II) to reduced sulfur in soil organic matter along upland-peat soil transects. *J. Environ. Qual.* 29:855–865. doi:10.2134/jeq2000.00472425002900030022x
- Stevenson, F.J. 1994. *Humus chemistry: Genesis, composition, reactions*. 2nd ed. John Wiley & Sons, Hoboken, NJ.
- Tomaszewski, J.E., R.P. Schwarzenbach, and M. Sander. 2011. Protein encapsulation by humic substances. *Environ. Sci. Technol.* 45:6003–6010. doi:10.1021/es200663h
- Vaughan, D., and R.E. Malcolm. 1985. Influence of humic substances on growth and physiological processes. In: D. Vaughan and R.E. Malcolm, editors, *Soil organic matter and biological activity*. Martinus Nijhof/Dr. W. Junk Publ., Dordrecht, the Netherlands. p. 37–75. doi:10.1007/978-94-009-5105-1_2
- Walter, W.D., D.P. Walsh, M.L. Farnsworth, D.L. Winkelman, and M.W. Miller. 2011. Soil clay content underlies prion infection odds. *Nat. Commun.* 2:200. doi:10.1038/ncomms1203
- Wingeyer, A.B., D.T. Walters, R.A. Drijber, D.C. Olk, T.J. Arkebauer, S.B. Verma, et al. 2012. Fall conservation deep tillage stabilizes maize residues into soil organic matter. *Soil Sci. Soc. Am. J.* 76:2154–2163. doi:10.2136/sssaj2012.0121
- Xia, K., W.F. Bleam, U. Skyllberg, P.R. Bloom, and E.A. Nater. 1999. XAS study of the binding of mercury(II) to reduced sulfur in soil organic matter. *Environ. Sci. Technol.* 33:257–261. doi:10.1021/es980433q
- Xia, K., F. Wessner, W. Bleam, P.R. Bloom, U.L. Skyllberg, and P.A. Helmke. 1998. XANES studies of oxidation states of S in soil and aquatic humic substances. *Soil Sci. Soc. Am. J.* 62:1240–1246. doi:10.2136/sssaj1998.03615995006200050014x
- Zang, X., J.D.H. van Heemst, K.J. Dria, and P.G. Hatcher. 2000. Encapsulation of protein in humic acid from a Histosol as an explanation for the occurrence of organic nitrogen in soil and sediment. *Org. Geochem.* 31:679–695. doi:10.1016/S0146-6380(00)00040-1
- Zheng, Y., M. Stute, A. van Geen, I. Gavrieli, R. Dhar, H.J. Simpson, et al. 2004. Redox control of arsenic mobilization in Bangladesh groundwater. *Appl. Geochem.* 19:201–214. doi:10.1016/j.apgeochem.2003.09.007