PYRENE AVAILABILITY AS DETERMINED BY THE
INTERACTIONS OF NATURAL ORGANIC MATTER
WITH THE SOIL MINERAL MATRIX

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ABSTRACT

DENISE MARIE BROWN: Pyrene Availability as Determined by the Interactions of Natural Organic Matter with the Soil Mineral Matrix.
(Under the direction of Professor Frederic K. Pfaender)

Soil organic matter (SOM), especially humic substances and biological material, profoundly affect polyaromatic hydrocarbon (PAH) movement, toxicity, and degradation when assessing acceptable end points for in situ remediation practices. The complex, interdependent relationship between soil chemical and physical attributes makes determining absolute PAH fate, transport, and availability a formidable task. This research attempted to correlate the impact known SOM components have on pyrene sorption as related to sorbent loading and type and mineral phase aggregation. The objective was achieved by generating a synthetic soil, composed of mineral matter, humic acid (HA), and peptidoglycan (PG), and comparing this matrix to freshly contaminated and aged natural soils, using pyrene as the representative hydrophobic organic contaminant.

In contrast to HA, a larger concentration of PG sorbed to both mineral phases (hematite and montmorillonite clay); thus, promoting further particle aggregation, and effectually resulting in greater pyrene sorption as evidenced by HPLC and fluorescence microscopy. The greater the concentration and number of constituents introduced into the synthetic matrix, culminating in a HA+PG mixture loaded onto clay, the greater the resulting pyrene sorption and similarity to spiked natural soils, both in chemical and physical attributes. The final result was a compiled fluorescence map visually detailing pyrene
location and percentage sorbed in relation to PG, HA, and the mineral phases for natural and synthetic soils, where the biological constituent held a significant influence over pyrene compared to that by the humic substances.

In summary, the type and concentration of SOM constituents, specifically humic substances and biological material, sorbed to a mineral phase significantly impact soil physical and chemical characteristics, and therefore, should be accounted for when determining pyrene fate, transport, and availability within contaminated soil systems. Potentially, when these observations are combined with fluorescence microscopy, consultants could determine the most effective remediation technology to target specific locations, such as pore spaces, or strong sorptive entities, such as PG, and achieve acceptable end-points.
This dissertation is dedicated to those that supported me the most.

To my parents, Robert and Sandy Brown, who taught me to persevere and strive for the best.

To my brother and sister-in-law, Darren and Angela Brown, who always knew when to ask “Aren’t you done yet?”

Finally, to my nephew, Austin, who always inspires me.

Finally, to my husband, Jeff Zborowski, who was kind enough to go to Iraq for seven months and leave me alone to finish this monstrosity, but also the one to make me smile through the frustration and love me through the tears.

Thank you.
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Thank you.
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<th>Description</th>
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<tr>
<td>BET</td>
<td>Brunauer, Emmett and Teller (surface area measurement method)</td>
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<tr>
<td>BF</td>
<td>Bright Field</td>
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<tr>
<td>CEC</td>
<td>Cation Exchange Capacity</td>
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<td>CLSM</td>
<td>Confocal Laser Scanning Microscopy</td>
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<td>CMN</td>
<td>Contaminated Minnesota (soil)</td>
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<td>CSEM</td>
<td>Cryogenic Scanning Electron Microscopy</td>
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<tr>
<td>DAPI</td>
<td>4’ 6-diamidino-2-phenylindole:2 HCl</td>
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<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<tr>
<td>FL</td>
<td>Fluorescence</td>
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<td>FI</td>
<td>Fluorescence Intensity</td>
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<td>FTIR</td>
<td>Fourier Transform Infrared</td>
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<td>HA</td>
<td>Humic Acid</td>
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<td>HOC</td>
<td>Hydrophobic Organic Compound</td>
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<tr>
<td>HPLC</td>
<td>High-Pressure Liquid Chromatography</td>
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<tr>
<td>HS</td>
<td>Humic Substances</td>
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<tr>
<td>I</td>
<td>Ionic Strength</td>
</tr>
<tr>
<td>KKY</td>
<td>Kentucky (soil)</td>
</tr>
<tr>
<td>LT</td>
<td>Light</td>
</tr>
<tr>
<td>MN</td>
<td>Minnesota (soil)</td>
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<tr>
<td>MW</td>
<td>Molecular Weight</td>
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<td>NAPL</td>
<td>Nonaqueous Phase Liquid</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<tr>
<td>NOM</td>
<td>Natural Organic Matter</td>
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<tr>
<td>OM</td>
<td>Organic Matter</td>
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<tr>
<td>PAH</td>
<td>Polyaromatic Hydrocarbon</td>
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<td>PG</td>
<td>Peptidoglycan</td>
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<tr>
<td>PMN</td>
<td>Pristine Minnesota (soil)</td>
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<tr>
<td>PSA</td>
<td>Particle Surface Area</td>
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<tr>
<td>RFI</td>
<td>Relative Fluorescence Intensity</td>
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<tr>
<td>SEM</td>
<td>Scanning Electron Microscope</td>
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<tr>
<td>SF</td>
<td>Schenck Forest (NC, soil)</td>
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<tr>
<td>SOM</td>
<td>Soil Organic Matter</td>
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<td>TOC</td>
<td>Total Organic Carbon</td>
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<td>TPEM</td>
<td>Two-Photon Excitation Microscopy</td>
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CHAPTER 1: INTRODUCTION AND OBJECTIVES

1.1 INTRODUCTION

Naturally occurring organic matter (OM) is a ubiquitous component of terrestrial and aquatic ecosystems, constituting one of the most abundant and oldest carbon forms on the surface of Earth [38, 67]. A residue resulting from the partial decomposition of plant tissues and microbial metabolism, soil organic matter (SOM) is a polydisperse, heterogeneous mixture of relatively recalcitrant entities placed in a poorly-defined state between degradation and biosynthesis [38]. The all-embracing term “humus” also describes this by-product mixture that is related to the vegetation, climate, composition, and properties of the particular soil or sediment environment where found [67]. These materials are defined by default – a category of natural substances that cannot be classified into any of the categories used for discrete materials, such as proteins, polysaccharides, and polynucleotides [67]. SOM comprises humic substances, lignin-derived components, carbohydrates, carbonaceous matter, and other organic compound classes, which confer a degree of environmental persistence lacking in discrete biomolecules [54]. Since these systems involve multiple interactions, SOM exerts a profound influence on aspects of soil nature and environmental processes, such as soil fertility, ion-exchange ability, water-holding capacity, structure, aggregation, sorption of metals and organics, and oxidation and reduction reactions of organic compounds [39, 47, 54, 90].
Polyaromatic hydrocarbons (PAHs) consist of natural and anthropogenic organic combustion products that pollute thousands of sites around the world. Though not well understood, sorption to natural solids is a widely accepted process that affects the fate, availability, transport, degradation, and biological activity of pollutants in the environment. Hydrophobic organic compounds, such as PAHs, that persist in soil or sediment become resistant to chemical and biological extraction, where the size of this desorption-resistant, nonavailable fraction dramatically increases with time [32, 33]. Therefore, nonavailability describes those compounds that are chemically, physically, and/or biologically inaccessible within the soil matrix and only accounted for in a mass balance equation by combustion/gas pyrolysis. Several mechanisms have been described for the aging of chemicals in soils and sediments, including diffusion and adsorption within SOM components, nonaqueous phase liquids (NAPL), or mineral solid pore systems [32]. Consequently, this nonavailable fraction is not necessarily recalcitrant to microbial degradation nor covalently bound to SOM. Elucidating these relationships between SOM, the soil matrix, and PAH binding and availability to microorganisms is essential to managing and remediating polluted sites.

Currently, research into PAH sequestration is limited to sorption/desorption studies and NMR and FTIR analyses, with little direct observation of component interactions within the soil matrix [62, 63]. Fluorescence microscopy provides a powerful, non-intrusive tool for visualizing and tracking the movement, storage locations, and degradation of organic chemicals within vegetation, soil, and water. It requires little to no sample modification or manipulation and uses the inherent autofluorescence of the chemical compound, plant, and/or NOM. For instance, Karimi-Lotfabad and Gray [45] used fluorescent confocal laser scanning (CLSM) and cryogenic scanning electron (CSEM) microscopies to examine nonaqueous
phase liquid (NAPL) distribution on and within the soil microstructure. The results indicated that NAPLs encased aggregate surfaces and filled accessible inter-aggregate pores to create a discrete phase, suggesting that contaminant diffusion out of NAPL-clay structures and filled pores was important to desorption from soil [45]. Another study by Wild et al. [99] determined PAH location and intercellular migration within plant roots and leaves via two-photon excitation microscopy (TPEM) [100, 101]. The study identified anthracene in five separate locations within the leaf, and when applied to the roots, highly focused compound "streams" formed over time and degraded into anthrone, anthraquinone, and hydroxyl-anthraquinone [99, 100, 101]. Although both studies are important, there is still no visual account of SOM component interaction and influence on PAH sorption and availability, which is essential to developing cost-effective and efficient in situ remediation technology.

1.2 OBJECTIVES

The first objective for this research was to determine the ability of synthetic clay to simulate natural soils, particularly the precision with which synthetic clay models the chemical, physical, and pyrene sorption properties of natural soils. The approach was to generate synthetic clays composed of known humic acid (HA) and peptidoglycan (PG) contents and compare them against two pristine natural soils in the following: cation exchange capacity (CEC), percent organic content, pH, particle size, BET (Brunauer, Emmett and Teller) surface area, and pyrene sorption. These properties were examined via total organic carbon (TOC) and spectroscopic analyses, a particle size analysis (PSA) program, high pressure liquid chromatography (HPLC), and pyrene fluorescence intensity measurements. The results allowed comparisons of soil physical and chemical attributes
regarding the ability of synthetic clay to accurately model pyrene sorption in natural environments and are addressed in Chapter 3.

Using the synthetic soil developed in Chapter 3, the second objective for this work was to determine the impact different SOM constituents, specifically HA and PG, have on soil tertiary structure and how these constituents interact with each other and the mineral phase. The goals for this study component included: (i) evaluation of HA sorption to mineral phases compared to PG, (ii) determining the impact HA and PG (individually and combined) have on particle aggregation, and (iii) observing the location of HA and PG (individually and combined) in relation to the mineral phase. The approach was to generate a clean, controllable soil system that could be manipulated to assess individual and combined interactions between SOM constituents and a mineral base. These interactions were examined via TOC analysis, spectrophotometry measurements, a particle size analysis program, and fluorescence microscopy, where direct visualization of SOM coatings is a novel approach to gaining information on soil structure. The result allowed known concentrations of two SOM components to be sequentially loaded onto a given solid phase, and thus, provided for an evaluation of their individual and combined affect on soil construction and resulting tertiary structure, which is discussed in Chapter 4.

Using the synthetic soil developed in Chapter 4, the objective for the pyrene spiking study was to examine HOC sorption, specifically the impact two different SOM constituents (HA and PG) have on sorbed pyrene concentrations and location, and compare the results with pyrene sorption to two natural soils. The goals for this component of the research included: (i) determining the affect PG and HA (individually and combined) have on pyrene sorption, (ii) correlating pyrene sorption data from the synthetic soils with that from natural
soils, (iii) evaluating the location of individual and combined soil constituents, PG and HA, in relation to pyrene and the mineral phase, and (iv) establishing from the fluorescence images whether pyrene has a preference for a specific SOM component. The approach was to generate a clean, controllable soil system that could be manipulated to assess visually and quantitatively the impact HA and PG have on pyrene sorption. These interactions were examined via HPLC and fluorescence microscopy, where direct visualization of SOM interactions with pyrene is a novel approach providing information that addresses fate, transport, and availability. The result allowed known concentrations of two SOM components to be sequentially loaded onto a given solid phase, and thus, provided for an evaluation of their individual and combined effect on pyrene sorption as compared to natural soils, which is described in Chapter 5.

The fourth objective for this research was to demonstrate if fluorescence intensity (combined with a calibration curve) may be used as a quick alternative technique to diagnose PAH presence and concentration in a contaminated soil and to preliminarily observe and measure pyrene fate and transport progress over time within a given natural soil. The goals for this objective included: (i) generating a calibration curve of sorbed pyrene concentration versus fluorescence intensity; (ii) using the calibration curve combined with fluorescence microscopy to accurately calculate the relative amount of individual and total PAHs in contaminated soils; and (iii) examining the dual-mode progression of freshly spiked and aged PAHs in natural soils. Using a calibration curve derived from the spiked soils in Chapter 5, two soils well-aged with creosote were analyzed for individual and total PAH amounts via fluorescence microscopy and compared to the values given by the traditional extraction and analysis method of a commercial laboratory. Also, the dual-mode progression of soil-bound
PAHs was observed and measured, using fluorescence microscopy and intensity. The result may be a more efficient and economical method to determine PAH content in soils compared to traditional protocols and visual groundwork for justification of the dual-mode model, which are both detailed in Chapter 6.

Chapter 7 states an overview of the results and conclusions for this research project.
CHAPTER 2: LITERATURE REVIEW

Soil particles are generated based on two distinct, yet interdependent attributes. The “chemical” aspect not only describes the organic adhesives used to bind individual mineral particles of all sizes together, but also the matrix necessary for hydrophobic organic contaminant (HOC) dissolution and sorption, potentially hindering HOC availability to degradation via microbial communities. Conversely, the “physical” title is used to label those soil characteristics inherent within the mineral matter itself, such as tertiary structure formation. Polyaromatic hydrocarbons (PAHs), a subset of HOCs, are a class of auto-fluorescent compounds that can be employed to probe into these mechanisms and phenomena related to soil construction and thereby understand bioavailability more profoundly.

2.1 SOIL CHEMICAL STRUCTURE

Soil chemistry and reactivity are significantly affected by the organic matter (OM) trapped therein or sorbed along the surface of the mineral components. Despite extensive research, the molecular structure, chemistry, and absolute role of this OM is not well understood [86]. Moreover, because of its variability and close relationship with clay minerals and metal oxides, the chemistry and reactions OM undergoes with metals, other natural organic matter, and anthropogenic chemicals are highly complex.
2.1.1 SOIL ORGANIC MATTER (SOM)

Humus and SOM are virtually synonymous and include the total organic compounds in soils, excluding nondecayed plant and animal tissues, their “partial decomposition” products, and soil biomass [75, 83, 86, 87]. SOM is a complex, amorphous mixture of highly heterogeneous, refractory molecules produced ubiquitously in the environment via chemical reactions among diverse species, early biomatter diagenesis, and random chemical alteration of precursor molecules [35, 54, 80, 83]. SOM contents range from 0.5–5% by weight in the surface horizon of mineral soils to 100% in histols and improve soil structure, aggregation, aeration, and water-holding capacity [23, 36, 86]. Further, SOM is an important source of H (3.3-4.8%), O (34-39%), N (3.7-4.1%), P (<1%), and S (<1%) [86]. SOM also contains large quantities of C (52-58%), which provide an energy source for soil macro- and microflora, and has a soil C/N ratio of approximately 10-12:1 [86]. Finally, SOM has a specific surface area up to 800-900 m²g⁻¹ and a cation exchange capacity (CEC) ranging from 150-300 cmol kg⁻¹; thus, the majority of surface soil CEC may be attributable to its SOM content [5, 86, 91, 93, 97].

SOM conformation influences soil ecosystem functions and reactivity, as well as organic substance and pollutant availability. The classical definition for SOM structure includes a polydisperse, linear polymeric concept in which SOM is the condensation product of polymers with varying molecular weight (MW) values, and these polymers are biologically synthesized from plant tissues, such as lignin, and cell death [67, 89]. Conversely, in the supra-molecular model neutral or alkaline SOM are self-assembling aggregations of predominantly small, apolar heterogeneous molecules (polymethyenic chains, fatty acids, steroid compounds, and aromatic moieties) stabilized by multiple weak
dispersive forces, such as hydrophobic interactions (van der Waals, charge transfer, $\pi-\pi$, and CH-$\pi$) and hydrogen bonds [14, 67, 68, 89]. This depicts SOM structure as a stable amphiphilic pseudomicelle in solution, where hydrophobic molecular entities reside within the core and hydrophilic functionalities remain exposed along the exterior, and hence, as a bilayer membrane when coating mineral grains [67, 89, 95, 96].

As shown in Figure 2.1, SOM is comprised of both nonhumic and humic substances (HS). The nonhumic substances have identifiable physical and chemical properties and include carbohydrates, proteins, peptides, amino acids, fats, waxes, and low molecular weight acids [83, 86, 91]. These compounds are transient in nature as they are easily metabolized by soil microorganisms. Conversely, HS, discussed in the next section, persist in soil for longer time periods due to recalcitrance.

**Figure 2.1.** Fractionation of SOM and humic substances [86].
**2.1.1.1 SOIL ORGANIC MATTER COMPONENTS**

**HUMIC SUBSTANCES (HS)**

Composing approximately 60-70% of soil organic carbon, HS are believed partially responsible for reduced contaminant availability [35, 59]. Humic acid (HA), in particular, is defined as a SOM fraction precipitating at pH 1 and characterized as a category of naturally occurring, biogenic, refractory, heterogeneous organic substances being yellow-to-black in color, of high molecular weight, and result from plant and animal residue decomposition [54, 67]. Scanning electron microscope (SEM) images of crystallized HA at total magnifications 100x and 900x are shown in Figure 2.2.

![Figure 2.2. SEM images of HA at 100x (left) and 900x (right) total magnifications.](image)

HS, comprising fulvic acid, HA, and humin generally bound to biomolecules, are macromolecular solids that contain highly functionalized aliphatic, alicyclic, aromatic, and heteroaromatic backbones cross-linked by metal ions or covalent bonds [103]. These aromatic and aliphatic structures are rich in oxygen-containing functional groups, e.g., −COOH, phenolic and/or enolic −OH, alcoholic −OH, and quinolic −C=O [59]. However, the heterogeneous nature of HS forbids a single molecular or structural representation, opting
instead for averages of many such formulae. Implemented as a means of communication, *pseudostructures* are theoretical molecular constructs having elemental, structural, and functional group features consistent with some or all the observed properties [54]. One such HA pseudostructure, based on recent research by Schulten and Schnitzer [82], is in Figure 2.3. These hypothetical representations are intended to suggest types of chemical systems that account for HS behavior observed in the laboratory and natural environment [54].

![Three-dimensional HA pseudostructure with molecular formula C\textsubscript{352}H\textsubscript{413}O\textsubscript{116}N\textsubscript{15}, elemental analysis 63.0% C, 6.0% H, 27.7% O, and 3.1% N, and MW 6710.16 g mol\textsuperscript{-1} [82].](image)

**Figure 2.3.** Three-dimensional HA pseudostructure with molecular formula C\textsubscript{352}H\textsubscript{413}O\textsubscript{116}N\textsubscript{15}, elemental analysis 63.0% C, 6.0% H, 27.7% O, and 3.1% N, and MW 6710.16 g mol\textsuperscript{-1} [82].

**PEPTIDOGLYCAN (PG)**

Leenheer *et al.* [52] and Jarusutthirak *et al.* [40] identified via Fourier Transform Infrared (FTIR) and \textsuperscript{13}CNMR spectroscopies that roughly one-third of SOM and dissolved organic matter (DOM) colloids is comprised of amino sugars, such as PG. As shown in Figure 2.4, the FTIR spectra for pure PG samples are similar to that for the Anaheim Lake colloids. PG, found in both Gram-negative and -positive bacteria, is a rigid layer that completely encases a cell, protects the plasma membrane, and conveys strength to the overlying cell wall [55, 94]. Though some bacteria have only a single PG layer, many
bacteria, especially Gram-positives, have up to 25 concentric PG layers, comprising up to 90% of the cell wall [55, 94]. The carbohydrate backbone (Figure 2.5) consists of alternating \( \beta(1 \rightarrow 4) \)-linked \( N \)-acetyl-D-glucosamine and \( N \)-acetylmuramic acid as linear chains [55, 94]. The lactic acid group of \( N \)-acetylmuramic acid in Figure 2.5 forms an amide bond with a D-amino acid-containing tetrapeptide, such as L-alanine, D-alanine, D-glutamic acid, and either lysine or diaminopimelic acid, to form the glycan repeating unit [55, 94]. Finally, neighboring parallel PG chains are covalently cross-linked in the third dimension through their tetrapeptide functional groups [55, 94].

**Figure 2.4.** FTIR spectra acquired by Dr. Leenheer of both peptidoglycans used for this experimental series.
Figure 2.5. The glycan tetrapeptide is a repeating unit in the PG structure. The structure above is for *E. coli* and most Gram-negative bacteria, while different amino acids are found in Gram-positives [55].

Though the glycosidic bonds connecting glycan sugars are strong, PG structural strength is realized only when these chains are cross-linked by amino acids, with greater rigidity coming from increased cross-linking [55]. For instance, cross-links in Gram-positive PG are usually by a peptide interbridge, where the kinds and numbers of amino acids vary among species [55, 94]. In the Gram-positive bacterium *Staphylococcus aureus*, Figure 2.6, each interbridge polypeptide consists of a pentaglycine chain that extends from the terminal carboxyl group of one tetrapeptide to the lysine ε-amino group in a neighboring tetrapeptide [55, 94].
2.1.1.2 DUAL MODE MODEL: GLASSY VERSUS AMORPHOUS CARBON

Pignatello and Xing [69] described a “soft or rubbery” versus “hard or glassy” carbon concept, the dual-mode model, to distinguish two broad SOM categories having dissimilar diagenetic histories and sorption properties. Based on the theory of SOM as a polymer mesh phase, ‘rubbery’ refers to a gel-like, expanded, flexible, and highly solvated state, while ‘glassy’ defines condensed, rigid, and less solvated fractions [107]. As substantiated by X-ray diffraction (XRD), wide-angle X-ray scattering (WAXS) nuclear magnetic resonance (NMR), and elemental data from soil and coal samples, condensed carbons are mainly aromatic in nature and contain internal, relatively long-lived, nanoscale holes equivalent to a crystallite thickness of approximately 25 –CH2– units [39, 60, 67, 104, 107]. Combined with a comparable amount of aliphatic, isotropically mobile poly-(methylene) carbon, these glassy and rubbery domains form SOM, HS, and humin aggregates [39, 67, 104]. To illustrate,
SOM in Figure 2.7 can be envisioned as a bulk partition medium consisting of rubbery (A) and glassy (B) regions. Dispersed among the glassy regions are adsorption sites (C) of varying energies called “holes,” where the concentration of such sites increases with SOM glassy character [69, 107]. These “holes” have high internal surface areas, are shallow or part of an extended network, mildly flexible due to relaxation via heat or plasticization by the penetrant, and based within the folds of single macromolecules, between aggregated macromolecules, or between the organic phase and anchored mineral surface [49, 69, 76, 103].

![Figure 2.7. Schematic of a natural organic matter particle or coating, illustrating a rubbery phase A, glassy phase B, and “holes” C.](image)

In this dual mode model parent soil sorption properties exhibit a combination of behaviors involving both linear partitioning and nonlinear, intramatrix, pore-filling retention [2, 15, 69, 103]. The adsorption domain, Figure 2.7, consists of the glassy, condensed moieties, such as black carbon (BC) and kerogen. The associated slow hole-filling mechanism yields nonlinear sorption isotherms, concentration dependence, sorbate planarity preference, multiphasic desorption kinetics, strongly elevated TOC-water distribution ratios ($K_{TOC}$), hysteretic behavior, and solute-solute competition [2, 3, 4, 15, 17, 29, 69, 103]. In contrast, amorphous SOM components, specifically HS and biopolymers, exhibit
simultaneous partitioning behavior associated with linear local isotherms, rapid diffusion, no sorption competition, and sorption reversibility [2, 3, 4, 15, 17, 69, 103, 107].

2.1.2 SOIL ORGANIC MATTER SORPTION TO MINERAL PHASES

There are numerous covalent, noncovalent, and electrostatic binding schemes to sorb SOM onto mineral phases, where the particular mechanism used affects SOM and pollutant transport and reactivity in soil and sedimentary systems [6, 78]. Since atoms on particle surfaces are not surrounded by oppositely charged ions, they are incompletely bonded, and therefore, the hydrated mineral surface layer is a “checkerboard” of electron rich (oxygen) and electron poor (metal) sites [24, 84]. As depicted in Figures 2.8 and 2.9, SOM coatings contain a heterogeneous mixture of proteins, carbohydrates, lipids, HS, and aromatic geopolymers, such as coal or soot, and bind to a mineral matrix, such as clay [51]. This retained SOM “organizes” into various conformations depending on the solid phase, including: macromolecular insoluble complexes, compounds bound together by protons and di- and trivalent ions, molecules adsorbed to clay or oxide surfaces, or organic matter in the interlayers of expanding-type clay minerals [65]. Further, the structure of sorbed SOM is controlled largely by the solvated configuration, pH, ionic strength, Ca$^{2+}$ concentration, the number of attachment points, degree of ionization for carboxylate and hydroxyl sites on SOM, hydroxyl site density, distribution, and reactivity, and other microtopographic surface features [63, 77].
Figure 2.8. SOM-mineral pseudostructure assembled from seven HA subunits, trisaccharides, hexapeptides, three iron oxide chains, phosphates, and alkali- and heavy metals [81].
Figure 2.9. Hypothetical chemical structure of SOM and associated contaminants on the surface of a clay mineral particle [51].

2.1.2.1  HUMIC SUBSTANCE SORPTION TO MINERAL PHASES

A bilayer membrane model was proposed for organic coatings in which hydrated, polar functionalities responsible for primary interactions with the mineral surface line the membrane exterior, and nonpolar hydrocarbon structures remain in the unsolvated interior portion, as in Figure 2.9 [51]. This HS bilayer masks the physicochemical properties of the underlying mineral substrate, e.g., electrophoretic mobility, colloidal stability, and transport, and renders hydrophilic surfaces hydrophobic, which are more conducive for sorbing organic contaminants [29, 51]. Therefore, HS adsorption to inorganic matter generally yields energetic differences and progresses via one or more of six mechanisms, including: (i) ligand
exchange-surface complexation, (ii) anion exchange (electrostatic interaction), (iii) cation exchange, (iv) cation and water bridging, (v) hydrogen bonding, and (vi) hydrophobic interactions [6, 29, 51]. In addition, the dominant mechanisms, van der Waals interactions, ligand exchange, and cation bridging, contribute approximately 60%, 35%, and 5%, respectively, to total sorption, where a combination of hydrophobic effects and ion exchange may supply half the adsorption attributed to van der Waals interactions [6, 7, 29, 51, 57, 62, 63, 77, 78].

As discussed, HS adsorption is dependent on solution parameters (pH, ionic strength, and bivalent cation concentrations), mineral surface and HS functional groups, and HS “quality” (size, aromaticity, and polarity) [63, 65, 77, 78]. For instance, sorption to hematite and kaolinite is positively correlated with HS aromaticity and molecular weight (MW) and negatively correlated with the O/C ratio, a measure of polarity [6, 78]. Adsorption affinity for larger molecules, such as HA vs. FA or soil OM vs. marine OM, is thermodynamically favored [6, 64, 78]. High performance size exclusion chromatography (HPSEC) and molar absorptivity data demonstrate that the MW of solvated HS decreases in the presence of adsorbing clay minerals and suggests that larger MW, hydrophobic, and aromatic moieties are preferentially sorbed, which presumably alters bulk chemical reactivity [6, 57, 64, 77, 78]. These larger molecules may have more functional groups or lay flat along the mineral surface, both allowing for maximum, simultaneous interaction points and a larger entropy gain than by adsorption of monofunctional, low-MW organic molecules [6, 29, 51, 78]. The resulting cumulative effect of multiple binding sites, or polydentate complex formation, between SOM and the particle surface can be very large, and adsorption becomes irreversible due to the improbability of complete, simultaneous bond breakage [6, 29, 63, 78].
2.1.2.2 PEPTIDOGLYCAN SORPTION TO MINERAL PHASES

Proteins, glycans (such as PG), and peptides are also highly surface reactive due to the hydrophobic effect, essential to forming organic coatings, and promote particle aggregation [6, 51, 74]. Non-covalent forces (e.g., electrostatic and van der Waals interactions, hydrogen bonding, and other forces dominated by $\pi-\pi$ bonds) act to stabilize these high MW glycoprotein-mineral surface interactions [20, 74]. Moreover, strong electrostatic attraction between a cationic amino group and a negatively charged clay surface may explain the high adsorption capacity of clay minerals for lysine as in Figure 2.10 and, by extension, poly-lysine and poly-arginine, which are basic PG components [20]. This indicates that both absorbent-adsorbate and adsorbate-adsorbate electrostatic interactions exert an important influence on PG-mineral phase adsorption.

![Figure 2.10. Hypothetical construct of PG sorption to a mineral surface.](image)

The amount of PG or protein adsorbed on the surface of montmorillonite clay is linearly related to the interlamellar/basal spacing, demonstrating that surface area
accessibility to macromolecules is important for adsorption [20]. The apparent adsorption partition coefficients for proteins are approximately $10^2$ L kg$^{-1}$ on illite and $10^3$-$10^4$ L kg$^{-1}$ on goethite and montmorillonite, which are sufficient to promote protein preservation [20]. Generally, basic polyamino acids have greater partition coefficients than acidic ones, and these coefficients indicate that electrostatic interactions dominate in protein adsorption, where very strong adsorption is likely due to binding at multiple sites just as for HS [20]. As illustrated in Figure 2.10, this strong sorption results because large biological molecules may exist in solution as flexible chains and attach to the mineral or HS surface at several points, leaving loops and trains in the solution or collapsing on the surface [20, 74].

2.1.3 SUMMARY

SOM is a complex composite of labile, recalcitrant, humic and non-humic substances, all affecting soil chemistry and reactivity by coating and aggregating individual particles via a pseudo-bilayer. Although determining a single molecular structure for SOM is almost impossible due to its vast heterogeneity, several of its distinct components have been identified, including PG, HS, and BC. These constituents yield the dual mode behavior inherent within SOM toward HOCs, where condensed organic domains are likely dominated by BC and other aromatic compounds and amorphous regions include more aliphatic carbon, such as HS and PG. Finally, though ligand exchange appears to be the major mechanism for sorbing SOM to mineral matrixes, other hydrophobic interactions, for example van der Waals forces, and electrostatic reactions, such as cation exchange, are significant depending on SOM composition, and bulk phase pH, temperature, ionic strength, and co-solute concentration.
2.2 SOIL PHYSICAL STRUCTURE

The pedosphere is an open system with fluxes of matter and energy from the lithosphere, biosphere, hydrosphere, and atmosphere [58]. The interaction of these Earth systems contributes to weathering processes which transform primary minerals to secondary minerals, such as clays and metal oxides [58]. The resulting mineral tertiary structure depends strongly on the soil particle size distribution, SOM content and quality, porosity or water storage capability, and the organic carbon content of individual size fractions [58, 81]. More fundamentally, mineral structure and the strength of atomic bonds in that structure are important determinants of mineral physical properties [58].

2.2.1 MINERAL PHASES

Clays are defined as fine-grained, crystalline, hydrous silicates with layer lattice structures [21]. A characteristic feature of clay is the extensive adsorptive surface for water, ions, and SOM. Specifically, surface area is described as the ratio of a particle surface to its volume, which is low for tightly-packed hematite (5.9 m² g⁻¹; Figure 2.11a) and high for expandable clays, such as montmorillonite (31.8 m² g⁻¹; Figure 2.11b) [46, 97]. Such a vast difference results from the additional surface area between the silicate sheets of expandable clays, called the interlayer area or internal surface [97]. In comparison, coarse sand has a specific surface area of approximately 0.01 m² g⁻¹, fine sand 0.1 m² g⁻¹, silt 0.1-1 m² g⁻¹, and humic acids 800-900 m² g⁻¹ [97]. Nonetheless, under natural conditions SOM intercalation within clay particles is rarely observed since macromolecules, such as HA, are larger than interlayer spaces, which are up to 1.8 nm for swelling smectites [81]. Hence, external surface
properties are more important for SOM sorption than the inter-particle crystal structure of clay minerals [81].

**Figure 2.11.** Three-dimensional models for (a) montmorillonite clay and (b) hematite [13, 18]. The molecule color coding includes: oxygen (red), iron (olive green for hematite and orange for clay), hydrogen (white), sodium (blue), magnesium (green), silica (gold), and aluminum (grey).

Furthermore, montmorillonite clay cation exchange capacity (CEC) is high at 80-120 cmol kg\(^{-1}\) (moles of exchangeable cation per kilogram of solid phase) compared to hematite, <2 cmol kg\(^{-1}\), and HA has the highest CEC at 150 - 300 cmol kg\(^{-1}\) [5, 91, 93, 97]. The two mechanisms driving this high montmorillonite CEC are both associated with the negative charges on silicate clays. The first mechanism involves unsatisfied valences at the broken edges of silica and alumina sheets and exposed oxygen and hydroxyl groups at external mineral surfaces, acting as negatively charged sites [97]. For instance, minerals such as hematite with the absence of isomorphous replacement have exchange sites confined to broken crystal edges, resulting in a low CEC [97]. Conversely, montmorillonite and other expandable clays have a relatively large amount of isomorphous replacement resulting in more exchange sites and a high CEC [97]. The second mechanism generating a high CEC for
mineral phase surfaces is hydrogen bonding [91]. Though both solid phases have a great deal of oxygen in their molecular formula, more oxygen atoms are exposed along the montmorillonite structure, as illustrated in Figure 2.12, than that for hematite due to the larger surface area and expandability of clay [97].

2.2.2 AGGREGATION

Aggregation and aggregate stabilization is a complex process that operates at a variety of spatial and temporal scales. Macroaggregates, >250 μm, are formed from microaggregates, 20-250 μm, by soil organism action and the binding forces of microbial metabolites or plant mucilages [81]. Macroaggregate stability is also conveyed by fine root presence, plant exudates released from growing roots, and rhizosphere microflora, such as glomalin released by the hyphae of vesicular-arbuscular mycorrhizal fungi, while microaggregate cores may include fragments of plant materials encrusted with inorganic matter [10, 28, 37, 72, 81, 102]. Finally, the smallest aggregates, <20 μm, are irregular arrangements of clay microstructures and biopolymers [81].
As discussed, SOM influences soil aggregation, porosity, and stability [10, 72, 81]. Conversely, aggregation affects SOM dynamics, and carbon storage and cycling as organic substances in micro- and nanopores may be biologically unavailable [10, 81]. More specifically, soil aggregation affects SOM formation, destruction, and stabilization, protects SOM from microbial breakdown, and influences decomposer micro-environment conditions [10, 28].

Aggregation is not due to SOM as a whole but specific components therein. Soil carbohydrates and microbial exocellular polysaccharides, representing only 10-20% of SOM composition, are a vital subset of organic constituents actively involved in the initial, transient formation of soil aggregates and decline as polysaccharides decompose [36, 37, 88, 90, 102]. Conversely, long-term aggregate stabilization is attributed to HS accumulation and organo-mineral complex formation [28, 36, 37]. For instance, when long-term pastures and arable sites were compared, the pasture aggregates had greater stabilization, SOM accretion, and hydrolysable and extractable carbohydrate content [36, 37]. Conversely, arable aggregates are weakly bound together due to low SOM content, entrapped air in pore spaces, and unequal pressures caused by differential swelling during rewetting [36]. These observations reflect an increase in humified binding agents under long-term pasture and a collapse under long-term arable conditions [36, 37].

2.2.3 PORE SPACES

Inorganic surfaces associated with geosorbent components include: external surfaces, swelling clay interlayer surfaces, and internal surfaces [53, 69]. These internal surfaces, classified in Figure 2.13 by sorbate behavior, contain: (i) macropores >500 Å that fill with
sorbate only when submerged; (ii) internal mesopores with diameters 20-500 Å that due to capillary condensation fill with sorbate at relative vapor pressures approaching saturation; and (iii) micropores with diameters <20 Å that influence sorbate sorption by the proximity of two solid surfaces and may connect larger cavities of mesopore size [53, 69]. Porosity, therefore, reflects the amount of pore space between grains and is the ratio of void space to total rock or soil volume [58, 79].

**Figure 2.13.** Cross-sectional schematic of a particle showing different sorption processes. “Particles” are usually aggregates of smaller grains cemented together by organic or inorganic materials, and porosity is due to spaces between and fractures within individual grains [69].
Some pores were originally either spaces between adjacent sedimentary particles or vesicles formed in igneous rocks as gases escaped from the cooling melts [58]. This type of porosity is called *primary porosity* as it developed at the same time the rock itself was created, whereas *secondary porosity*, such as fractures, is generated after rock formation [58]. Moreover, the size of individual primary pore spaces does not affect water storage capacity since the total pore space volume is the same regardless of pore size [58]. However, grain size and surface tension do affect water quantities that flow through pore spaces, where smaller grains and fractures have more surface area per unit volume than larger grains and fractures [58]. As a result, smaller particles or fractures sorb more water, SOM, and/or pollutants to the soil or rock.

2.3 POLYAROMATIC HYDROCARBONS

Polyaromatic compounds are ring structures held together by stable bonds containing delocalized $\pi$ electron clouds that grant resonance stabilization across the entire molecule [56]. Polyaromatic hydrocarbons (PAHs), of which pyrene is an example, are a class of such compounds that contain at least two condensed rings, emit autofluorescence under UV light, and are synthesized by the incomplete combustion of saturated hydrocarbons under oxygen-deficient conditions and by diagenesis [31, 56, 98, 109].

![Pyrene](image)

Even hydrocarbons with low MWs, such as ethane in the reaction below, act as PAH precursors via pyrosynthesis, which occurs at temperatures exceeding 500° C when C–H and C–C bonds are broken to form free radicals [56]. These radicals dehydrogenate and
chemically combine to develop aromatic ring structures resistant to thermal degradation, where higher temperatures generate more highly condensed PAHs [98].

The hydrocarbon tendency to form PAHs via pyrosynthesis varies in the order aromatics > cycloolefins > olefins > paraffins, where an existing ring structure is conducive to PAH formation [56]. This process occurs naturally in vegetation fires, volcanic activity, and diagenesis, yielding a low, ubiquitous concentration between 1-100 μg kg\(^{-1}\) throughout the surface of Earth [98]. However, due to several anthropogenic partial combustion and pyrolysis processes that favor PAH production, these compounds are encountered more abundantly in urban atmosphere, soil, and water bodies from sources that include engine exhausts, wood stove and cigarette smoke, internal combustion engines, charbroiled food, creosote, and petroleum residues, such as road and roofing asphalt [56].

PAH soil concentration increases in the order arable soils < mineral soils under forest < permanent grassland < urban soils < specifically contaminated sites, where the most abundant individual PAHs include benzo(b+j+k)fluoranthenes, chrysene, fluoranthene, and pyrene [9, 41, 98]. Although PAH concentrations generally decrease at greater soil depths due to degradation, sequestration, and DOM-mediated leaching, high PAH concentrations may occur in deeper horizons from sequential depositions of contaminated material,
particularly at industrial sites for coke ovens, gasworks, petroleum refineries, and wood preservation plants [34, 98]. Further, evidence exists that climate substantially influences PAH deposition patterns as soil temperature and moisture, and land use applications and duration influence PAH decomposition and volatilization [98].

2.3.1 DUAL MODE SORPTION MODEL

Understanding PAH sorption mechanisms has moved from correlations between sorption and OM content to relationships with specific OM structures and domains [75, 98]. As illustrated by Schulten and Schnitzer [82] in Figure 2.14, PAH association with SOM sorbed to a mineral phase depends on the polarity, aromaticity, molecular size, configuration, and chemical composition of the carrier molecule [1, 27, 42, 50, 75, 98]. Therefore, PAH bioavailability, i.e. the potential transfer from soil to living organisms, depends on hydrophilicity and binding of the compound within the soil, where an increasing soil residence time decreases PAH extractability, degradability, and toxicity [50, 98].
Figure 2.14. Pseudostructure describing SOM binding to the inner surface of silica sheets via \( \text{Al}^{3+} \) and \( \text{Fe}^{3+} \) bridges, pesticide (hydroxyatrazine) immobilization, and biological molecule sorption [81].

Prompted by recent geosorbent studies the simplistic partitioning model underwent re-evaluation into a dual-mode mechanism, illustrated in Figure 2.13, and is defined as simultaneous slow, nonlinear condensation in (or adsorption on the walls of) pores and rapid, linear partitioning into an organic phase [8, 69, 105, 106]. This dual mode model encompasses the following attributes: (i) nonlinear sorption isotherms, requiring that the activity coefficient for the dissolved compound inside SOM be concentration-dependent; (ii) competition in multisolute systems, implying site specificity; (iii) concentration-dependent heat of sorption, indicating that sorption potential is not constant; (iv) increasing apparent hysteresis and decreasing HOC desorption rates and extractabilities as a function of sorbate size, concentration, and residence time on geosorbents; and (v) recognition that the intrinsic
heterogeneity of natural geosorbents may be augmented by adherent or entrapped NAPLs, anthropogenic organic matter, diagenic materials (kerogen and low-rank coals), catagenic matter (hard coals), and BC (soot and charcoal) [4, 8, 27, 53, 69, 103, 106].

Even for classically “noninteracting” compounds, such as aromatic hydrocarbons and chlorinated solvents, sorption by a solid matrix requires solute transfer from bulk solution to a site of immobilization, thermodynamically driven as illustrated in Figure 2.15 by hydrophobic expulsion from water and the affinity between sorbate and HS [8, 48, 69, 77, 91, 105, 106]. Once transported, the intermolecular interactions available to neutral organic compounds, such as van der Waals and aromatic ($\pi$) electron forces, dipole-dipole, dipole-induced dipole, and hydrogen bonding, are common to both the adsorption and partitioning mechanisms [16, 17, 34, 69, 103]. For instance, nonionic organic contaminants with aromatic $\pi$ electrons may form charge-transfer complexes with electron donating or accepting SOM moieties, as illustrated in Figure 2.16 by the strong $\pi$ complex between pyrene and a SOM aromatic functional group [27, 51].

Figure 2.15. Cartoon depicting the two driving forces of hydrophobic sorption: water expulsion and affinity between sorbate and HS [48].
Figure 2.16. Hypothetical chemical structure of a natural organic coating and associated pyrene contaminant.

Resulting from this heterogeneity in sorbate interaction potential, dual mode sorption isotherms are operationally separated into a “fast” fraction, the amount sorbed after 24 hours, and a kinetically “slow” fraction, the amount sorbed thereafter [105, 106]. An empirical relationship known as the Freundlich isotherm can be used as a surrogate for this dual-mode isotherm:

\[
C_s = K_F \cdot C_w^n
\]  

[1]

where \(K_F\) and \(n\) are constants [84, 106]. Relationship [1] assumes there are multiple types of sorption sites (linear and non-linear) acting in parallel, with each site exhibiting a different sorption free energy and total abundance [53, 84, 104]. In particular, \(n\) is an index of site free energy distribution, i.e., the smaller the \(n\) the broader the energy distribution and the greater the hole-filling mechanism contribution [84, 106]. When \(n = 1\), the isotherm is linear, and constant sorption free energies at all sorbate concentrations are inferred; when \(n < 1\), the isotherm is concave downward, and adsorption likely involves weaker and weaker free
energies; finally when \( n > 1 \), the isotherm is convex upward, and sorbate presence in the sorbent encourages further dissolution [84].

### 2.3.1.1 PAH DISSOLUTION INTO SOM

Diffusion is defined as “random movement under the influence of a concentration gradient” [69]. For hydrophobic solutes and when molecular propensity toward mineral sorption is low, diffusion mechanisms are dominated by SOM as in Figure 2.17, where diffusion is the rate-limiting step and SOM exists as surface coatings or discreet particles [8, 53, 63, 69, 77]. This dissolution domain contains thermally dynamic sites, meaning they are constantly disappearing and reforming due to thermal deformation of the humic backbone, swelling, and co-solvent effects, causing an averaged energy profile as for a liquid [53, 69, 105, 106]. Sorption under dilute conditions in the amorphous, lipophilic organic carbon domain (Figure 2.17) exhibits partitioning behavior associated with linear local isotherms, rapid diffusion, no competition, sorption reversibility, and obeys Fick’s law of concentration-independence [8, 27, 53, 69, 104, 106]. The apparent, sorbed-phase diffusivity and resulting rate are, therefore, dependent on intra-organic matter viscosity and electronic properties, the particle size distribution, chemical interactions between diffusing molecules and SOM moieties, diffusant structure and concentration, temperature, number of co-solutes, and exposure history, where mixtures of polymer sphere sizes can lead to bimodal diffusion curves [8, 69, 105, 106].
Figure 2.17. Trapping of the xenobiotic pesticide hydroxyatrazine metabolite, circled for clarity, by SOM [81].

Although several studies designate SOM aromatic carbon domains [2, 3, 11, 15, 16, 17, 25, 30, 43], such as BC or glassy HS, as the primary moieties governing PAH sorption and distribution, results by Salloum et al. [75] indicate substantial dissolution into “rubbery” SOM paraffinic components, where a decrease in sorbent aromaticity was not met with an equal decline in PAH sorption [28]. These less recognized polymethylenic structures may be physically constrained by soil minerals or various SOM structures, such as lignin and cellulose, and therefore, may not be as accessible or competitive against other SOM functionalities [75]. Studies further demonstrate that plant exudates (aliphatic carbon associated with SOM, humin, and kerogen) sorbed as much or more PAHs than highly aromatic components, such as HA and lignin [28, 75].
2.3.1.2 FILLING SOM AND MINERAL PHASE HOLES WITH PAHS

Analogous to the glassy state of a polymer, condensed SOM, such as BC and hard HS, exhibit a combination of transient sorption sites (Figure 2.18). These sites include relatively rigid, planar, aromatic surfaces and internal meso- and micropores within SOM, which likely encompass “slow” and “very slow” kinetics, respectively [4, 17, 27, 53, 104, 105, 106]. In addition to SOM as the sorptive phase, “slow” and “very slow” HOC adsorption may occur on exposed or HS-lined external surfaces and internal porous regions of mineral particles [4, 27, 53, 69]. The overall sorption process is typified by nonlinear (Freundlich $n$ values from 0.30 to 0.70) isotherms, concentration-dependent dissolution, bimodal kinetics, sorption-desorption hysteresis, sorbate-sorbate competition, planarity or molecular size selectivity, and correlates with the N$_2$ (or CO$_2$)-determined microvoid sorbent volumes [1, 16, 17, 27, 42, 53, 61, 63, 69, 104, 105, 106, 107]. Nitrogen isotherms at 77 K, and more recent 273 K CO$_2$ data, indicate that adsorption “sites” are less abundant on external SOM regions readily available to bulk solution and more profuse within internal regions, suggesting uneven site distribution [49, 105, 106]. These pores (Figure 2.18) are local regions of rigid, free volume, where one or a few sorbate molecules undergo interactions via adsorptive van der Waals complexation with the internal surface [16, 27, 105, 106]. Pores are limited in population, span a range of steric and electronic characteristics, confer sorption specificity, involve tortuous pathways, interconnectivity, dead-end pores, and variability in pore diameter, where a greater volume yields a larger contribution by the hole-filling mechanism [1, 8, 69, 107]. Therefore, at low aqueous PAH concentrations (ng L$^{-1}$ and less), such as that found in marine systems, sorption within shallow to extended pore networks is thermodynamically preferred [2, 3, 15, 17]. Conversely, in heavily polluted and
OM-rich systems nonlinear sorption sites become saturated, inducing more competition, and are replaced by sorption to nonpyrogenic SOM constituents [2, 15].

Figure 2.18. Dual-mode conceptualization, where SOM is depicted as an amalgam of rubbery and glassy phases. Both phases have dissolution domains, but the glassy phase also has holes where specific interactions occur [106].

Another factor affecting the pore-filling mechanism is when a solid shows enhanced affinity for a sorbate during a second sorption cycle, the “conditioning effect”, which is more pronounced at higher sorbate concentrations because at low concentrations HOCs only fill existing voids without inducing significant deformation [76]. Slow pore-deformation hysteresis occurs by sorbate-induced, quasi-reversible alteration of the sorbent away from its thermodynamic state through an increase in the unrelaxed free volume [76]. This occurs via two methods: dilation of existing holes due to the motion of sorbate molecules exerting pressure on polymer strands lining the hole, and hole formation by which incoming sorbate molecules create new holes from proto-hole sites [76]. These larger or “new” holes are partially conserved during desorption as the structural rigidity impairs relaxation back to the thermodynamic state, resulting in the manifestation of hysteresis [76].
2.4 SUMMARY

The schematic in Figure 2.19 illustrates conceptual PAH-geosorbent interactions. In summary, there are at least five geochemical processes that affect SOM and HOC distribution and fate: sorption/partition, precipitation, volatilization, chemical and biochemical oxidation/reduction, and complexation, where *sequestration* refers to a combination of diffusion limitations [53, 91]. Presumably, no single sorption mechanism dominates, and in natural systems multiple processes likely contribute to rate-limited sorption behavior, complicating the interpretation of macroscopic data [69].

The mineral domain has surface reactivity attributable to: (i) exposed external mineral surfaces at the particle scale and surfaces within macropores; (ii) surfaces within mesopores and micropores of inorganic mineral matrices; and (iii) the interlayer surfaces of swelling clays at the nanometer scale [53, 69, 81]. Rubbery and glassy SOM components constitute a second sorption domain, where adherent or entrapped nonaqueous phase liquids (NAPLs) function as rubbery OM and combustion residue, e.g., BC, functions as hard carbon [3, 15, 53, 69, 107].

By analogy to the polymeric glassy state, condensed SOM carbon exhibits a combination of sorption behaviors involving both linear partitioning and nonlinear intramatrix, pore-filling retention [53, 104, 105, 106]. Adsorption of solute molecules, Figure 2.19, within condensed organic matter (case B) or hydrophobic microporous regions of minerals (case E) may require protracted time scales, be nonlinear, hysteretic, and subject to solute-solute competition [53, 61, 63, 69, 104, 105, 106, 107]. In contrast, and by correlation with a rubbery polymer, the amorphous SOM domain, cases A, C, and D in Figure 2.19, may exhibit partitioning behavior associated with linear local isotherms, rapid diffusion, no
competition for sorption, and sorption reversibility [8, 53, 69, 104, 106]. Also, like a polymer, SOM may exhibit transition from a condensed state to a loosely-knit rubbery state as temperature or cosolvent concentration is increased [53, 103, 105, 106].

**Figure 2.19.** Conceptual model of geosorbent domains, including different forms of SOM, combustion residue, and anthropogenic carbon, such as NAPLs [53].
CHAPTER 3: SYNTHETIC CLAY AS A SURROGATE FOR MODELING PYRENE SORPTION TO NATURAL SOIL

3.1 INTRODUCTION

Synthetic soil is a matrix constructed by individually sorbing SOM components, such as humic acid (HA) and/or peptidoglycan (PG), of known character and quantity onto a single bare mineral phase, of which kaolinite and montmorillonite clays are regularly used. What results is a “synthesized” cleaner, controllable soil devoid of the extraneous variables and unknowns inherent to natural soils and which commonly cause experimental interferences that complicate data interpretation. For instance, SOM from natural soils contains multiple components (HA, humin, biological constituents, carbonaceous material, and non-humic compounds) of varying and unknown chemical composition and quantities, which are responsible for many of the unique properties of soil. In comparison, the synthetic soil used for research in the succeeding chapters contained only one or two of these components, specifically HA and/or PG, at known amounts and chemical composition. It is due to this “clean, known, and controllable” characteristic that use of synthetic clays has been a common and preferred matrix to study hydrophobic organic contaminant (HOC) sorption relationships and mechanisms [6, 7, 29, 57, 61, 62, 63, 78, 81, 82].

However, synthetic soil is often criticized as solely portraying the “perfect” sorption scenario and, as a result, is not applicable to the natural environment. Therefore, the objective of this chapter was to determine the ability of synthetic clay to simulate natural soils,
particularly the precision with which synthetic clay modeled the chemical, physical, and pyrene sorption properties of natural soils. The approach was to generate synthetic clays composed of known HA and PG contents and compare them against two pristine natural soils in the following: CEC (cation exchange capacity), percent organic content, pH, particle size, BET (Brunauer, Emmett and Teller) surface area, and pyrene sorption. These properties were examined via total organic carbon (TOC) and spectroscopic analyses, a particle surface area (PSA) analysis program, high pressure liquid chromatography (HPLC), and pyrene fluorescence intensity measurements. The results allowed comparisons of soil physical and chemical attributes regarding the ability of synthetic clay to accurately model pyrene sorption in natural environments.

3.2 EXPERIMENTAL SECTION

All chemicals and solvents were either HPLC grade or higher. Glassware used in the preparation of any solution, whether stock, diluted, buffer, or sample, were cleaned thoroughly using an acid-washing regimen. Metal and plasticware were cleaned in the same manner as for glass, except an acid bath was not used.

3.2.1 NATURAL SOILS

Two uncontaminated natural soils were employed for the research described in Chapters 3 through 6. PMN (pristine Minnesota soil) was the unspoiled representative of CMN (contaminated Minnesota soil), a soil from a wood preservation facility historically contaminated by creosote and diesel fuel for approximately 50-55 years. PMN was from an
uncontaminated field adjacent to the CMN site. SF was the second pristine soil used for this research and was collected from Schenck Forest, NC.

### 3.2.2 SYNTHETIC SOILS

A generalized schematic of the SOM loading procedure is shown in Figure 3.1. The solid phases, hematite (J.T. Baker) and Na-saturated montmorillonite clay (SWy-2; Source Clay Minerals Repository) were first baked at 500° C in a muffle furnace for 24 hrs [63]. To prevent degradation, fresh 5000 mg L\(^{-1}\) stock solutions of HA and PG were generated in pH 8.00 buffer (pHydrion; \(I \approx 0.014\) M) using an IHSS soil HA standard (1S102H) and freeze-dried \(S.\) aureus (77140; BioChemika) PG, respectively. Diluted HA (0-800 mg L\(^{-1}\)) and/or PG (0-200 mg L\(^{-1}\)) solutions were generated by pipetting a calculated volume of stock into 25 mL fresh pH 7.00 buffer (pHydrion; \(I \approx 0.105\) M) made with ultra-purified water. The diluted solutions were added to 0.500 g ± 0.0005 g of room temperature hematite or clay in disposable 50-mL Corning centrifuge tubes and vortexed for approximately 1 min [50, 63]. The tubes were mounted at a 45° angle on a New Brunswick orbital shaker, covered with a black plastic bag to avoid UV degradation, and equilibrated at ambient lab temperature (22° C) for 20 hrs at 200 rpm [63]. Each loading concentration was prepared in duplicate, and to monitor for TOC contamination, duplicate blank buffer and solid phase samples were carried throughout the entire experiment with the loaded specimens. After equilibration the samples were centrifuged at 2416 g at 22° C, hematite for 20 min and clay for 30 min [63]. The supernatants were then transferred via disposable glass Pasteur pipets to rinsed 40-mL amber vials and analyzed for TOC [63]. If sequential loadings occurred, the entire process was repeated with the second standard, either PG or HA. Once the loadings were complete,
the solid phase was dried in a vacuum desiccator with indicating DriRite (Fisher Scientific) and transferred to 1.8-mL amber bottles until microscopic analysis.

**Figure 3.1.** This brief schematic details the loading procedure when HA was added first. The SOM component order was opposite if PG was added first.

### 3.2.3 AQUEOUS PHASE PG AND HA ANALYSIS

Sample collection, storage, handling, and analysis, with the exception of inorganic carbon (IC) purging via acid addition and bubbling, were conducted in accordance with Standard Method #5310B Combustion-Infrared Method for TOC [22]. No further dilution was required for any analyzed samples as a universal sensitivity catalyst was employed by the instrument, a Shimadzu TOC-5000 Total Organic Carbon Analyzer equipped with an ASI-5000 autosampler. The sorbed fraction of HA and/or PG was calculated by the
difference between the initial concentration and the TOC remaining in solution [63, 75]. Sorption distribution coefficients (K_D) were calculated using linear regression [2] of the respective Freundlich isotherm [1] and the K_D formula [3] [84].

\[ C_s = K_F \cdot C_w^n \]  
\[ \log C_s = n \log C_w + \log K_F \]  
\[ K_D = K_F \cdot C_w^{n-1} \]

To correct for the amount of HA that desorbed from the mineral matrix upon addition of the PG aqueous solutions (when PG was added after HA), samples were analyzed by a Hitachi U-3300 UV/Vis Spectrophotometer at 254 nm using a 1.0-mL quartz, semimicro cuvette (10-mm pathlength; Fisherbrand QS 282). Supernatant samples with a 50 mg L\(^{-1}\) or greater starting concentration were diluted, using a 100 µL supernatant : 1000 µL pH 7.0 buffer ratio, for the result to be within a measurable range and reasonably free of inner-filter effects [44, 59, 66, 109]. Blank pH 7.0 buffer was employed in the reference cell and automatically subtracted from the base graph. The dissolved HA concentration was calculated via a calibration curve and subtracted from the amount sorbed to the mineral phase (determined from the TOC data).

3.2.4 PARTICLE SURFACE AREA (PSA) ANALYSIS

For all samples 5-10 mg of dried soil were mounted onto an acetone-rinsed, glass microscope slide (Fisherbrand Superfrost Plus; 25 x 75 x 1.0 mm) with a raised coverslip (CoverWell Incubation Chambers; 0.50 µL; Bio-Labs) placed overtop. Samples were observed at 100x total magnification using an OPELCO Olympus IX71 research inverted fluorescence microscope outfitted with an Olympus MicroFire camera. Soils were visualized
without perturbation using conventional bright-field light, and a total of ten images per sample were acquired. The images were captured in binary colors with a 1.000 gain and 687 ms exposure time, batch transformed from 12-bit TIFF files to 640x480 pixel TIFF images at 72 resolution using Adobe Photoshop Elements CS software, and analyzed for PSA using BioQuant Nova Prime software. The BioQuant program removed particle background shadows, highlighted yellow individual grains or aggregates as in Figure 3.2, ignored periphery particles by outlining those to be measured in red, numbered and calculated the surface area of each outlined particle, and exported the values to an Excel spreadsheet.

![Figure 3.2. Representative image of PSA analysis using a BioQuant software program.](image)

### 3.2.5 PYRENE APPLICATION TO SYNTHETIC AND NATURAL SOILS

Pyrene (>99%, Sigma Aldrich), used without further purification, was dissolved in HPLC-grade dichloromethane (DCM) and stored in an amber bottle at -20°C [50, 63, 75]. In 30-cm³ clear glass centrifuge tubes (Kimax), varying concentrations of the pyrene stock, 100-5000 mg L⁻¹, were added to triplicate natural soils or synthetic clays loaded with HA and/or PG as described previously [71]. The samples were vortexed for 1 min, and the DCM was allowed to evaporate in a hood [71]. One soil sample was sacrificed for immediate solvent...
extraction and HPLC analysis to determine the amount of pyrene volatilized [71]. To the other two samples 5-mL fresh pH 7.00 buffer (pHyrion; $I \approx 0.105$ M) made with ultra-purified water were added, and the tube screw caps were covered with aluminum foil over the Teflon-lined septa [12, 50, 63, 71, 75, 108]. To prevent photodegradation light was excluded by covering the tubes with a black plastic bag [63]. The suspensions were equilibrated at a 45° angle on an orbital shaker at 150 RPM for 48 hrs at 22° C [12, 50, 63, 75]. The tubes were centrifuged in rubber adapters at 2415 g at 22° C, natural soil for 20 min and clay for 30 min. The supernatants were removed via disposable glass Pasteur pipets to rinsed 40-mL amber vials for solvent extraction, and the screw caps were covered with aluminum foil over the Teflon-lined septa [12, 50, 63, 71, 75, 108]. The soils were dried in a vacuum desiccator with indicating DriRite (Fisher Scientific) and transferred to 1.8-mL amber bottles until microscopic analysis.

### 3.2.6 AQUEOUS PHASE PYRENE ANALYSIS

Pyrene in the aqueous supernatant and volatilization samples was solvent extracted and quantified by HPLC to determine via subtraction the amount sorbed by the soil matrix. Within the storage vial, the sample was extracted with three 10-mL volumes of HPLC-grade DCM, and each extraction was equilibrated fully upright on an orbital shaker in the dark at 150 RPM for 48 hours at 22° C [70]. The three extracts per sample were combined in one 40-mL amber vial, evaporated over a 24-hr period to dryness using a gentle stream of UHP-grade N$_2$ gas, and re-dissolved in 5 mL HPLC-grade acetonitrile (ACN) [70, 73]. The reconstituted samples were transferred via disposable glass Pasteur pipets to 8-mL amber vials and stored in a -4° C freezer until HPLC analysis.
Supernatant extracts were analyzed by a Waters™ 600E HPLC System controller and 717 Plus autosampler, a Supelco LC-PAH column (250 mm x 4.6 mm, ID), and an 85% ACN : 15% water isocratic mode with 1.5 mL min$^{-1}$ flow rate [70]. Pyrene was identified fluorescently using a Perkin Elmer LS40 fluorescence detector, operating at excitation and emission wavelengths 336 nm and 398 nm, respectively [70]. The resulting chromatography data were analyzed via Millennium 2010 software and plotted in an Excel spreadsheet [70]. Sorbed pyrene was calculated from the difference between the quantity added (determined from the sacrificed samples) and that in the equilibrated solutions, minus blanks for loss to volatilization and sorption to glassware [63, 75, 108].

3.2.7 FLUORESCENCE MICROSCOPY

Using the mounted soils described in Section 3.2.4, samples were observed at 100x total magnification via an OPELCO Olympus IX71 research inverted fluorescence microscope outfitted with an Olympus MicroFire camera. Pyrene fluorescence was viewed with 300-400 nm UV light using a filter specific for PAHs. Images for fluorescence intensity analysis were captured in grayscale colors with a 1.000 gain and 4.99 sec exposure time as 8-bit uncompressed TIFF files. Images of buffer solutions, empty glass slides, crystallized HA and PG, and blank solid phases were acquired as evidence that no significant background fluorescence interference was present and needed to be digitally subtracted from the sample images.
3.2.8 FLUORESCENCE INTENSITY ANALYSIS

Fluorescence intensity is a unit-less value assigned to either an individual fluorescing body or an entire image describing the brightness of the observed fluorescence, where areas or images with a higher concentration of fluorescence yield greater intensities. This value ranges from 0-255 for an 8-bit image and was measured by Fovea Pro 3.0, where ‘0’ describes an image that is completely black (devoid of all light) and ‘255’ represents a pure white image. Intensity was the chosen RGB component as all grayscale images have the same hue and saturation, and grayscale images were used due to their higher resolution compared to colored images. When graphed against the spiked pyrene concentration, the resulting fluorescence intensity increase corresponded with an increase in the amount of pyrene sorbed to the soil.

3.3 RESULTS

3.3.1 COMPARISON OF PHYSICAL AND CHEMICAL ATTRIBUTES

The purpose of this work was to develop a synthetic clay consisting of only the primary components that may influence PAH sorption, and thus, eliminate the extraneous variables common to natural soils that complicate interpretation of sorption relationships and mechanisms. This synthetic clay was then examined for its ability and accuracy to act as a clean, controllable surrogate for natural soils. As shown in Table 3.1, the synthetic clay CEC, N₂ BET surface area, pH, and percent organic matter fell within the range given by the two pristine natural soils used for this research, PMN and SF. Moreover, when comparing blank
clay (montmorillonite not loaded with SOM) versus HA clay, it appeared that addition of HA caused clay to exhibit physical and chemical characteristics analogous to the natural soils.

**Table 3.1. Characteristics of Natural and Synthetic Soil Samples**

<table>
<thead>
<tr>
<th></th>
<th>PMN</th>
<th>SF</th>
<th>Blank Clay</th>
<th>HA Clay</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Soil Class</strong> (a)</td>
<td>Mineral Soil</td>
<td>Mineral Soil</td>
<td>Mineral Soil</td>
<td>Mineral Soil</td>
</tr>
<tr>
<td><strong>Textural Class</strong> (a)</td>
<td>Sandy Loam</td>
<td>Sandy Clay Loam</td>
<td>Clay</td>
<td>Clay</td>
</tr>
<tr>
<td><strong>Total PAH (ppm)</strong> (b)</td>
<td>20.4 ± 4.6</td>
<td>0.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>Pyrene Conc. (ppm)</strong> (b)</td>
<td>3.5 ± 1.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>CEC (meq 100 cm⁻¹)</strong> (a)</td>
<td>13</td>
<td>46.2</td>
<td>76.4 (e)</td>
<td>38.0</td>
</tr>
<tr>
<td><strong>N₂ BET Surface Area (m² g⁻¹)</strong> (c)</td>
<td>7.6 ± 2.3</td>
<td>10.0</td>
<td>25.2 ± 0.2 (d)</td>
<td>7.9 ± 0.1 (f)</td>
</tr>
<tr>
<td><strong>pH</strong> (e)</td>
<td>8.1</td>
<td>6.8</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td><strong>% Sand</strong> (a)</td>
<td>63</td>
<td>58</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>% Silt</strong> (a)</td>
<td>24</td>
<td>22</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>% Clay</strong> (a)</td>
<td>13</td>
<td>21</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><strong>% Organic Matter</strong> (a)</td>
<td>1.5</td>
<td>10.9</td>
<td>0.0 (f)</td>
<td>3.0</td>
</tr>
</tbody>
</table>

(a) Soil class and texture, cation exchange capacity (CEC), pH, percent sand/silt/clay, and percent organic matter were analyzed by the University of Wisconsin Soil Science Extension Service, Madison, WI.
(b) PAH content was determined by Triangle Laboratories, Inc., Durham, NC, using EPA method 8270. The total PAH content includes the 16 priority compounds listed by EPA (acenaphthene, acenaphthylene, anthracene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(g,h,i)perylene, benzo(k)fluoranthene, chrysene, fluoranthene, fluorene, naphthalene, phenanthrene, pyrene, dibenz(a,h)anthracene, and indeno(1,2,3-cd)pyrene).
(c) Surface area was measured by Clear Science, Inc., Baton Rouge, LA.
(d) Mean values are presented with one standard deviation calculated from triplicate samples.
(e) CEC and percent organic matter for blank montmorillonite clay was determined by the Clay Minerals Society Source Clay Repository, Aurora, CO.

### 3.3.2 PARTICLE SURFACE AREA (PSA) ANALYSIS

In Figure 3.3 the PSA values for both synthetic clays were within the range of those given for PMN and SF and diverged significantly from the blank clay PSA. Just as for the characteristics summarized in Table 3.1, it appeared that the addition of SOM caused clay to behave more like a natural soil. Hence, the PSA measurements, in combination with data from Table 3.1, indicated that the physical and chemical characteristics of synthetic clay were similar to those for the natural soils, yielding strong evidence to the ability for synthetic clay to model the structure of natural soils.
Figure 3.3. Comparison of PSA data for natural soils, HA+PG synthetic clays, and blank clay, where the synthetic clays fell within the range observed for the pristine natural soils (PMN and SF).

3.3.3 PYRENE SORPTION TO NATURAL AND SYNTHETIC SOILS

As mentioned previously, the primary goal of this section was to determine if the synthetic clay was an acceptable surrogate for modeling pyrene sorption, and thus, eliminate many of the unknowns or complicating factors when using natural soils. As depicted in Figure 3.4 and the $K_D$ values in Table 3.2, pyrene sorption to HA+PG synthetic clay was statistically similar to that within natural soils ($P = 0.05$ and 0.06 for SF and PMN, respectively). When comparing the synthetic and blank clay graphs, it appeared that the addition of SOM caused clay to behave more like the natural soils in regard to the resulting pyrene sorption.
Table 3.2. Distribution Coefficients for Pyrene Sorbed to Synthetic Clay, Blank Clay, and Natural Soils

<table>
<thead>
<tr>
<th>Soil Sample:</th>
<th>$R^2$:</th>
<th>Freundlich $n$ Values:</th>
<th>Average $K_D$: (mL g$^{-1}$)</th>
<th>One Std. Dev.: (mL g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$100$ mg L$^{-1}$ PG + (100, 400, and 800 mg L$^{-1}$) HA on Clay</td>
<td>0.98</td>
<td>1.17</td>
<td>284</td>
<td>58</td>
</tr>
<tr>
<td>PMN Soil</td>
<td>0.85</td>
<td>0.98</td>
<td>306</td>
<td>11</td>
</tr>
<tr>
<td>SF Soil</td>
<td>0.93</td>
<td>1.26</td>
<td>327</td>
<td>91</td>
</tr>
<tr>
<td>Blank Clay</td>
<td>0.93</td>
<td>0.53</td>
<td>491</td>
<td>438</td>
</tr>
</tbody>
</table>

**Pyrene Sorption on Synthetic and Natural Soils**

![Graph showing Pyrene Sorption](image)

**Figure 3.4.** Freundlich isotherms comparing the sorption of pyrene on natural soils, blank and synthetic clays, where the addition of SOM caused clay to behave more like natural soil in regard to pyrene sorption.

To complement the HPLC results in Figure 3.4, pyrene fluorescence intensity measurements were taken for each sorbed pyrene concentration. In Figure 3.5 graphs of the pyrene fluorescence intensities for the spiked synthetic and natural soils were similar in curvature and plateau, as compared to the linear character of the blank clay data. Therefore,
the sorbed pyrene results in Figures 3.4 and 3.5 indicated that synthetic clay was a reasonable surrogate for natural soils when researching sorption mechanisms.

![Synthetic Clay, Blank Clay, and Natural Soils Freshly Spiked with 1.0-50.0 mg g\(^{-1}\) Pyrene](image)

**Figure 3.5.** Comparison of fluorescence intensities for pyrene sorbed to natural soils and synthetic clays, where the resulting graphs were similar in curvature and plateau.

### 3.4 DISCUSSION

According to the data presented in this chapter, synthetic clay appeared to accurately simulate the physical, chemical, and pyrene sorption characteristics of natural soils. In Table 3.1 the synthetic clay CEC, \(N_2\) BET, pH, and percent organic matter fell within the range given by the two pristine natural soils, PMN and SF. However, these attributes were considerably different compared to those for blank clay (montmorillonite not loaded with SOM). This trend was also noted for the PSA results in Figure 3.3, where the synthetic clays
fell within the range set by the natural soils and deviated from blank clay. Finally, according to the Freundlich isotherms in Figure 3.4 and calculated $K_D$ values in Table 3.2, pyrene sorption to both natural soils and the synthetic clay was statistically similar ($P = 0.05$ and 0.06 for SF and PMN, respectively). To substantiate this observation, the graphs of pyrene fluorescence intensities in Figure 3.5 had relatively the same curvature and plateau, indicating comparable behavior between the natural soils and synthetic clay. When pyrene sorption to blank clay was compared to that for synthetic clay (Figures 3.4 and 3.5) the resulting graphs varied appreciably.

This trend of synthetic soil deviating from blank clay and embracing the characteristics of natural soils appeared to stem from the introduction of SOM components to clay. SOM assimilation changed the chemical properties of montmorillonite clay by satisfying valences at broken edges of silica and alumina sheets and exposed oxygen and hydroxyl groups along external mineral surfaces. SOM effectually masked these negative charges with a hydrophobic coating that had a greater adhesive character, surface area (800-900 m$^2$ g$^{-1}$), and CEC (150-300 cmol kg$^{-1}$); thus, making the surface more conducive to the approach by and sorption of other hydrophobic compounds [5, 91, 93, 97]. While this addition of SOM to clay promoted pyrene sorption and particle aggregation similar to that for natural soils, SOM also partially or completely blocked pore spaces and the interlayer area of expandable clays, thereby decreasing the CEC and N$_2$ BET surface area compared to blank clay [49, 76].

In summary, once SOM was added to clay, the “synthesized” matrix deviated significantly from the characteristics of blank clay and instead, behaved more like a natural soil in regard to chemical and physical properties, particle aggregation, and pyrene sorption.
Therefore, based on similarities in a variety of properties, synthetic clay was an acceptable surrogate for modeling pyrene sorption to natural soils, eliminating the potential interferences and confusion caused by the inherent unknowns of natural soils, and thus, was used in experiments detailed in the following chapters.
CHAPTER 4: IMPACT OF HUMIC ACID AND PEPTIDOGLYCAN ON THE TOTAL ORGANIC CARBON CONCENTRATION AND PARTICLE AGGREGATION OF SYNTHETIC SOIL

4.1 INTRODUCTION

SOM, encompassing nonhumic and humic substances (HS), is a complex, amorphous mixture of highly heterogeneous, refractory molecules produced ubiquitously in the environment via biomatter diagenesis and random chemical alteration of diverse precursor molecules [35, 54, 80, 83]. HS compose approximately 60-70% of soil organic carbon and, therefore, are a primary factor influencing soil physical and chemical dynamics, such as water and nutrient retention, particle aggregation, porosity, stability, surface area, cation exchange capacity (CEC), and contaminant availability [23, 35, 36, 59, 86]. HS, comprising fulvic acid, humic acid (HA), and humin generally bound to remnant biomolecules, are macromolecular solids that contain highly functionalized aliphatic, alicyclic, aromatic, and heteroaromatic backbones cross-linked by metal ions or covalent bonds [103]. HA, in particular, is defined as a SOM fraction precipitating at pH 1 and characterized as a category of naturally occurring, biogenic, refractory, heterogeneous organic substances being yellow-to-black in color, of high molecular weight, and resulting from plant and animal residue decomposition [54, 67].

Though not well researched, biological materials, specifically amino sugars such as peptidoglycan (PG), are a significant component of SOM, representing up to one third of soil
organic carbon [40, 52]. PG, found in both Gram-negative and -positive bacteria, is a rigid layer constructed from a carbohydrate backbone and amino acid side chains that completely encases a cell, protects the plasma membrane, and conveys strength to the overlying cell wall [55, 94]. The overall strength of this compound is only realized when the tetrapeptide functional groups are cross-linked, and the degree of cross-linking determines the recalcitrance of the overall molecule [55, 94].

Pores are present in mineral phases and SOM (most notably glassy HS and black carbon) and theorized to be the primary factor influencing hydrophobic organic contaminant (HOC) non-availability. For either media, pores are local regions of relatively rigid free volume where one or a few sorbate molecules undergo sorptive interactions [16, 27, 105, 106]. These pores may be shallow or connected to a larger internal network, macro- to nanosized in diameter, associated with SOM or comprise a pristine surface, confer HOC size and conformation specificity, and depending on their population, may contribute profoundly to the dual-mode mechanism [1, 4, 8, 27, 53, 69, 107]. This dual-mode model is defined as simultaneous slow, nonlinear condensation in (or adsorption on the walls of) pores and rapid, linear partitioning into an organic phase [8, 69, 105, 106]. An empirical relationship known as the Freundlich isotherm [1] is often used to represent the dual mode model, where $K_F$ is the Freundlich constant and $n$ is a measure of sorption nonlinearity [84, 106].

$$C_s = K_F \cdot C_w^n$$  \hspace{1cm} [1]

Soil aggregates are mineral particles of various sizes “cemented” together by the inherent adhesive characteristics of SOM to form particles with greater surface area. It is well known that SOM influences soil aggregation, porosity, and aggregate stability, as documented by numerous studies comparing long-term pastures to arable sites [10, 36, 37, 55]
72, 81]. Accordingly, soil aggregation affects SOM formation, destruction, and stabilization, protects SOM from microbial breakdown, and influences micro-environment conditions, specifically the availability of sorbed organic substances such as HOCs [10, 28, 81].

Using the synthetic soil developed in Chapter 3, the purpose of this chapter was to determine the impact different SOM constituents, specifically HA and PG, have on soil tertiary structure and how these constituents interacted with each other and the mineral phase. The goals for this research included: (i) evaluation of HA sorption to mineral phases compared to PG, (ii) determining the impact HA and PG (individually and combined) have on particle aggregation, and (iii) observing the location of HA and PG (individually and combined) in relation to the mineral phase. The approach was to generate a clean, controllable soil system that could be manipulated to assess individual and combined interactions between SOM constituents and a mineral base. These interactions were examined via total organic carbon (TOC) analysis, spectrophotometry measurements, a particle surface area (PSA) analysis program, and fluorescence microscopy, where direct visualization of sorbed SOM was a novel approach addressing soil structure. The result allowed known concentrations of two SOM components to be sequentially loaded onto a given solid phase; thus, providing for an evaluation of their individual and combined affect on soil construction and resulting tertiary structure.

4.2 EXPERIMENTAL SECTION

All chemicals and solvents were either HPLC grade or higher. Glassware used in the preparation of any solution, whether stock, diluted, buffer, or sample, were cleaned
thoroughly using an acid-washing regimen. Metal and plasticware were cleaned in the same manner as for glass, except an acid bath was not used.

4.2.1 SYNTHETIC SOILS

The method used to generate the synthetic soils was identical to that stated in Section 3.2.2. The one addition to this method was that ratios of hematite and montmorillonite clay (such as 20:80, respectively) were also used as a solid phase.

4.2.2 AQUEOUS PHASE PG AND HA ANALYSIS

The methods used to determine the amount of PG and/or HA sorbed to a mineral phase were identical to those stated in Section 3.2.3.

4.2.3 PARTICLE SURFACE AREA (PSA) ANALYSIS

The method used to analyze soil PSAs was identical to that discussed in Section 3.2.4, with no additions.

4.2.4 FLUORESCENT LABELING OF PEPTIDOGLYCAN

To induce fluorescence in PG, an alternative Gram staining technique was used that takes advantage of lectin selective binding, wheat germ agglutinin, to N-acetylglucosamine, a main component within the PG structure [85]. The entire procedure was performed in the dark so as to preserve the fluorescein isothio-cyanate (FITC) probe fluorescence. Freeze-dried wheat germ agglutinin labeled with FITC (L4895; Sigma Aldrich) was diluted to 100 μg mL⁻¹ with fresh pH 7.2 phosphate buffer (pHydrion) made with ultra-purified water and
stored in a -20º C freezer in a 125-mL amber bottle covered with aluminum foil until needed [85]. Approximately 10-20 mg of each soil sample were placed in 4-mL (10x75 mm; Fisher Scientific) disposable glass test tubes. The soil was completely covered with the lectin solution for 30 seconds and then gently diluted with the pH 7.2 phosphate buffer [85]. The diluted lectin solution was immediately decanted from the sample, the soil was scraped onto an acetone-rinsed, glass microscope slide (Fisherbrand Superfrost Plus; 25 x 75 x 1.0 mm), covered with a raised coverslip (CoverWell Incubation Chambers; 0.50 μL; Bio-Labs), and placed in a vacuum desiccator overnight with indicating DriRite (Fisher Scientific) to dry the sample. The soil was visualized fluorescently via a FITC filter, where the resulting labeled PG fluoresced a bright yellow-green as shown in Figure 4.1 [85].

![Image](image.jpg)

**Figure 4.1.** Freeze-dried *S. aureus* PG sample labeled with a FITC probe via an alternative Gram staining method.

### 4.2.5 FLUORESCENCE MICROSCOPY

Using the mounted soils described in Section 4.2.3, all samples were observed at 100x total magnification using an OPELCO Olympus IX71 research inverted fluorescence...
microscope outfitted with an Olympus MicroFire camera. Both mineral phases were visualized without perturbation using conventional bright-field light, as pure hematite and montmorillonite clay do not fluoresce [45]. Sorbed HA produced blue autofluorescence upon excitation by UV light at around 365 nm and was detected using a DAPI (4’ 6-diamidino-2-phenylindole) filter [19, 47, 59]. Lectin-labeled PG required a FITC filter to observe the green fluorescence at an emission wavelength of 522 nm.

Images for the purpose of compilation and mapping the location of individual components were taken with a 1.000 gain as 12-bit uncompressed TIFF files at a maximum exposure time of 4.99 sec, so as not to saturate the camera CCD. Conversely, images for fluorescence intensity analysis were captured as 8-bit uncompressed TIFF files in grayscale colors with a 1.000 gain and 4.99 sec exposure time. Images of buffer solutions, empty glass slides, crystallized HA and PG, and blank solid phases were acquired as evidence that no significant background fluorescence interference was present and had to be digitally subtracted from the sample images.

4.2.6 IMAGE COMPILATION

Fluorescence microscope images were digitally overlaid using Adobe Photoshop 7.0 or CS. For multiple-component systems as in Figure 4.2, a light microscope image of the mineral matrix was acquired first, followed by the fluorescence images for each SOM component. The images were reduced to 50% transparency by Adobe Photoshop 7.0 or CS, ensuring that neither the solid phase texture nor fluorescence was diminished. These images were then interfaced by the same software to gain a composite photograph, such as that for
the two-component system in Figure 4.2. This combined image could subsequently be enhanced or further analyzed using Adobe Photoshop 7.0 or CS.

**Figure 4.2.** Schematic of how images were interfaced to determine where SOM constituents were located in relation to the mineral phase.
4.2.7 FLUORESCENCE INTENSITY ANALYSIS

The method used to determine soil fluorescence intensity was identical to that stated in Section 3.2.8. When graphed against the loaded HA and/or PG concentration, the resulting fluorescence intensity increase corresponded with an increase in the amount of SOM sorbed to the soil.

4.3 RESULTS

4.3.1 HUMIC ACID (HA) SORPTION

Using the synthetic soil described in Chapter 3, the purpose of this research was to determine the impact different SOM constituents, specifically HA and PG, have on soil tertiary structure and how these constituents interact with each other and the mineral phase. The first sorption experiment examined a two-component system consisting of HA (0-200 mg L\(^{-1}\)) and one mineral phase. Figure 4.3 and the \(K_D\) values in Table 4.1 illustrated a distinct difference between HA loadings on both mineral phases, where montmorillonite clay sorbed 1.7 times more HA than hematite due to the larger, expandable surface area of clay compared to hematite [97]. Freundlich \(n\) values (Table 4.1) were statistically different from 1.00 and indicated a nonlinear sorption mechanism [83, 84].

<table>
<thead>
<tr>
<th>OM and Mineral Phase:</th>
<th>R(^2):</th>
<th>Freundlich (n): Values:</th>
<th>Average (K_D): (mL g(^{-1}))</th>
<th>One Std. Dev.: (mL g(^{-1}))</th>
<th>Does (n = 1.0)?</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA on Clay</td>
<td>0.95</td>
<td>1.03</td>
<td>19.7</td>
<td>0.7</td>
<td>P = 0.063</td>
</tr>
<tr>
<td>HA on Hematite</td>
<td>0.92</td>
<td>0.94</td>
<td>11.8</td>
<td>0.8</td>
<td>P = 0.0001</td>
</tr>
</tbody>
</table>

Table 4.1. Distribution Coefficients for HA Sorption Experiments
Figure 4.3. Freundlich isotherms depicting montmorillonite clay as the stronger HA sorbent compared to hematite due to the larger surface area of clay.

Due to the malleable nature of pure hematite, the PSA analyses gave skewed results and were omitted. As shown in Figure 4.4, increasing the HA loading concentration from 50 to 500 mg L\(^{-1}\) increased the average surface area, indicating that clay aggregation increased with the addition of adhesive (in the form of HA) [36, 37]. The PSA for blank clay (montmorillonite not loaded with HA) was included in Figure 4.4 for comparison to the loaded clays.
Comparison of PSAs for HA-Loaded Clays

**Figure 4.4.** Comparison of PSAs for HA clays, where increasing the HA concentration caused the average surface area to also increase.

Furthermore, when HA was introduced to a two-mineral-phase system, the result was sorption and particle aggregation beyond what was observed with hematite or clay alone. As demonstrated in Figure 4.5a, adding 20%-30% hematite to a 100 mg L\(^{-1}\) HA on clay matrix yielded 1.2 times more HA sorption than by 100% clay. As shown by the PSA analyses in Figure 4.5b, sorption of 100 mg L\(^{-1}\) HA to an 80% clay : 20% hematite mixture increased the average PSA 2.5-fold compared to that by clay alone, where PSAs for the blank clay and clay:hematite mixture (no HA added) were included for comparison. Both phenomena were likely caused by hematite particles inserting themselves into the clay crystalline structure, resulting in an overall increase in the available clay surface area [92]. This additional surface area provided for greater HA sorption, and thus, increased the amount of adhesive available in the soil matrix to favor particle aggregation [36, 37].
100 mg L\(^{-1}\) HA Sorbed to Single and Mixed Mineral Phases

![Graph showing sorption of HA on different mineral phases.](image)

Comparison of Clay and Clay:Hematite PSAs

![Graph showing particle surface areas for different mixtures.](image)

**Figure 4.5.** (a) Addition of 100 mg L\(^{-1}\) HA to various ratios of hematite and clay, where an 80% clay : 20% hematite ratio yielded the greatest sorbed amount. (b) Comparison of PSAs for HA on clay and an 80% clay : 20% hematite mixture, where the addition of hematite caused a 2.5-fold increase in particle aggregation compared to 100% clay.

As stated, one of the goals for this research was to directly observe via fluorescence microscopy the location of HA in relation to the mineral phase, a novel approach to
examining sorption relationships. As shown in Figure 4.6a, the diffuse, blue HA (800 mg L$^{-1}$) fluorescence encompassed whole particles and suggested that sorption generally occurred along the surface. This observation was confirmed by the lack of variable fluorescence intensities in the composite image, as fluorescent bodies trapped within pores concentrate and yield larger intensity values compared to diffuse entities along the surface. The graph in Figure 4.6b demonstrated that an increase in the sorbed concentration of HA, an autofluorescent macromolecule, caused a predictable increase in the fluorescence intensity of the soil.
Fluorescence (FL) microscope image of HA using DAPI filter. Light (LT) microscope image of clay particles.

Composite FL & LT Image
(Fluorescence intensity values are in white.)
Fluorescence Intensities for 0-200 mg L$^{-1}$ HA on Clay

\[ y = 4.94x + 60.28 \]
\[ R^2 = 0.86 \]

Figure 4.6. (a) Combined DAPI and light images of 800 mg L$^{-1}$ HA sorbed to montmorillonite clay at 100x total magnification. (b) Graph of sorbed HA concentration versus the resulting fluorescence intensity. Items that are boxed within the FL and LT images were examples; for instance, in (a) three examples of clay particles were highlighted in boxes. In the composite image, the boxed areas were used to generate the corresponding fluorescence intensity value.

4.3.2 PEPTIDOGLYCAN (PG) SORPTION

Although Leenheer et al. [52] and Jarusutthirak et al. [40] have determined that amino sugars, such as PG, constitute up to one third of DOM colloids, biological SOM components have remained largely un-researched with respect to their impact on soil structure and functions. As depicted in Figure 4.7 and the corresponding $K_D$ values in Table 4.2, montmorillonite clay retained 3.5 times more PG than hematite. This difference in affinity possibly originated from the larger, expandable surface area of clay compared to hematite and the charge distribution along the hematite surface, which tends to repel other
negatively charged moieties such as on PG [97]. Further, Freundlich $n$ values (Table 4.2) were statistically different from 1.00 and denote a nonlinear sorption mechanism [83, 84].

Table 4.2. Distribution Coefficients for PG Sorption Experiments

<table>
<thead>
<tr>
<th>Mineral Phase:</th>
<th>OM and R²:</th>
<th>Freundlich $n$ Values:</th>
<th>Average $K_D$:</th>
<th>One Std. Dev.:</th>
<th>Does $n = 1.0$?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(mL g⁻¹)</td>
<td>(mL g⁻¹)</td>
<td></td>
</tr>
<tr>
<td>PG on Clay</td>
<td>1.00</td>
<td>1.43</td>
<td>414</td>
<td>130</td>
<td>$P = 0.0001$</td>
</tr>
<tr>
<td>PG on Hematite</td>
<td>0.99</td>
<td>1.15</td>
<td>119</td>
<td>17</td>
<td>$P = 0.0001$</td>
</tr>
</tbody>
</table>

![PG Sorption Isotherms](image)

**Figure 4.7.** Isotherms depicting montmorillonite clay as the stronger PG sorbent compared to hematite.

The graph in Figure 4.8 showed that the concentration of PG exerted a profound influence over particle aggregation, where an increase in sorbed PG (from 0-200 mg L⁻¹) generally caused an increase in the clay surface area. This relationship potentially resulted from the presence of additional adhesive character in the matrix as more PG was loaded onto clay.
Figure 4.8. A comparison of PSAs showed that increasing the sorbed PG concentration generally caused an increase in the average particle size.

As stated, one goal of this research was to visualize via fluorescence microscopy the location of PG in relation to clay. Although green PG fluorescence (100 mg L\(^{-1}\)) emitted from the montmorillonite clay surface in Figure 4.9a, a substantial amount appeared to settle between aggregated particles and within pore spaces and edges, where the potential for noncovalent bonding was the greatest [97]. This observation was evidenced by the varying fluorescence intensities in the composite image, where the largest intensity values, or brightest FITC green color, came from pore spaces, cracks, edges, and between aggregated particles (Figure 4.9a). The graph in Figure 4.9b demonstrated that an increase in the amount of sorbed PG caused a predictable increase in the fluorescence intensity of the soil.
Fluorescence (FL) microscope image of PG using FITC filter.

Light (LT) microscope image of clay particles.

Composite FL & LT Image
(Fluorescence intensity values are in red.)
4.3.3 HUMIC ACID (HA) AND PEPTIDOGLYCAN (PG) COMBINED SORPTION

Another purpose of this work was to examine the combined effect different SOM constituents, specifically HA and PG, have on soil tertiary structure and how these constituents interacted with each other and the mineral phase. Sequential addition of SOM components was adopted to determine each concentration loaded onto the solid phase and the resulting effect, and therefore, provide a “pseudo-map” of synthetic soil construction. As shown in Figure 4.10, a noticeable difference in total SOM content developed when the PG concentration was increased compared to that for HA. For instance, a concentration increase of 50 mg L\(^{-1}\) PG caused a 6.0-fold total loading increase compared to a 0.8-fold increase for 50 mg L\(^{-1}\) of HA. However, as the total SOM concentration increased the \(K_D\) and Freundlich
$n$ values decreased (Table 4.3), potentially indicating that optimum sorption sites were becoming saturated with greater amounts of added SOM. Though more data points were necessary, it also appeared that total sorption onto 80% clay : 20% hematite was greater than for clay alone ($P = 0.033$), likely resulting from a greater surface area caused by smaller hematite particles inserting themselves into the clay framework [92].

**Table 4.3. Distribution Coefficients for HA + PG Sorption Experiments**

<table>
<thead>
<tr>
<th>SOM and Mineral Phase:</th>
<th>$R^2$:</th>
<th>Freundlich $n$ Values:</th>
<th>Average $K_D$: (mL g$^{-1}$)</th>
<th>Std. Dev.: (mL g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mg/L PG + 100 mg/L HA on Clay</td>
<td>1.00</td>
<td>1.61</td>
<td>11.6</td>
<td>0.2</td>
</tr>
<tr>
<td>50 mg/L PG + 400 mg/L HA on Clay</td>
<td>1.00</td>
<td>1.61</td>
<td>20.7</td>
<td>0.2</td>
</tr>
<tr>
<td>50 mg/L PG + 800 mg/L HA on Clay</td>
<td>1.00</td>
<td>1.61</td>
<td>29.6</td>
<td>0.8</td>
</tr>
<tr>
<td>100 mg/L PG + 100 mg/L HA on Clay</td>
<td>1.00</td>
<td>0.71</td>
<td>63.7</td>
<td>0.6</td>
</tr>
<tr>
<td>100 mg/L PG + 400 mg/L HA on Clay</td>
<td>1.00</td>
<td>0.71</td>
<td>45.3</td>
<td>1.3</td>
</tr>
<tr>
<td>100 mg/L PG + 800 mg/L HA on Clay</td>
<td>1.00</td>
<td>0.1</td>
<td>37.6</td>
<td>0.2</td>
</tr>
<tr>
<td>100 mg/L PG + 400 mg/L HA on 80% clay : 20% hematite</td>
<td>1.00</td>
<td>0.71</td>
<td>46.1</td>
<td>0.3</td>
</tr>
</tbody>
</table>

![Figure 4.10. Sorption isotherm for PG and HA on montmorillonite clay, where an increase in PG concentration had a more profound impact on the total SOM content as shown by the large space between the lines.](image-url)
In Figure 4.11 HA+PG synthetic clays yielded greater particle aggregation compared to blank and HA or PG clays. This likely resulted for two reasons: (1) the aggregational forces for two SOM components were combined compared to only one; and (2) the increased total sorbed SOM content introduced additional adhesive character into the matrix. Furthermore, as recognized in Section 4.3.2, PG again appeared to influence aggregation more than HA. For instance, an increase of 20 mg L\(^{-1}\) PG caused a 1.5-fold increase in PSA, a considerable jump for such a small amount of additional TOC. This may have resulted from the greater sorption potential of PG compared to HA, where PG contributed more to the total SOM content than HA.

**Figure 4.11.** Comparison of PSAs for blank, HA-, PG-, and HA+PG-loaded clays, where combining the SOM constituents resulted in the largest surface areas.

As discussed, a goal of this research was to examine by fluorescence microscopy the location of HA and PG in relation to each other and the mineral phase. In Figure 4.12a there
was considerable overlap and interconnection of green PG and blue HA fluorescence, an observation that may be significant when studying HOC sorption in later chapters. Although 100 mg L\(^{-1}\) PG and 400 mg L\(^{-1}\) HA sorption to montmorillonite clay (Figure 4.12a) occurred along the clay surface, the brightest green PG and blue HA colors, indicating the greatest sorption extent, resided within pore spaces, edges, cracks, and between aggregated particles, where the potential for noncovalent bonding was the largest [97]. This observation was supported by a substantial variation in fluorescence intensity values from the composite image, where fluorescent bodies trapped within small areas concentrate and yield larger intensities compared to diffuse surficial sorption. The graph in Figure 4.12b demonstrated that an increase in the amount of sorbed HA and PG caused a predictable increase in the fluorescence intensity of the soil.
Fluorescence (FL 1) microscope image of PG using FITC filter.

Fluorescence (FL 2) microscope image of HA using DAPI filter.

Composite FL 1 & FL 2 & LT Image
(Fluorescence intensity values are in red.)
Figure 4.12. (a) Combined FITC, DAPI, and light images of 100 mg L\(^{-1}\) PG and 400 mg L\(^{-1}\) HA sorbed to montmorillonite clay at 100x total magnification. (b) Graph of sorbed PG and HA concentration versus the resulting fluorescence intensity.

4.4 DISCUSSION

From the data presented in this chapter, the synthetic matrix behaved in a predictable manner, where PG potentially played a vital, yet relatively unstudied, sorptive role within soil systems. Overall, clay sorbed approximately 1.7 and 3.5 times more HA and PG, respectively, than hematite (Figure 4.13), as determined from the corresponding \(K_D\) values in Table 4.4. This potentially resulted from the greater surface area (31.8 m\(^2\) g\(^{-1}\)) and CEC (80-120 cmol kg\(^{-1}\)) of expandable clays compared to metal oxides (5.9 m\(^2\) g\(^{-1}\) and <2 cmol kg\(^{-1}\), respectively) [5, 91, 93, 97].

\[
y = 20.83x + 71.54
\]

\(R^2 = 0.65\)
Table 4.4. Distribution Coefficients for HA and PG Sorption Experiments

<table>
<thead>
<tr>
<th>OM and Mineral Phase:</th>
<th>R²:</th>
<th>Freundlich n Values:</th>
<th>Average K₉: (mL g⁻¹)</th>
<th>One Std. Dev.: (mL g⁻¹)</th>
<th>Does n = 1.0?</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA on Clay</td>
<td>0.95</td>
<td>1.03</td>
<td>19.7</td>
<td>0.7</td>
<td>P = 0.063</td>
</tr>
<tr>
<td>HA on Hematite</td>
<td>0.92</td>
<td>0.94</td>
<td>11.8</td>
<td>0.8</td>
<td>P = 0.0001</td>
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<tr>
<td>PG on Clay</td>
<td>1.00</td>
<td>1.43</td>
<td>414</td>
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<tr>
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<td>1.15</td>
<td>119</td>
<td>17</td>
<td>P = 0.0001</td>
</tr>
</tbody>
</table>

HA and PG Sorption Isotherms

Figure 4.13. Sorption isotherms for PG and HA on montmorillonite clay and hematite, where PG had the greatest resulting sorption.

However, PG sorption to hematite and clay was 10.1 and 21.0 times greater, respectively, than that for HA as calculated from the K₉ data in Table 4.4. This significant difference in sorption affinity potentially stemmed from the polar nature of PG (H/C ratio 1.60) compared to HA (H/C ratio 1.15), where the PG structure provided a greater number of external oxygen and amino functional groups for approaching and binding to a negatively-
charged mineral surface (Figure 4.14), such as clay or other silicates [82]. Furthermore, PG sorption generated stronger noncovalent bonds, such as ligand exchange and electrostatic interaction, with clay compared to HA, which used hydrogen bonding and van der Waals forces [6, 7, 20, 29, 51, 57, 62, 63, 74, 77, 78]. SOM assimilation, in general, changed the chemical properties of montmorillonite clay by satisfying valences at the broken edges of silica and alumina sheets and exposed oxygen and hydroxyl groups along the external mineral surfaces [97].
In correspondence with this larger sorbed PG amount, the resulting clay aggregation for PG was greater than that for HA (Figure 4.15). Moreover, increasing the loaded concentration for both SOM components (from blank to 200 or 500 mg L⁻¹), in general, caused an increase in clay surface area. This was due to the increased adhesive character within the soil matrix, for HA and PG sorption alike, and has been documented in the field for non-tilled soils when SOM was allowed to accumulate [36, 37].
Figure 4.15. Comparison of PSAs for blank, HA-, and PG-loaded clays, where PG resulted in the largest surface areas due to its greater sorbed concentration.

Addition of 20-30% hematite into the clay system (Figure 4.5a) provided for 1.2 times greater HA sorption. This increased sorption was possibly caused by small hematite particles inserting themselves within the regular crystalline structure of clay (Figure 4.16a), thereby effectively increasing the overall surface area available to sorb HA (Figure 4.16b) [92]. As a result of this increased capacity for HA, the adhesive character of the matrix was also heightened and, thus, promoted particle aggregation beyond that for clay alone (Figure 4.16b).
Comparison of Clay and Clay:Hematite PSAs

Figure 4.16. (a) Theoretical representation of hematite particles inserted within the regular crystalline structure of clay, where white arrows indicate potential noncovalent bonds. (b) Comparison of PSAs for clays versus clay : hematite mixtures.
When both SOM components were introduced to clay, PG maintained a profound influence over the total sorbed SOM concentration and particle aggregation, where an increase of 50 mg L$^{-1}$ PG caused a 6.0-fold increase in the total sorbed SOM amount (Figure 4.10) compared to a 0.8-fold increase for 50 mg L$^{-1}$ HA. As suggested for the sorption of single components, this was potentially due to the polar nature of PG compared to HA, making its approach and sorption to clay more conducive. Furthermore, sorption of PG and HA to clay caused a corresponding increase in particle aggregation (Figure 4.11) compared to that for PG or HA alone on clay. The reason for this was likely twofold: (1) the sum of two aggregational forces (HA and PG) was greater than that for one, regardless of the strength the singular forces were known to exert; and (2) the overall clay TOC content was greater compared to sorption of single components (Figure 4.17), meaning the matrix adhesive character was heightened and allowed for more (or larger) particles to “cement” together. Finally, based on the limited data in Figure 4.17, when 20% hematite was introduced into the HA+PG clay matrix total SOM sorption was greater than for clay alone. As stated, this was possibly due to small hematite particles inserting within the clay crystalline structure (Figure 4.16a), causing an increase in the amount of available surface area able to accommodate a greater total sorbed SOM concentration [92].
Comparison of HA, PG, and HA+PG Sorption Isotherms

**Figure 4.17.** Sorption isotherms for PG and/or HA on montmorillonite clay and an 80% clay : 20% hematite mixture, where HA+PG had the largest total sorbed amount.

Autofluorescent blue HA and FITC-labeled green PG were viewed via fluorescence microscopy, and the resulting images were overlapped to determine the location of both constituents in relation to the mineral phase and each other. Although the fluorescence intensities for both SOM components increased with an increase in TOC (Figure 4.18), PG sorbed more extensively compared to HA at any given loading concentration (Figure 4.13) and caused a steeper rise in the fluorescence intensity graph with concentration. This “excess” PG and accompanying fluorescence concentrated between clay particles (aggregated more effectively by the greater sorbed PG concentration; Figure 4.13) and within pore spaces, cracks, and edges, where a larger density of oxygen- and nitrogen-containing moieties were known to exist and the potential for noncovalent bonding was higher.
compared to the surface [97]. This was confirmed by the significant difference between intensity measurements for specific fluorescing entities in the PG clay composite image (Figure 4.9a), whereas the apparent lack of variation in the HA clay composite (Figure 4.6a) indicated only diffuse surficial sorption. The theory was further supported by the HA+PG clays, where an increase in the sorption of two SOM components caused a greater response in the fluorescence intensity measurements (Figure 4.18). Again, this was due to “excess” SOM and its accompanying green and blue fluorescence concentrating between clay particles (aggregated by a greater sorbed TOC compared to PG or HA alone; Figure 4.17) and within mineral pore spaces, cracks, and edges, as supported by the varying intensities in Figure 4.13a, causing the overall fluorescence intensity of the image to also increase.

**Figure 4.18.** Graph of sorbed SOM concentration versus the resulting fluorescence intensity for PG, HA, and HA+PG on montmorillonite clay.
Finally, in regard to the synthetic soil in Figure 4.12a, HA and PG appeared to form a well-integrated green-blue coating on clay, whether along the surface, between aggregated particles, or within pores spaces, cracks, and edges. As shown by the theoretical schematic in Figure 4.19a, this could theoretically occur via a desorption-rearrangement-resorption mechanism within the soil slurry upon addition of the second SOM component. This hypothesis was based on preliminary HA sorption experiments, where up to 13% of previously sorbed HA was found to disassociate from clay upon addition of fresh buffer. This rearrangement may also explain similarities (P = 0.003 and 0.006 for clay and hematite, respectively) between the final sorbed TOC values (Figure 4.19b) when various loading procedures were attempted: (i) HA sorbed to clay first and PG second, (ii) PG sorbed to clay first and HA second, (iii) HA and PG simultaneously sorbed to clay, and (iv) direct addition of PG to the equilibrated solution of HA and clay. Using HA sorbed first as the example procedure, the mechanism would progress as follows: (1) HA was added to the clay first per the method in Section 4.2.1; (2) upon introduction of the second solution containing PG, loosely or non-sorbed HA re-suspended into the buffer and allowed for PG (the stronger sorbent per TOC results in Figure 4.13) to effectively alter the clay surface chemistry into a hydrophobic matrix that was more conducive to HA sorption; (3) the PG configuration rearranged to accept sorption with HA and thus, a highly interconnected SOM coating resulted. Essentially, PG became a bridge between HA and montmorillonite clay.
Figure 4.19. (a) Theoretical schematic depicting the integrated sorption of HA and PG on montmorillonite clay. (b) Regardless of the order used to load HA and PG onto clay, the resulting TOC was statistically similar.
In summary, PG had a more profound affect on the TOC content and surface area of soils compared to HA, indicating that this particular SOM constituent should be examined more closely and specifically with respect to its impact on HOC fate. This particular finding is significant as PG has rarely been examined before as a major contributor to SOM character, where using an alternative Gram staining technique to fluorescently label PG was a second novel approach. The composite fluorescence images developed within this research constitute a third point of significance as such visual representations of soil structure have never been attempted before. In general, as the TOC of a given soil system increased particle aggregation also increased, yielding greater intra-particle space, and resulting in larger fluorescence intensity values due to “excess” TOC concentrating within these and other confined spaces, such as pores.
CHAPTER 5: INFLUENCE OF SOM COMPONENTS ON PYRENE FRESHLY SPIKED IN SYNTHETIC AND NATURAL SOILS

5.1 INTRODUCTION

It is well documented that the nature of SOM, specifically humic substances (HS) and black carbon (BC), significantly impacts the availability and toxicity of hydrophobic organic contaminants (HOCs), such as polyaromatic hydrocarbons (PAHs). PAH association with SOM sorbed to a mineral phase depends on the polarity, aromaticity, molecular size, configuration, and chemical composition of the carrier molecule, as well as the total surface area created via SOM-particle aggregation [1, 27, 42, 50, 75, 98]. The dual-mode model is the generally accepted mechanism by which HOCs sorb within soil and is defined as simultaneous slow, nonlinear condensation in (or adsorption on the walls of) pores and rapid, linear partitioning into an organic phase [8, 69, 105, 106]. This model assumes the following attributes: (i) nonlinear sorption isotherms, (ii) competition in multisolute systems, (iii) concentration-dependent heat of sorption, and (iv) decreased HOC extractability as a function of sorbate size, concentration, and residence time within geosorbents [4, 8, 27, 53, 69, 103, 106]. Furthermore, an empirical relationship known as the Freundlich isotherm can be used as a surrogate for the dual-mode model, where $K_F$ is the Freundlich constant and $n$ is a measure of sorption nonlinearity [84, 106].

$$C_s = K_F \cdot C_w^n$$  \[1\]
Currently, research into PAH sequestration is limited to sorption/desorption studies and nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR) analyses, with little direct observation of component dynamics in the soil matrix [62, 63]. Karimi-Lotfabad and Gray [45] used fluorescent confocal laser scanning (CLSM) and cryogenic scanning electron (CSEM) microscopies to examine nonaqueous phase liquid (NAPL) contaminant distribution on and within the soil microstructure. Another study by Wild et al. [99] determined PAH location and intercellular migration within plant leaves via two-photon excitation microscopy (TPEM) [100, 101]. Although both studies are important, there is still no visual account of SOM component interaction and influence on PAH sorption and availability, which is essential to developing cost-effective and efficient *in situ* remediation technology.

Using the synthetic soil described in Chapter 4, the purpose of this chapter is to examine pyrene sorption, specifically the impact two different SOM constituents (HA and PG) have on sorbed pyrene concentration and location, and compare the results with pyrene sorption to two natural soils. The goals for this research include: (i) determining the affect PG and HA (individually and combined) have on pyrene sorption, (ii) correlating pyrene sorption data from the synthetic soils with that from natural soils, (iii) evaluating the location of individual and combined soil constituents, PG and HA, in relation to pyrene and the mineral phase, and (iv) establishing from the fluorescence images whether pyrene has a preference for a specific SOM component. The approach was to generate a clean, controllable soil system that could be manipulated to assess visually and quantitatively the impact HA and PG have on pyrene sorption. These interactions were examined via high pressure liquid chromatography (HPLC) and fluorescence microscopy, where direct
visualization of SOM interactions with pyrene is a novel approach providing information that addresses fate, transport, and availability. The result allowed known concentrations of two SOM components to be sequentially loaded onto a given solid phase; thus, providing for an evaluation of their individual and combined affect on pyrene sorption as compared to natural soils.

5.2 EXPERIMENTAL SECTION

All chemicals and solvents were either HPLC grade or higher. Glassware used in the preparation of any solution, whether stock, diluted, buffer, or sample, were cleaned thoroughly using an acid-washing regimen. Metal and plasticware were cleaned in the same manner as for glass, except an acid bath was not used.

5.2.1 NATURAL AND SYNTHETIC SOILS

The uncontaminated, natural and synthetic soils described in Chapters 3 and 4 were employed for the research discussed in this chapter. The two pristine natural soils are the same as detailed in Section 3.2.1 and Table 3.1 The synthetic soils were generated by methods in Sections 3.2.2 and 3.2.3 and examined for their physical and chemical properties in Table 3.1 and Section 4.3. The mineral base was either Na-saturated montmorillonite clay (SWy-2; Source Clay Minerals Repository) or an 80% clay : 20% hematite (J.T. Baker) ratio, and the SOM coating consisted of 50 or 100 mg L\(^{-1}\) freeze-dried \textit{S. aureus} PG (77140; BioChemika) and/or 100, 400, or 800 mg L\(^{-1}\) soil HA standard (1S102H; International Humic Substances Society).
5.2.2 PYRENE APPLICATION TO SYNTHETIC AND NATURAL SOILS

The method used to spike natural and synthetic soils with pyrene was identical to that discussed in Section 3.2.5. Additions to this method included: (i) spiking glass beads (<40-μm diameter; Potters Industries, Inc.) and molecular sieves (13X, 100-120 mesh; Supelco) with pyrene; (ii) replacing the dicholormethane (DCM) carrier solvent with diesel fuel; and (iii) employing packed soil columns in place of the common slurry method for spiking contaminants. For the diesel fuel the exact same concentration and method was used, although gentle heating was required to force the pyrene crystals into solution. To construct the soil columns 9.0-in. disposable glass pipets underwent an acid-washing regimen, 0.5-in. of glass wool was compacted at the neck of each pipet, and a 2.0-in. plug of soil was added on top of the wool. The columns remained vertical throughout the experiment via clamps, and 40-mL amber vials were placed under each column to collect pyrene solutions that passed through. The soil was kept moist by covering the open end of the pipet with aluminum foil, which also restricted any pyrene evaporation. These additions were required to verify the original procedure stated in Section 3.2.5.

5.2.3 AQUEOUS PHASE PYRENE ANALYSIS

The method used to determine the amount of pyrene sorbed to various soils was identical to that in Section 3.2.6.

5.2.4 FLUORESCENT LABELING OF PEPTIDOGLYCAN

The alternative Gram-staining method used to fluorescently label PG was identical to that stated in Section 4.2.4.
5.2.5 FLUORESCENCE MICROSCOPY

The methods used to mount and view contaminated soil samples were identical to those discussed in Section 4.2.5. Blue pyrene fluorescence was viewed with 300-400 nm UV light using a filter specific for PAHs.

5.2.6 IMAGE COMPILATION

The method used to overlap images was identical to that stated in Section 4.2.6. However, since HA fluorescence under the DAPI filter was the same blue color as the pyrene fluorescence with the PAH filter, to differentiate the compounds within the composite image the HA image was pseudo-colored with a palette of red hues using Adobe Photoshop CS. Composite images were, therefore, layered with the following scheme, starting with the bottom layer: (i) white light mineral phase image, (ii) pseudo-colored red HA fluorescence image, (iii) green PG image, and (iv) blue pyrene image.

5.2.7 FLUORESCENCE INTENSITY ANALYSIS

The method used to determine soil fluorescence intensity was identical to that stated in Section 3.2.8.

5.2.8 FLUORESCENCE HISTOGRAMS TO DETERMINE PYRENE-SOM ASSOCIATIONS

To determine which SOM component preferentially interacted with pyrene in the multiple-component synthetic and natural soils, RGB (red-blue-green) histograms for each filter color were generated via Fovea Pro 3.0. A histogram is a graphical representation detailing the number of pixels in an image corresponding to each of 255 brightness levels.
When two such histograms were plotted together the overlap was a measurement of the equivalent overlap in the fluorescence image, much like generating a Venn diagram. For instance, Figure 5.1 contains three images: pyrene (blue) observed by the filter specific for PAHs; PG (green) via the FITC filter; and HA (pseudo-colored red via Adobe Photoshop CS2 to differentiate it from pyrene fluorescence) as viewed through the DAPI filter. Fovea Pro 3.0 digitally converted each image into a corresponding histogram plotted in Microsoft Excel. When all three were graphed together, the overlapping values for the pyrene (blue) and HA (red) or pyrene and PG (green) histograms were summed and divided by 1,920,000 (the total number of pixels for each 1600x1200 pixel image used to generate an individual histogram). The resulting decimal was converted to a percent, which was a measure of the pyrene percentage associated with HA or PG fluorescence.
Figure 5.1. Sample histograms used to determine the percentage of pyrene associated with specific SOM components in fluorescence images.
5.3 RESULTS

5.3.1 BACKGROUND INTERFERENCE AND PYRENE SORPTION TESTS

Prior to employing fluorescence analysis, samples were analyzed for interferences. An empty slide and coverslip, buffer solutions, and baked mineral phases showed no detectable background fluorescence. Crystallized HA gave the brightest fluorescence via the DAPI filter, lectin-labeled PG by the FITC filter, and pyrene with the non-specific PAH filter. Before adding pyrene to the natural or synthetic soils, sorption tests were performed, including: (i) spiking pyrene onto glass beads and molecular sieves, (ii) using diesel fuel as the carrier solvent, and (iii) employing packed soil columns in place of the common slurry method for spiking pyrene. Pyrene was spiked onto glass beads to evaluate whether attachment occurred on relatively inert surfaces (Table 5.1). At a low 2.5 mg g\(^{-1}\) concentration pyrene sorbed to irregularities along the glass bead surface (Figure 5.2a), as shown by small points of blue pyrene fluorescence. Beads that appeared to be completely encased in blue fluorescence were adjacent to precipitated pyrene and, thus, acted as a magnifying glass. Conversely, when 25.0 mg g\(^{-1}\) were applied the pyrene precipitated (Figure 5.2b), as shown by the lack of fluorescence within the glass beads and the presence of crystalline pyrene. This difference in sorption between the high and low concentrations was further evidenced by the drop in fluorescence intensity of the glass beads (Table 5.1) and indicated that with increasing concentrations, pyrene preferred to precipitate rather than attach to inert surfaces.
Table 5.1. Pyrene Sorption on Glass Beads

<table>
<thead>
<tr>
<th>Spiked Pyrene Conc. (mg g⁻¹)</th>
<th>Sorbed Pyrene Conc. (mg g⁻¹) (a)</th>
<th>Fluorescence Intensity (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.0 ± 0.0</td>
<td>6.6 ± 1.7</td>
</tr>
<tr>
<td>2.5</td>
<td>2.86 ± 0.1</td>
<td>104.6 ± 17.6</td>
</tr>
<tr>
<td>25.0</td>
<td>24.5 ± 0.1</td>
<td>49.7 ± 7.1</td>
</tr>
</tbody>
</table>

(a) Averages reported with one standard deviation based on duplicate samples.
(b) Averages reported with one standard deviation based on replicates of ten.

Figure 5.2. Sorption of (a) 2.5 mg g⁻¹ and (b) 25.0 mg g⁻¹ pyrene on glass beads at 100x total magnification, where precipitation became evident at the higher concentration. Items that are boxed within the images are examples; for instance, in (a) five examples of glass beads are highlighted in two boxes.

Molecular sieves of known pore diameter and distribution were spiked with pyrene to demonstrate sorption within pore spaces. At the 2.5 mg g⁻¹ concentration (Figure 5.3a) pyrene diffusely covered the surface, as shown by the generalized dark blue fluorescence covering each particle, and settled randomly within a few pore spaces, denoted by the bright blue fluorescent points. In contrast, when spiked with 25.0 mg g⁻¹, pyrene sorption into pore spaces became more important, as demonstrated by the prevalence of bright blue dots (indicating pore openings) in Figure 5.3b and the significant change in the average fluorescence intensity (Table 5.2). This difference in sorption likely resulted from the presence of additional pyrene residing within the same amount of space, meaning the
“excess” pyrene was forced to occupy pore spaces as available surface sites became saturated. Moreover, this experiment demonstrated the appearance of pyrene sorption within pore spaces, a necessary observation when assessing sorption to soils.

Table 5.2. Pyrene Sorption on Molecular Sieves

<table>
<thead>
<tr>
<th>Spiked Pyrene Conc. (mg g⁻¹)</th>
<th>Sorbed Pyrene Conc. (mg g⁻¹) (a)</th>
<th>Fluorescence Intensity (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.0 ± 0.0</td>
<td>8.6 ± 1.0</td>
</tr>
<tr>
<td>2.5</td>
<td>2.7 ± 0.0</td>
<td>150.5 ± 45.5</td>
</tr>
<tr>
<td>25.0</td>
<td>24.6 ± 0.8</td>
<td>237.6 ± 23.1</td>
</tr>
</tbody>
</table>

(a) Averages reported with one standard deviation based on duplicate samples.  
(b) Averages reported with one standard deviation based on replicates of ten.

Figure 5.3. Sorption of (a) 2.5 mg g⁻¹ and (b) 25.0 mg g⁻¹ pyrene on molecular sieves at 100x total magnification. Although surficial pyrene was apparent in both images (dark blue in ‘a’ and light blue in ‘b’), sorption within pores became more apparent with a larger spiked concentration, as indicated by bright blue dots of fluorescence.

A second test examined use of a soil column, versus slurries, for spiking pyrene. The purpose of a soil column was to simulate the leaching phenomena common to uncontained hazardous waste sites. Soil slurries are the generally accepted technique used to administer pyrene but are criticized as not representing processes within the natural environment. On comparison of the soil column and slurry samples (Figure 5.4), the pyrene distribution appeared very dissimilar, which was supported by the considerable variation between the
sorbed pyrene concentrations and resulting fluorescence intensities (Table 5.3). The standard deviations for the soil column data indicated vast heterogeneity within the sample in contrast to that for the slurries. This heterogeneity may not be a significant concern for long-term experiments (months to years) but causes severe problems for short-term spiking (hours to days) and microscale analysis, as were used for this research.

Table 5.3. Comparison of Pyrene Sorption in Soil Slurries and Columns

<table>
<thead>
<tr>
<th>Spiked Pyrene Conc. (mg g⁻¹)</th>
<th>Sorbed Pyrene Conc. (mg g⁻¹) (a)</th>
<th>Fluorescence Intensity (b)</th>
<th>Sorbed Pyrene Conc. (mg g⁻¹) (a)</th>
<th>Fluorescence Intensity (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.0 ± 0.0</td>
<td>2.0 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>2.5</td>
<td>0.8 ± 0.3</td>
<td>2.5 ± 0.5</td>
<td>2.6 ± 0.0</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td>25.0</td>
<td>11.3 ± 0.0</td>
<td>8.3 ± 3.4</td>
<td>24.4 ± 0.0</td>
<td>21.5 ± 0.3</td>
</tr>
</tbody>
</table>

(a) Averages reported with one standard deviation based on duplicate samples. (b) Averages reported with one standard deviation based on replicates of ten.

Figure 5.4. Sorption of 25.0 mg g⁻¹ pyrene on 400 mg L⁻¹ HA clay using the (a) soil column and (b) slurry techniques (100x total magnification), where pyrene sorption was more homogeneous via slurries.

A third sorption test employed diesel fuel as the carrier solvent, rather than DCM. While diesel fuel is often the carrier for PAHs in creosote operations it generally degrades much faster than the PAHs and is not present in highly aged contamination. Therefore, the purpose of this experiment was to simulate how contamination is generally delivered to the
natural environment, specifically the method by which CMN and KKY (two aged soils used in this work) were generated. Two spiking procedures were employed to apply pyrene, (1) soil columns, as discussed for Figure 5.4, and (2) slurries, the commonly accepted protocol [12, 50, 63, 71, 75]. Unfortunately, for both methods the resulting images and HPLC data were ineffective as the fuel emitted a non-specific fluorescence over the entire sample (Figure 5.5 and Table 5.4) and masked the pyrene fluorescence, making quantification and visual observation nearly impossible.

Table 5.4. Pyrene Sorption on Synthetic Clay via a Diesel Fuel Carrier

<table>
<thead>
<tr>
<th>Spiked Pyrene Conc. (mg g⁻¹)</th>
<th>HA on Clay COLUMN</th>
<th>HA on Clay SLURRY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fluorescence Intensity (a)</td>
<td>Fluorescence Intensity (a)</td>
</tr>
<tr>
<td>0.0</td>
<td>20.3 ± 4.3</td>
<td>17.3 ± 2.0</td>
</tr>
<tr>
<td>2.5</td>
<td>55.2 ± 9.9</td>
<td>45.4 ± 2.8</td>
</tr>
<tr>
<td>25.0</td>
<td>209.6 ± 13.5</td>
<td>188.5 ± 19.3</td>
</tr>
</tbody>
</table>

(a) Averages reported with one standard deviation based on replicates of ten.

Figure 5.5. Images depicting 2.5 mg g⁻¹ pyrene sorbed to 400 mg L⁻¹ HA on clay within a (a) soil column and (b) slurry at 100x total magnification, where the diesel fuel carrier solvent masked the pyrene fluorescence.

Finally, polarized light images were taken of pyrene (or PAH)-contaminated natural and synthetic soils (Figure 5.6). Polarized light differentiated between precipitated pyrene (or
PAHs) laying on the soil surface and pyrene that was actually sorbed within the matrix. As shown in Figure 5.6a, the multi-colored particles on CMN are PAHs that have not sorbed within the soil. In comparison, a lack of color in the spiked synthetic soil image (Figure 5.6b) indicated that most of the 50 mg g\(^{-1}\) pyrene, a potentially saturating concentration, sorbed to 400 mg L\(^{-1}\) HA clay without crystallizing.

**Figure 5.6.** Polarized light images of (a) CMN and (b) 50.0 mg g\(^{-1}\) pyrene on synthetic clay, where the multi-colored particles were crystallized PAHs that did not sorb within the soil matrix.

### 5.3.2 PYRENE SORPTION TO HA SYNTHETIC SOILS

One goal of this work was to determine the impact singular SOM components have on pyrene sorption, where the spiked concentration range (0-50.0 mg g\(^{-1}\) pyrene) was chosen to simulate minimally to highly contaminated sites. As shown in Figure 5.7 and the corresponding \(K_D\) data in Table 5.5, pyrene sorption yielded a case III nonlinear Freundlich isotherm for 100, 400, and 800 mg L\(^{-1}\) HA-coated clays, in which previously sorbed molecules led to a modification of the soil surface that favored further contaminant sorption [83]. In comparison, blank clay (no SOM present) followed a case I nonlinear Freundlich curve, where the frequency and energy of sorption sites within clay were finite [83].
indicated that adding HA to clay increased the number of available sorption sites capable of sequestering pyrene, which likely resulted from the large surface area and hydrophobicity of HA [97].

Table 5.5. Distribution Coefficients for Pyrene Sorption on Blank and HA-Loaded Clays

<table>
<thead>
<tr>
<th>OM and Mineral Phase:</th>
<th>$R^2$:</th>
<th>Freundlich $n$:</th>
<th>Average $K_D$: (mL g$^{-1}$)</th>
<th>One Std. Dev.: (mL g$^{-1}$)</th>
<th>Does $n = 1.0$?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank Clay</td>
<td>0.93</td>
<td>0.53</td>
<td>491</td>
<td>438</td>
<td>$P = 0.462$</td>
</tr>
<tr>
<td>Avg. of 100, 400, and 800 mg L$^{-1}$ HA Clay</td>
<td>0.89</td>
<td>1.98</td>
<td>100</td>
<td>53</td>
<td>$P = 0.066$</td>
</tr>
</tbody>
</table>

Figure 5.7. Freundlich isotherms depicting pyrene sorption on blank and HA-loaded clays, where adding HA increased the clay sorption capacity.

A second goal of this research was to directly observe the location of sorbed pyrene in relation to single SOM constituents, such as HA, and the mineral phase. As discussed, pyrene was spiked onto clays loaded with 100, 400, and 800 mg L$^{-1}$ HA, representing sorption to soils of low, moderate, and high HS content. As shown in Figure 5.8a, the diffuse, blue
pyrene (2.5 mg g$^{-1}$) fluorescence primarily encompassed whole particles and suggested that the majority of the sorption was surficial. This observation was confirmed by the lack of variable fluorescence intensities in the composite image. This likely stemmed from the surficial sorption of HA onto clay, Section 4.3.1, and pyrene dissolution into the HA. Moreover, the graph in Figure 5.8b demonstrated that an increase in the sorbed pyrene concentration yielded a predictable increase in the fluorescence intensity of the soil, confirming the HPLC data in Figure 5.7.
Fluorescence (FL) microscope image of pyrene using PAH filter.

Light (LT) microscope image of HA clay particles.

Composite FL & LT Image
(Fluorescence Intensity values are in white.)
5.3.3 PYRENE SORPTION TO PG SYNTHETIC SOILS

Although contributing up to one third of total SOM content, biological materials have generally not been examined for their capacity to sorb contaminants. As depicted in Figure 5.9, addition of 50 or 100 mg L\(^{-1}\) PG, representing soils of low and moderate biological content, respectively, caused a significant increase in the pyrene sorption capacity compared to that for blank clay. To support this observation, \(K_D\) values were calculated (Table 5.6), where PG-loaded clay sorbed 1.9 times more pyrene than blank clay. This difference in affinity likely resulted from PG masking the clay particles with a hydrophobic coating that offered greater surface area and an environment conducive to the approach and sorption of pyrene [97]. Further, Freundlich \(n\) values were statistically different from 1.00 and denoted nonlinear sorption mechanisms, case I for blank clay and case III for PG-loaded clay [83, 84].
Table 5.6. Distribution Coefficients for Pyrene Sorption on Blank and PG-Loaded Clays

<table>
<thead>
<tr>
<th>OM and Mineral Phase:</th>
<th>R²:</th>
<th>Freundlich $n$ Values:</th>
<th>Average $K_D$: (mL g⁻¹)</th>
<th>One Std. Dev.: (mL g⁻¹)</th>
<th>Does $n = 1.0$?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank Clay</td>
<td>0.93</td>
<td>0.53</td>
<td>491</td>
<td>438</td>
<td>P = 0.462</td>
</tr>
<tr>
<td>Avg. of 50 and 100 mg L⁻¹ PG Clay</td>
<td>0.99</td>
<td>1.51</td>
<td>934</td>
<td>452</td>
<td>P &lt; 0.0001</td>
</tr>
</tbody>
</table>

**Figure 5.9.** Freundlich isotherms depicting pyrene sorption on blank and PG-loaded clays, where the addition of PG increased the clay sorption capacity.

An additional goal of this research was to visualize via fluorescence microscopy the location of pyrene in relation to PG and clay. Although pyrene (Figure 5.10a) appeared to sorb along the surface of 100 mg L⁻¹ PG-loaded clay, the brightest pyrene blue color emanated from between aggregated particles and within pore spaces, cracks, and edges, where PG sorption was potentially greatest due to noncovalent bonding with exposed clay hydroxyl groups [97]. This observation was supported by varying fluorescence intensities in
the composite image. The graph in Figure 5.10b demonstrated that an increase in the amount of sorbed pyrene caused a predictable increase in the fluorescence intensity of PG clays.

Fluorescence (FL) microscope image of pyrene using PAH filter.  
Light (LT) microscope image of PG clay particles.

Composite FL & LT Image  
(Fluorescence Intensity values are in white.)
Figure 5.10. (a) Combined fluorescence and light images of 2.5 mg g\(^{-1}\) pyrene sorbed to 100 mg L\(^{-1}\) PG on clay at 100x total magnification. (b) Graph of sorbed pyrene concentration versus the resulting fluorescence intensity.

5.3.4 PYRENE SORPTION TO HA + PG SYNTHETIC SOILS

Another objective of this work was to examine the combined impact SOM constituents, specifically HA and PG, have on pyrene sorption. Sequential addition of HA and PG was used to determine each amount loaded onto the solid phase and the resulting effect, and therefore, generate a “pseudo-map” of soil construction. Pyrene was sorbed to clay and an 80% clay : 20% hematite mixture, both coated with 100 mg L\(^{-1}\) PG and 100, 400, or 800 mg L\(^{-1}\) HA to represent soils with a low to high total SOM content. As shown in Figure 5.11, increasing the HA concentration from 100 to 800 mg L\(^{-1}\) generally increased the amount of sorbed pyrene, a likely result of heightened hydrophobic content and surface area from the extra HA. This observation was supported by the \(K_D\) values in Table 5.7, where
adding 700 mg L⁻¹ HA to the matrix yielded a 1.4-fold increase in pyrene sorption.

Furthermore, the Freundlich $n$ values (Table 5.7) denoted nonlinear sorption mechanisms for the HA+PG-loaded soils [83, 84]. Though more data points were necessary, it also appeared that pyrene sorption onto the 80% clay : 20% hematite mixed solid phase was greater than for clay alone. This difference in affinity potentially resulted from three interconnected phenomena: (i) smaller hematite particles inserting themselves into the clay crystalline framework, (ii) a larger sorbed SOM content, and (iii) increased particle aggregation [92]. This observation was supported by the $K_D$ values in Table 5.7, where 100 mg L⁻¹ PG + 400 mg L⁻¹ HA on a mixed solid phase sorbed 2.8-times more pyrene than the same HA+PG concentrations on clay alone.

### Table 5.7. Distribution Coefficients for Pyrene Sorbed to HA+PG Synthetic Soils

<table>
<thead>
<tr>
<th>OM and Mineral Phase:</th>
<th>$R^2$:</th>
<th>Freundlich $n$ Values:</th>
<th>Avg. $K_D$: (mL g⁻¹)</th>
<th>Std. Dev.: (mL g⁻¹)</th>
<th>Does $n = 1.0$?</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mg L⁻¹ PG + 100 mg L⁻¹ HA on Clay</td>
<td>0.99</td>
<td>1.23</td>
<td>255.0</td>
<td>63.6</td>
<td>P = 171</td>
</tr>
<tr>
<td>100 mg L⁻¹ PG + 400 mg L⁻¹ HA on Clay</td>
<td>0.89</td>
<td>0.79</td>
<td>287.4</td>
<td>87.2</td>
<td>P = 0.0003</td>
</tr>
<tr>
<td>100 mg L⁻¹ PG + 800 mg L⁻¹ HA on Clay</td>
<td>0.87</td>
<td>1.10</td>
<td>355.0</td>
<td>44.3</td>
<td>P = 0.031</td>
</tr>
<tr>
<td>100 mg L⁻¹ PG + 400 mg L⁻¹ HA on 80% Clay : 20% Hematite</td>
<td>1.00</td>
<td>1.20</td>
<td>796.0</td>
<td>201.0</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Pyrene Sorption on HA+PG Synthetic Soils

- 100 mg/L PG + 400 mg/L HA on 80% Clay : 20% Hematite
- 100 mg/L PG + 100 mg/L HA on Clay
- 100 mg/L PG + 400 mg/L HA on Clay
- 100 mg/L PG + 800 mg/L HA on Clay

**Figure 5.11.** Freundlich isotherms depicting pyrene sorption on HA+PG-coated synthetic soils, where the mixed soil (an 80% clay : 20% hematite ratio) resulted in the greatest affinity.

Although some surfaces of the 100 mg L\(^{-1}\) PG + 800 mg L\(^{-1}\) HA-coated clay appeared encased in diffuse blue pyrene (Figure 5.12a), the brightest points emanated from pore spaces, broken edges, and between aggregated particles, where SOM sorption was likely greatest for noncovalent bonding with exposed oxygenated functional groups on clay [97]. This observation was supported by varying fluorescence intensities in the composite image, where fluorescent bodies trapped within small areas concentrated and yielded larger intensity values compared to diffuse, surficial entities. The graph in Figure 5.12b demonstrated that an increase in the amount of sorbed pyrene caused a predictable increase in the soil fluorescence intensity. However, as compared to the HPLC data (Figure 5.11), increasing the HA content did not detectably influence the pyrene fluorescence intensities. However, any change in the
pyrene intensities may have been masked by the increased HA autofluorescence interfering with the PAH filter measurements, as pyrene and HA both require UV light to fluoresce.
5.3.5 PYRENE SORPTION TO NATURAL SOILS

Using the synthetic soil model discussed in Chapter 4, one purpose of this work was to compare sorbed pyrene concentration and location within two different SOM constituents (HA and PG) to that within natural soil. As stated, the spiked concentration range (0-50.0 mg g\(^{-1}\) pyrene) was chosen to simulate low to high site contamination. Regardless of the physical and chemical differences between PMN and SF (Table 3.1), pyrene sorption in Figure 5.13 and the statistically similar \(K_D\) values in Table 5.8 (\(P = 0.587\)) indicate a standardized pyrene behavior in two natural soils. Furthermore, the Freundlich \(n\) values denoted a nonlinear sorption mechanism for both natural soils [84].
Table 5.8. Distribution Coefficients for Pyrene Sorbed to Natural Soils

<table>
<thead>
<tr>
<th>Mineral Phase:</th>
<th>$R^2$:</th>
<th>Freundlich $n$ Values:</th>
<th>Average $K_D$: (mL g$^{-1}$)</th>
<th>One Std. Dev.: (mL g$^{-1}$)</th>
<th>Does $n = 1.0$?</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMN Soil</td>
<td>0.85</td>
<td>0.98</td>
<td>306</td>
<td>11</td>
<td>$P = 0.315$</td>
</tr>
<tr>
<td>SF Soil</td>
<td>0.93</td>
<td>1.26</td>
<td>327</td>
<td>91</td>
<td>$P = 0.026$</td>
</tr>
</tbody>
</table>

Figure 5.13. Freundlich isotherms depicting sorption of pyrene on two natural soils, PMN and SF.

As portrayed in Figure 5.14a, the apparent prevalence of bright blue pyrene fluorescence between aggregated particles and within pore spaces, cracks, and edges exceeded the diffuse surficial blue. This pyrene sorption preference corresponded with heightened SOM presence, as the potential for noncovalent bonding with exposed functional groups in these areas was the greatest [97]. Moreover, variable fluorescence intensities in the composite image lend further evidence to this theory, where fluorescent entities trapped within small regions, such as pores, concentrated and yielded higher intensity values. The graph in Figure 5.14b demonstrated that fluorescence intensity predictably increases with an
increased in sorbed pyrene and reinforced observations from Figure 5.13 regarding the potential similar behavior of pyrene in natural soils.

Fluorescence (FL) microscope image of pyrene using PAH filter. Light (LT) microscope image of SF soil.

Composite FL & LT Image
(Fluorescence Intensity values are in white.)
Figure 5.14. (a) Combined PAH and light images of 2.5 mg g⁻¹ pyrene sorbed to SF soil at 100x total magnification. (b) Graph of sorbed pyrene concentration versus the resulting fluorescence intensity.

5.3.6 PYRENE PREFERENCE AND “MAPPING” CONTAMINATED SOILS

Finally, one of the primary goals for this research was to construct a “pseudo-map” of pyrene location in relation to HA, PG, and the mineral phases, and determine the percentage of pyrene associated with each SOM component. Presented in Figure 5.15 are individual and combined fluorescence images for a synthetic soil composed of 2.5 mg g⁻¹ pyrene sorbed to 400 mg L⁻¹ HA and 100 mg L⁻¹ PG on clay. As a comparison, Figure 5.16 includes individual and combined fluorescence images for PMN soil, depicting the location of 2.5 mg g⁻¹ pyrene in relation to HS, amino sugars (primarily PG), and mineral particles. The DAPI images were pseudo-colored red to distinguish HA (or HS) autofluorescence from that for pyrene (blue), PG (or amino sugars) was labeled with FITC (green), and exposed mineral phases were
brown to black in color. As discussed in Section 5.2.6, the composite images were generated by individually overlapping fluorescence images from each filter (DAPI, PAH, and FITC) with a light microscope image of the mineral phase. The fluorescence images were adjusted to 40% opacity so the color and texture of each layer could be seen in the composite.
**Figure 5.15.** “Pseudo-Map” depicting sorption of 2.5 mg g\(^{-1}\) pyrene in relation to 400 mg L\(^{-1}\) HA and 100 mg L\(^{-1}\) PG on clay. PG was labeled green with FITC, DAPI autofluorescence from HA was pseudo-colored red, pyrene appeared blue via the PAH filter, and clay was brown to black in color under white light. All images were taken unperturbed at a 100x total magnification.
Fluorescence (FL 1) microscope image of pyrene using PAH filter.

Light (LT) microscope image of PMN soil.

Fluorescence (FL 2) microscope image of HS using DAPI filter.

Fluorescence (FL 3) microscope image of amino sugars (PG) using FITC filter.
Figure 5.16. “Pseudo-Map” depicting sorption of 2.5 mg g⁻¹ pyrene to PMN soil components. Amino sugars (PG) were labeled green with FITC, DAPI autofluorescence from HS was pseudo-colored red, pyrene appeared blue via the PAH filter, and the mineral phases were brown to black in color under white light. All images were taken unperturbed at a 100x total magnification.

To determine which SOM component pyrene preferred in the multiple-component synthetic and natural soils, RGB (red-blue-green) histograms for each fluorescence filter were generated (Section 5.2.8). A histogram is a graphical representation detailing the number of pixels in an image corresponding to each of 255 brightness levels, where pseudo-colored HA (or HS), PG (or amino sugars), and pyrene yielded red, green, and blue histograms, respectively (Figure 5.17). When the histograms for each sample were graphed together the resulting overlap estimated the equivalent color overlap in the fluorescence
image, much like a Venn diagram. More simplistically, the overlap between the red (HA) and blue (pyrene) graphs (Figure 5.17a) versus the green (PG) and blue (pyrene) graphs (Figure 5.17b) indicated the percentage of pyrene associated with HA and PG, respectively.

**Figure 5.17.** Using histograms from the PMN images as an example, measuring the overlap between (a) the red (HS) and blue (pyrene) graphs versus (b) the green (PG) and blue (pyrene) graphs estimated the percentage of pyrene associated with HA and PG, respectively.
RGB histograms were generated for the following samples spiked with 2.5 mg g$^{-1}$ pyrene: (i) 400 mg L$^{-1}$ HA on clay, (ii) 100 mg L$^{-1}$ PG on clay, (iii) 400 mg L$^{-1}$ HA and 100 mg L$^{-1}$ PG on clay, (iv) 400 mg L$^{-1}$ HA and 100 mg L$^{-1}$ PG on 80% clay : 20% hematite, and (v) PMN. The percentage of pyrene associated with HA (or HS) and PG (or amino sugars) for each sample were presented in Figure 5.18. For the multi-component synthetic soils pyrene appeared to primarily associate with PG, where both of the HA and PG soils yielded significantly different percentages compared to the individual PG and HA clays. Results for PMN were also different from the HA+PG synthetic soils, in that pyrene fluorescence associated with HS more than the amino sugars. One explanation for this observation was that PMN HS included humin and HA, both strong sorptive components for pyrene, whereas the multi-component synthetic soils contained only HA and were, hence, dominated by PG.

![Pyrene Association with SOM Components](image)

**Figure 5.18.** Graph of fluorescence overlap deduced from individual filter images. The overlap estimated the percentage of pyrene associated with HA (or HS) and PG (or amino sugars) in the synthetic and natural soils.
To substantiate the results in Figure 5.18, histograms were generated of individual sorbed pyrene points within the composite images. For example, in the 2.5 mg g$^{-1}$ pyrene sorbed to 100 mg L$^{-1}$ PG + 400 mg L$^{-1}$ HA clay image (Figure 5.19a), thin strips around three visible pyrene points were analyzed via Adobe Photoshop CS to yield three RGB histograms (Figure 5.19b). Thin strips were used instead of blocks to obtain a more straightforward (one-dimensional) examination of pyrene sorption preference within a particular row of pixels. As shown in Figure 5.19b, the three blue pyrene graphs resembled the green PG peaks more closely in structure and axis placement than the red HA graphs, and thus, indicated that the pyrene points coincided with PG, which supports the data in Figure 5.18.
Figure 5.19. Strips of the composite 2.5 mg g\(^{-1}\) pyrene sorbed to 100 mg L\(^{-1}\) PG + 400 mg L\(^{-1}\) HA clay image (a) were taken to generate RGB histograms (b) of pyrene points and the surrounding linear area. The pyrene peaks coincided well with those from green PG, and thus, confirmed the preference data in Figure 5.18.

5.4 DISCUSSION

In general, the test cases from Section 5.3.1 behaved as expected and included: (i) spiking pyrene onto glass beads and molecular sieves, (ii) using diesel fuel as the carrier solvent, and (iii) employing packed soil columns in place of the common slurry method for spiking pyrene. Low amounts of pyrene adhered to “rough” or “irregular” patches on inert glass surfaces, (Figure 5.2; Table 5.1), whereas large concentrations caused precipitation rather than attachment. In contrast, pyrene application to molecular sieves visually demonstrated sorption within pore spaces (Figure 5.3; Table 5.2), where increasing the spiked concentration yielded brighter points of blue fluorescence. Soils trapped in a column
and spiked with pyrene via a leaching mechanism yielded heterogeneous fluorescence
intensity and HPLC data compared to the corresponding slurry procedure (Figure 5.4; Table
5.3), making any microscale observation difficult and subject to skepticism. Finally, using
diesel fuel as the carrier solvent caused a non-specific fluorescence to blanket entire particles
(Figure 5.5; Table 5.4) and masked any fluorescence from SOM and pyrene. For the purpose
of this research, these test cases supported using a slurry method with a DCM carrier solvent
as the optimal technique for spiking and visually observing pyrene sorbed within soil.

As shown in Figure 5.20, the experimental system behaved in a predictable manner,
and different SOM types and loaded concentrations caused variations in pyrene sorption,
specifically PG appeared to have a more profound influence than previously recognized.
Pyrene sorption to SOM-coated soils produced case III nonlinear Freundlich isothersms
(Table 5.9), in which previously sorbed molecules led to a modification of the soil surface
that favored further contaminant sorption [83]. In comparison, pyrene sorption on bare
montmorillonite clay followed a case I nonlinear Freundlich curve, where the frequency and
energy of sorption sites within the clay were finite [83]. Considering the greater
hydrophobicity and surface area of SOM (800-900 m² g⁻¹) compared to clay (31.8 m² g⁻¹),
these results indicated that the introduction of additional and/or greater concentrations of
SOM components to a mineral phase correspondingly increased the total available surface
area and yielded a matrix more favorable for pyrene dissolution [97].
Figure 5.20. Freundlich isotherms depicting pyrene sorption on multiple- and single-component synthetic soils, where the type and total amount of SOM sorbed to the mineral phase exerted a profound influence.

Table 5.9. Distribution Coefficients for Pyrene Sorbed to Multiple- and Single-Component Synthetic Soils

<table>
<thead>
<tr>
<th>OM and Mineral Phase:</th>
<th>R²</th>
<th>Freundlich n Values:</th>
<th>Average K_d: (mL g⁻¹)</th>
<th>One Std. Dev.: (mL g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank Clay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(100, 400, and 800 mg L⁻¹) HA on Clay</td>
<td>0.93</td>
<td>0.53</td>
<td>491</td>
<td>438</td>
</tr>
<tr>
<td>(50 and 100 mg L⁻¹) PG on Clay</td>
<td>0.89</td>
<td>1.98</td>
<td>100</td>
<td>53</td>
</tr>
<tr>
<td>100 mg L⁻¹ PG + (100, 400, and 800 mg L⁻¹) HA on Clay</td>
<td>0.99</td>
<td>1.51</td>
<td>935</td>
<td>452</td>
</tr>
<tr>
<td>Avg. of 100 mg L⁻¹ PG + (100, 400, and 800 mg L⁻¹) HA on Clay</td>
<td>0.98</td>
<td>1.17</td>
<td>284</td>
<td>58</td>
</tr>
<tr>
<td>100 mg L⁻¹ PG + 400 mg L⁻¹ HA on 80% Clay : 20% Hematite</td>
<td>1.00</td>
<td>1.20</td>
<td>796</td>
<td>201</td>
</tr>
</tbody>
</table>

Although pyrene should have more affinity for HA due to its high aromatic carbon content (50.0%), PG presence (Figure 5.20) resulted in greater pyrene sorption for two potential reasons: (i) the sorbed PG concentration on clay was higher than that for HA,
resulting in a larger hydrophobic matrix for pyrene dissolution; and (ii) this greater amount of sorbed PG also produced more particle aggregation compared to HA, which provided a larger total surface area available for pyrene sequestration (Chapter 4). However, the anomaly was that PG alone on clay yielded the largest sorbed pyrene concentration over those soils with two SOM constituents and/or mineral phases (Figure 5.20), indicating that the soil TOC content was not the only factor influencing sorption. One possible theory is that upon addition of PG to the HA-sorbed clay, the desorption-rearrangement-resorption mechanism occurred (Chapter 4), and HA masked some of the available sorption sites on PG, making them either partially or completely unavailable to pyrene (Figure 5.21). Such a phenomenon was shown for naphthalene sorption to environmental BCs, where SOM presence caused a decline in BC $K_D$ values compared to those for the pristine surfaces [76].
As depicted in Figure 5.22 and the $K_D$ values in Table 5.10, pyrene sorption to HA+PG synthetic clay behaved in a manner significantly similar to that shown by two natural soils ($P = 0.048$ and 0.062 for SF and PMN, respectively). In comparison, the 80% clay : 20% hematite soil appeared to yield the greatest pyrene sorption. This potentially resulted from a larger particle surface area and greater total SOM sorption compared to clay (Chapter 4), spiking only two pyrene concentrations versus six for clay, and/or the presence of hematite in general, which was not an inclusion of the natural soils used for this work. These results indicated that the HA+PG synthetic clay behaved in a manner similar to natural
soil, and that HS and amino sugars (corresponding to HA and PG in the synthetic clay) were important SOM components in natural soils influencing pyrene sorption.

**Figure 5.22.** Freundlich isotherms comparing the sorption of pyrene on natural and multiple-component synthetic soils.

**Table 5.10. Distribution Coefficients for Pyrene Sorbed to Synthetic and Natural Soils**

<table>
<thead>
<tr>
<th>OM and Mineral Phase:</th>
<th>R²:</th>
<th>Freundlich n Values:</th>
<th>Average K&lt;sub&gt;D&lt;/sub&gt;: (mL g&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>One Std. Dev.: (mL g&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avg. of 100 mg L&lt;sup&gt;-1&lt;/sup&gt; PG + (100, 400, and 800 mg L&lt;sup&gt;-1&lt;/sup&gt;) HA on Clay</td>
<td>0.98</td>
<td>1.17</td>
<td>243</td>
<td>120</td>
</tr>
<tr>
<td>100 mg L&lt;sup&gt;-1&lt;/sup&gt; PG + 400 mg L&lt;sup&gt;-1&lt;/sup&gt; HA on 80% Clay : 20% Hematite</td>
<td>1.00</td>
<td>1.20</td>
<td>531</td>
<td>481</td>
</tr>
<tr>
<td>PMN Soil</td>
<td>0.85</td>
<td>0.98</td>
<td>306</td>
<td>11</td>
</tr>
<tr>
<td>SF Soil</td>
<td>0.93</td>
<td>1.26</td>
<td>327</td>
<td>91</td>
</tr>
</tbody>
</table>

The graph in Figure 5.23 indicated that regardless of the soil, synthetic or natural, an increase in pyrene concentration caused a predictable rise in fluorescence intensity values. Furthermore, as substantiated by the HPLC data, fluorescence intensity was contingent on the
particular soil matrix examined, as these values reflected the amount of sorbed pyrene. Specifically, PG presence in the soil matrix exerted a profound impact on the pyrene sorption capacity and its resulting fluorescence intensity compared to HA and HA+PG on clay, again indicating that TOC content was not the only influencing factor and that masking in the HA+PG soils potentially reduced pyrene sorption. Moreover, pyrene fluorescence intensities for the spiked synthetic and natural soils were similar in curvature and plateau compared to the linear character of the blank clay (montmorillonite clay not loaded with SOM) data, indicating that SOM addition caused clay to behave more like the natural soils in regard to pyrene sorption. Therefore, the fluorescence intensities supported observations regarding the HPLC results in Figures 5.20 and 5.22, and implicated intensity measurements as a quick, alternative method to determine PAH incidence and concentrations in the environment.
Fluorescence Intensities of 0-50.0 mg g\(^{-1}\) Pyrene Sorbed to Natural and Synthetic Soils

![Fluorescence Intensities Graph](image)

**Figure 5.23.** Graph of sorbed pyrene concentration versus the resulting fluorescence intensity for synthetic and natural soils.

Currently, research into PAH sequestration was limited to sorption/desorption studies and NMR and FTIR analyses on bulk soil samples with contaminant concentrations determined by destructive and arduous techniques, such as solvent extraction and HPLC [62, 63]. As a non-destructive, facile, and rapid alternative method, fluorescence microscopy has the capability to determine contaminant presence, concentration, and interactions *in situ* and regardless of whether the compound was free or sorbed. For instance, Karimi-Lotfabad and Gray [45] used CLSM and CSEM microscopies to examine NAPL contaminant distribution.
on and within the soil microstructure. Another study by Wild et al. [99] determined PAH location and intercellular migration within plant leaves via TPEM [100, 101].

For the fluorescence images blue pyrene diffusely coated HA clay surfaces (Figure 5.8), as evidenced by a lack of varying fluorescence intensities in the composite image. This was likely due to pyrene dissolution within HA, where HA has been shown to cover entire particle surfaces (Chapter 4). For PG clays the pyrene fluorescence primarily formed bright blue dots along clay edges, within pores, and between aggregated particles (Figure 5.10), as verified by the significantly varying intensity measurements. Since a greater amount of PG sorbed to clay, compared to HA, the “excess” concentrated within pores, edges, and between aggregates (Chapter 4), and as a result, pyrene also sorbed within these areas due to the abundance of PG, the preferred sorbent as shown by HPLC and fluorescence intensity data (Figures 5.20 and 5.23). For the HA+PG clays pyrene sorption appeared to include characteristics from both the HA and PG clays (Figure 5.12), likely the consequence of HA and PG placement and abundance. Although considerable overlap and interconnection of green PG and blue HA fluorescence occurred along the clay surface (Chapter 4), the brightest colors, indicating the greatest sorption extent, resided within pore spaces, edges, and between aggregated particles, and were the same locations where the brightest pyrene fluorescence was noted per intensity measurements on the composite image.

The purpose of soil “pseudo-maps” was to generate a visual representation of how soil was constructed at the microscale, a novel approach to examining pollutant fate and transport within natural soil. Qualitatively, these composite images were meant to illustrate where individual SOM components were in relation to the mineral phase and any spiked contamination, in other words, a particle-sized “road map” detailing the location of specific
sites (Figure 5.24). Quantitatively, histograms generated from the individual filter images that comprised the composite image, measured pollutant preference for specific SOM components, such as PG versus HA. Together, the qualitative and quantitative observations theoretically provided a more explicit analysis of pollutant fate than what sorption/desorption studies have shown thus far and may help develop cost-effective and efficient in situ remediation technology that targets these specific sequestration sites.

Figure 5.24. Schematic of a soil “pseudo-map” that details where specific SOM entities were in relation to the mineral phase and pyrene.

In Figures 5.15 and 5.16 it was shown that images from three different fluorescence filters and a light microscope image could be overlaid to yield a composite image “mapping” the location of individual SOM components in relation to pyrene and the mineral phase. Pyrene association percentages were deduced for the synthetic and natural soil samples by
measuring the blue/green (pyrene/PG) and blue/red (pyrene/HA) histogram overlap for the individual filter images. These “pseudo-maps” indicated that pyrene fluorescence primarily affiliated with PG (9.7% and 75.2%) over HA (3.6% and 53.8%) in the synthetic clay and hematite:clay mixed soil, respectively (Figure 5.15). This result was expected from the HPLC data in Figure 5.20, where PG has a significant impact on pyrene sorption compared to HA. Conversely, for the composite PMN image in Figure 5.16, pyrene appeared to associate predominantly with the red HS fluorescence (53.3%) compared to amino sugars (47.4%). One explanation was that PMN HS included humin and HA, both strong sorptive components for pyrene, whereas the HA+PG synthetic soils contained only HA and were, therefore, dominated by PG.

Significant findings in this chapter included: (i) the profound influence PG (and potentially other biological constituents) had over pyrene sorption, a previously unrecognized or under-appreciated source for HOC sequestration; (ii) using an alternative Gram-staining method to fluorescently visualize PG within the soil matrix; and (iii) the construction of soil “pseudo-maps,” which are a novel approach to addressing contaminant in situ fate and availability. In summary, as shown by HPLC and fluorescence intensity measurements, pyrene sorption was influenced by the type and concentration of SOM present in the soil system as well as any inhibitory affects, such as masking available sorption sites. Moreover, use of fluorescence microscopy to document pyrene location and matrix preference was a valuable tool to further knowledge of pollutant fate and transport in soils and could potentially make remediation technology more effective.
CHAPTER 6: FLUORESCENCE INTENSITY AS A TECHNIQUE TO STUDY PAH CONCENTRATIONS, FATE, AND TRANSPORT WITHIN SOILS

6.1 INTRODUCTION

Currently, research into PAH sequestration is limited to arduous, destructive, and possibly hazardous techniques, such as solvent extraction, chromatography, radioisotope labeling, and NMR and FTIR analyses [42, 62, 63, 76]. Although beneficial for quantitative measurements, these approaches offer little direct observation of particle-scale PAH dynamics relative to the soil matrix and fail to describe the overall fate and transport mechanisms, of which the dual-mode model is an example [8, 69, 105, 106]. Combining qualitative and quantitative analysis methods could potentially provide a more comprehensive representation of pollutant movement, sorption, and degradation, which is essential to developing efficient and cost-effective in situ remediation technology.

Fluorescence microscopy provides a powerful, non-intrusive tool for visualizing and tracking the movement, storage locations, and degradation of organic chemicals within vegetation, soil, and water. It requires little to no sample modification or manipulation and uses the inherent autofluorescence of the chemical compound, plant, and/or NOM. For instance, Karimi-Lotfabad and Gray [45] used fluorescent confocal laser scanning (CLSM) and cryogenic scanning electron (CSEM) microscopies to examine nonaqueous phase liquid (NAPL) distribution on and within the soil microstructure. The results indicated that NAPL
encased aggregate surfaces and filled accessible inter-aggregate pores to create a discrete phase, suggesting that contaminant diffusion out of NAPL-clay structures and filled pores was important to desorption from soil [45]. Another study by Wild et al. [99] determined PAH location and intercellular migration within plant roots and leaves via two-photon excitation microscopy (TPEM) [100, 101]. The study identified anthracene in five separate locations within the leaf, and when applied to the roots, highly focused compound "streams" formed over time and degraded into anthrone, anthraquinone, and hydroxyanthraquinone [99, 100, 101].

The objective of this research was two-fold and intended to help advance current remediation technology. The first purpose was to show that fluorescence intensity (combined with a calibration curve) could be used as a rapid, alternative technique to diagnose PAH concentration in a contaminated soil. The second purpose was to preliminarily observe and measure the progress of pyrene fate and transport over time within a given natural soil, using fluorescence microscopy techniques. The specific goals for this research included: (i) generating a calibration curve of sorbed pyrene concentration versus fluorescence intensity; (ii) using the calibration curve combined with fluorescence microscopy to diagnose PAH presence and total amount in contaminated soils; and (iii) examining the dual-mode succession of PAHs in a natural soil. Using a calibration curve derived from the spiked soils described in Chapter 5, two soils well-aged with creosote were analyzed for individual and total PAH amounts via fluorescence microscopy and compared to the values given by the traditional extraction and analysis methods of a commercial laboratory. Also, the dual-mode progression of soil-bound PAHs was observed and measured, using fluorescence microscopy and intensity. The result may be a more efficient and economical method to determine PAH
content in soils compared to traditional protocols, and visual groundwork for justification of the dual-mode model.

6.2 EXPERIMENTAL SECTION

All chemicals and solvents were either HPLC grade or higher. Glassware used in the preparation of any solution, whether stock, diluted, buffer, or sample, were cleaned thoroughly using an acid-washing regimen. Metal and plasticware were cleaned in the same manner as for glass, except for using an acid bath.

6.2.1 NATURAL AND SYNTHETIC SOILS

A total of four natural soils and an HA+PG synthetic clay discussed in Chapters 4 and 5 (Table 6.1) were employed for the research described in this chapter. Two of the natural soils (CMN and KKY) were from wood preservation facilities historically contaminated by creosote and diesel fuel for approximately 50-55 years. Conversely, the remaining two soils were pristine representatives, where PMN was from an uncontaminated field adjacent to the CMN site and SF was from Schenck Forest, NC.

The HA+PG synthetic clay was generated as detailed in Sections 4.2.1 and 4.2.2, spiked with pyrene alongside PMN and SF using methods explained in Sections 5.2.2 and 5.2.3, and examined for its sorption properties as described in Section 5.3. The mineral base was Na-saturated montmorillonite clay (SWy-2; Source Clay Minerals Repository), and the SOM coating consisted of 100 mg L\(^{-1}\) *S. aureus* PG (77140; BioChemika) and 100, 400, or 800 mg L\(^{-1}\) IHSS soil HA standard (1S102H).
Table 6.1: Characteristics of Natural and Synthetic Soil Samples

<table>
<thead>
<tr>
<th></th>
<th>KKY</th>
<th>CMN</th>
<th>PMN</th>
<th>SF</th>
<th>Blank Clay</th>
<th>Synthetic Clay</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Textural Class</strong></td>
<td>Sandy Loam</td>
<td>Sand</td>
<td>Sandy Loam</td>
<td>Sandy Clay Loam</td>
<td>Clay</td>
<td>Clay</td>
</tr>
<tr>
<td><strong>Total PAH</strong> (ppm)</td>
<td>12407.6 ± 3464.6 (d)</td>
<td>4393.1 ± 693.1 (d)</td>
<td>20.4 ± 4.6 (d)</td>
<td>0.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>Pyrene Conc.</strong> (ppm)</td>
<td>1825.8 ± 367.7 (d)</td>
<td>462.6 ± 100.3 (d)</td>
<td>3.5 ± 1.0 (d)</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>CEC</strong> (meq 100 cm⁻¹)</td>
<td>28</td>
<td>22</td>
<td>13</td>
<td>46.2</td>
<td>76.4 (d)</td>
<td>38.0</td>
</tr>
<tr>
<td><strong>N₂ BET</strong> (m²/g)</td>
<td>4.0 ± 0.2 (d)</td>
<td>8.9 ± 1.4 (d)</td>
<td>7.6 ± 2.3 (d)</td>
<td>10.0</td>
<td>25.2 ± 0.2 (d)</td>
<td>7.9 ± 0.1 (d)</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>7.8</td>
<td>7.5</td>
<td>8.1</td>
<td>6.8</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td><strong>% Sand</strong></td>
<td>63</td>
<td>89</td>
<td>63</td>
<td>58</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>% Silt</strong></td>
<td>30</td>
<td>8</td>
<td>24</td>
<td>22</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>% Clay</strong></td>
<td>7</td>
<td>3</td>
<td>13</td>
<td>21</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><strong>% Organic Matter</strong></td>
<td>10.4</td>
<td>2.7</td>
<td>1.5</td>
<td>10.9</td>
<td>0.0 (e)</td>
<td>3.0</td>
</tr>
</tbody>
</table>

(a) Soil class and texture, cation exchange capacity (CEC), pH, percent sand/silt/clay, and percent organic matter were analyzed by the University of Wisconsin Soil Science Extension Service, Madison, WI.
(b) PAH content was determined by Triangle Laboratories, Inc., Durham, NC, using EPA method 8270. The total PAH content includes the 16 priority compounds listed by EPA (acenaphthene, acenaphthylene, anthracene, benzo(a)anthracene, benzo(j)fluoranthene, benzo(g,h,i)perylene, benzo(k)fluoranthene, chrysene, fluoranthene, fluorene, naphthalene, phenanthrene, pyrene, dibenz(a,h)anthracene, and indeno(1,2,3-cd)pyrene).
(c) Surface area was measured by Clear Science, Inc., Baton Rouge, LA.
(d) Mean values are presented with one standard deviation calculated from triplicate samples.
(e) CEC and percent organic matter for blank montmorillonite clay was determined by the Clay Minerals Society Source Clay Repository, Aurora, CO.

6.2.2 FLUORESCENCE MICROSCOPY

The methods used to mount and view contaminated soil samples were identical to those discussed in Section 4.2.5. Blue PAH fluorescence was viewed with 300-400 nm UV light using a filter specific for PAHs.

6.2.3 IMAGE COMPILATION

The method used to overlap images was identical to that stated in Section 4.2.6, with one exception that the fluorescence images were of PAHs or pyrene and not SOM.
6.2.4 FLUORESCENCE INTENSITY ANALYSIS

The method used to determine soil fluorescence intensity was identical to that stated in Section 3.2.8. When graphed against the sorbed pyrene concentration, the resulting fluorescence intensity increase corresponded with an increase in the amount of pyrene sorbed to the soil.

6.2.5 ESTIMATING PAH CONTENT BY FLUORESCENCE INTENSITY

The ‘pyrene’ filter was named as such by the manufacturer because it was intended for use in assays where pyrene was introduced as a selective chromagen. In contrast, environmental PAH contamination is commonly found as a complex mixture of compounds, and the filter excitation and emission wavelengths are not specific for pyrene alone but yield a degree of fluorescence from each of the priority PAHs. Therefore, the relative fluorescence intensity (RFI) was a measure of this fluorescence per milligram of a given PAH, normalized to pyrene (Table 6.2). For example, fluoranthene and benzo(b)fluoranthene produced the greatest fluorescence per mole (404%), more than four times that of pyrene (100%).
Table 6.2. Relative Fluorescence Intensities (RFIs) for 16 Priority PAHs

<table>
<thead>
<tr>
<th>PAHs Present:</th>
<th>Relative FI: (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acenaphthene</td>
<td>9</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>4</td>
</tr>
<tr>
<td>Anthracene</td>
<td>80</td>
</tr>
<tr>
<td>Benzo(a)anthracene</td>
<td>200</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>30</td>
</tr>
<tr>
<td>Benzo(b)fluoranthene</td>
<td>404</td>
</tr>
<tr>
<td>Benzo(g,h,i)perylene</td>
<td>109</td>
</tr>
<tr>
<td>Benzo(k)fluoranthene</td>
<td>15</td>
</tr>
<tr>
<td>Chrysene</td>
<td>57</td>
</tr>
<tr>
<td>Fluoranthe</td>
<td>404</td>
</tr>
<tr>
<td>Fluorene</td>
<td>3</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>38</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>2</td>
</tr>
<tr>
<td>Pyrene</td>
<td>100</td>
</tr>
<tr>
<td>Dibenzo(a,h)anthracene</td>
<td>33</td>
</tr>
<tr>
<td>Indenopyrene</td>
<td>20</td>
</tr>
</tbody>
</table>

To determine the total and individual PAH concentrations of an unknown contaminated soil, it was proposed that a calibration curve and the soil fluorescence intensity could be used to estimate these amounts. Therefore, the known soil samples used for the curve consisted of two natural soils (PMN and SF; Table 6.1) and synthetic clays loaded with 100 mg L\(^{-1}\) PG and 100, 400, and 800 mg L\(^{-1}\) HA freshly spiked with pyrene and analyzed via HPLC (as described in Sections 5.2.2 and 5.2.3) and fluorescence microscopy (Section 6.2.2). A calibration curve was produced by plotting the amount of sorbed pyrene versus the corresponding fluorescence intensity. As described in Section 6.2.4, the fluorescence intensity for a designated soil was measured in triplicate, and using the linear regression equation from the calibration curve, the amount of sorbed pyrene was calculated. This pyrene amount was then multiplied by the RFI decimal (Table 6.2) for each of the 16 priority PAHs to estimate their individual concentrations in the soil. To gain a total PAH content, the individual concentrations were summed.
6.3 RESULTS

6.3.1 FLUORESCENCE INTENSITY CALIBRATION CURVES TO DETERMINE UNKNOWN PAH CONTENTS

One purpose of this research was to test the accuracy of a rapid, alternative method for determining PAH presence and general concentration in natural soils compared to traditional extraction and analysis protocols. The log-log relationship of the calibration curve in Figure 6.1 yielded a relatively linear plot ($R^2 = 0.94$), indicating that fluorescence intensity directly correlated with the amount of visible pyrene sorbed to the mineral phase. The aged contaminated natural soils (CMN and KKY) employed to test the curve were previously analyzed by a commercial laboratory (Table 6.1; Triangle Laboratories, Inc., Durham, NC), using an EPA-approved solvent extraction and GC/MS method. As shown in Table 6.3, the fluorescence intensity of both test soils confirmed pyrene presence and yielded an estimated content that was significantly similar to the extraction data.
Figure 6.1. Calibration curve generated by plotting freshly spiked pyrene concentrations against their respective fluorescence intensities.

Table 6.3. Comparison of Measured and Estimated Sorbed Pyrene Amounts

<table>
<thead>
<tr>
<th></th>
<th>CMN</th>
<th>KKY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence Intensity</td>
<td>19 ± 2</td>
<td>100 ± 16</td>
</tr>
<tr>
<td>Estimated Concentration (ppm)</td>
<td>480 ± 43</td>
<td>1531 ± 166</td>
</tr>
<tr>
<td>Measured Concentration (ppm)</td>
<td>463 ± 100</td>
<td>1561 ± 392</td>
</tr>
<tr>
<td>Similarity of estimated and measured concentrations.</td>
<td>P = 0.796</td>
<td>P = 0.872</td>
</tr>
</tbody>
</table>

Although this method did denote PAH presence and a generalized total concentration within the test soils, the estimated individual concentrations severely departed from the extracted ones (Table 6.4). This deviation potentially stemmed from singular or combined sources. For instance, the RFI for each PAH was determined via its dissolved or crystalline form in an ideal aqueous environment. This likely introduced bias by yielding a larger fluorescence value compared to the same PAH sorbed to a mineral phase that contains
interferences from the particles themselves or other soil organics. Another possibility was that soils used to generate the calibration curve were freshly spiked with pyrene alone, whereas the test soils were naturally aged with a complex PAH mixture and diesel fuel. Finally, compared to extraction of a three-dimensional soil matrix, fluorescence intensity analysis used a two-dimensional image, where the amount of pyrene within pores and other spaces hidden from direct view could not be determined.

Table 6.4. Estimated and Extracted PAH Concentrations for Aged Contaminated Soils

<table>
<thead>
<tr>
<th>PAHs Present:</th>
<th>GC/MS CMN (ppm)</th>
<th>Estimated CMN (ppm)</th>
<th>GC/MS KKY (ppm)</th>
<th>Estimated KKY (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acenaphthene</td>
<td>193.8</td>
<td>43.2</td>
<td>796.5</td>
<td>137.8</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>12.2</td>
<td>19.2</td>
<td>31.3</td>
<td>61.2</td>
</tr>
<tr>
<td>Anthracene</td>
<td>446.1</td>
<td>384.0</td>
<td>318.4</td>
<td>1224.8</td>
</tr>
<tr>
<td>Benzo(a)anthracene</td>
<td>297.2</td>
<td>960.1</td>
<td>562.8</td>
<td>3062.1</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>288.8</td>
<td>144.0</td>
<td>260.2</td>
<td>459.3</td>
</tr>
<tr>
<td>Benzo(b)fluoranthene</td>
<td>377.1</td>
<td>1939.4</td>
<td>436.3</td>
<td>6185.4</td>
</tr>
<tr>
<td>Benzo(g,h,i)perylene</td>
<td>190.4</td>
<td>523.2</td>
<td>115.1</td>
<td>1668.8</td>
</tr>
<tr>
<td>Benzo(k)fluoranthene</td>
<td>105.3</td>
<td>72.0</td>
<td>122.4</td>
<td>229.7</td>
</tr>
<tr>
<td>Chrysene</td>
<td>352.2</td>
<td>273.6</td>
<td>447.5</td>
<td>872.7</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>723.0</td>
<td>1939.4</td>
<td>2292.5</td>
<td>6185.4</td>
</tr>
<tr>
<td>Fluorene</td>
<td>184.7</td>
<td>14.4</td>
<td>1019.5</td>
<td>45.9</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>64.3</td>
<td>182.4</td>
<td>280.3</td>
<td>581.8</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>451.2</td>
<td>9.6</td>
<td>3053.1</td>
<td>30.6</td>
</tr>
<tr>
<td>Pyrene</td>
<td>462.6</td>
<td>480.0</td>
<td>1560.9</td>
<td>1531.0</td>
</tr>
<tr>
<td>Dibenz(a,h)anthracene</td>
<td>69.4</td>
<td>158.4</td>
<td>15.3</td>
<td>505.2</td>
</tr>
<tr>
<td>Indenopyrene</td>
<td>174.9</td>
<td>96.0</td>
<td>98.6</td>
<td>306.2</td>
</tr>
<tr>
<td><strong>Totals:</strong></td>
<td><strong>4393.1</strong></td>
<td><strong>7239.0</strong></td>
<td><strong>11410.5</strong></td>
<td><strong>23087.9</strong></td>
</tr>
</tbody>
</table>

6.3.2 DUAL-MODE PROGRESSION OF PYRENE SOPTION WITH AGING

The second purpose of this section was to generate a preliminary visual and analytical examination of the dual-mode mechanism for pyrene (or PAH) sorption within natural soil, yielding direct observations in support of this theoretical sorption model. The soils in Figures 6.2-6.4 appear dissimilar because they consisted of three different soil samples: (i) PMN freshly spiked with pyrene, (ii) PMN aged with pyrene for six months, and (iii) CMN aged
for 50-55 years with creosote, a PAH mixture, respectively. The PMN particles in Figure 6.3 were larger than those in Figure 6.2 due to the technique used to separate the liquid and solid phases (Section 5.2.2), where upon centrifugation the incubation vessels broke and much of the fine-grade soil (<0.2 μm) was lost. Moreover, the particles in Figure 6.4 appeared larger than those portrayed in Figure 6.2 due to the aggregational forces exerted by partially degraded diesel fuel in the sample, where the fuel was used to apply a creosote coating to wood products.
Figure 6.2. PMN freshly spiked with 2.5 mg g⁻¹ pyrene at 100x total magnification, where a majority of the blue pyrene fluorescence coated entire particle surfaces as supported by the similar intensity values in the composite image (white values).
**Figure 6.3.** PMN spiked with 2.5 mg g$^{-1}$ pyrene and incubated for six months at 100x total magnification. Surficial blue fluorescence was largely limited to patches as pyrene increasingly resided within pores, inter-particle spaces, and edges, which was supported by the varying intensities in the composite image (white values).
Figure 6.4. Well-aged CMN soil contaminated with a PAH mixture and diesel fuel for 50-55 years at 100x total magnification, where the majority of blue pyrene fluorescence resided within pores, inter-particle spaces, and edges as evidenced by the wide variation in intensity values for the composite image (white values).
Although pyrene appeared to settle within rough areas on PMN (Figure 6.2), such as macropore openings, cracks, and inter-particle spaces, a majority of the blue fluorescence completely coated particle surfaces. This observation was consistent with the low, similar fluorescence intensity values in the composite image, indicating diffuse fluorescence coverage along the surface. In contrast, blue surficial pyrene fluorescence decreased from whole particles in Figure 6.2 to patches in Figure 6.3 when spiked PMN was incubated for six months, where pyrene increasingly resided within pores, inter-particle spaces, and edges. This was evidenced by the greater variation between fluorescence intensity data in the composite image for Figure 6.3 relative to Figure 6.2. Such alteration potentially resulted for two reasons: (i) a longer allotted time frame (6 months versus 48 hours) for pyrene to move into the tortuous pathways of the soil matrix; and/or (ii) initializing the assimilation of easily-obtainable pyrene carbon sources by a degrader community. Finally, compared to the freshly spiked and incubated soils, examination of the well-aged CMN soil in Figure 6.4 suggested that PAHs increasingly reside within pores and inter-particle spaces over time, as supported by the significant deviation between fluorescence intensities in the composite image. These PAHs likely compose the non-available fraction resulting from aging/weathering processes and partial degradation by indigenous bacterial communities [8, 29, 61, 75, 98, 101].

To further examine the dual-mode model the fluorescence intensities of entire PAH-filter images were measured as described in Section 6.2.4, rather than individual entities as presented in the composite images for Figures 6.2-6.4. According to Figure 6.5, as pyrene (or PAHs) naturally aged within the soil matrix the fluorescence intensity increased. This possibly occurred because freshly spiked pyrene, Figure 6.2, had a more diffuse surficial sorption pattern compared to the incubated and well-aged MN soils, Figures 6.3 and 6.4,
respectively, which caused diffuse fluorescence and a low intensity value. As pyrene aged and increasingly migrated into pore spaces and other small areas, such as cracks or between aggregated particles, the fluorescence concentrated into brighter points and, thus, increased the resulting intensity data, as was depicted for molecular sieves in Section 5.3.

![Fluorescence Intensity of Soil-Bound Pyrene or PAHs over Time](image)

**Figure 6.5.** Comparison of fluorescence intensities for MN soil samples spiked with pyrene (or PAHs for CMN) and aged for various time periods.

### 6.4 DISCUSSION

Predicted pyrene data from the fluorescence calibration curve corresponded with the measured concentrations for two well-aged, creosote-contaminated soils ($P = 0.796$ for CMN and $P = 0.872$ for KKY). Although effective when diagnosing PAH presence and relative total concentration, especially pertaining to pyrene, using a calibration curve to quickly and efficiently measure individual concentrations in contaminated soils must be evaluated further. Deviations in the RFIs potentially resulted for three reasons. First, the RFI for each PAH was determined via its dissolved or crystalline form in an ideal aqueous environment. This
potentially introduced bias by yielding a larger fluorescence value compared to the same PAH sorbed to a mineral phase, which contains interferences from the particles themselves or other soil organics. Perhaps when determining RFI percentages individual PAHs sorbed to PMN or synthetic clay should be analyzed instead via the non-specific PAH filter. Second, soils used to generate the calibration curve were freshly spiked with pyrene, whereas the test soils were well-aged naturally with creosote and diesel fuel. Third, compared to extraction of a three-dimensional soil matrix, fluorescence intensity analysis used a two-dimensional image, where the amount of pyrene within pores and other spaces hidden from direct view could not be determined.

However, the main objective of Section 6.3.1 was to demonstrate that contaminant analysis via fluorescence microscopy may serve as a straightforward and non-destructive alternative method to traditional extraction and analysis protocols. Fluorescence intensity has the capability to determine contaminant presence and relative concentrations in situ and regardless of whether the compound is free or sorbed. In essence, this method can be used to rapidly examine a particular soil matrix, where the apparent contaminant location yields information regarding soil age and ease with which it may be remediated. The method, therefore, may have broad application to mechanistic studies of PAH fate in soils.

The dual-mode model is defined as simultaneous slow, nonlinear condensation in (or adsorption on the walls of) pores and rapid, linear partitioning into an organic phase [8, 69, 105, 106]. The partitioning fraction is labeled kinetically “fast” because it encompasses the amount of contaminant sorbed within 24 hours, and the “slow” or “very slow” adsorption fraction is the amount sorbed thereafter [105, 106]. Preliminary examination of pyrene sorption within natural soils strongly suggested that after 48 hours the majority of the
contaminant blanketed whole particles, while migrating into some easily accessible or shallow pores and inter-particle spaces as well (Figure 6.2) [53, 69, 104, 106]. After six months to decades (Figure 6.3 and 6.4, respectively), pyrene and PAHs increasingly appeared to occupy soil pores, edges, and inter-particle spaces, as sufficient time had passed to allow slow migration deeper into the tortuous intra- and inter-particle pathways. A hypothetical schematic is portrayed in Figure 6.6.

As suggested by the dual-mode model this gradual movement from surface to pore sorption potentially occurred for two reasons. After adapting to the presence of pyrene, the indigenous biological community preferentially used carbon sources that were easily
accessible, including surficial pyrene and that within large, shallow macropores. The second reason was that diffusion and capillary action via rainwater and/or NAPLs within the intra- and inter-particle pore network possibly promoted contaminant transfer from the surface or entrance of macropores into internal micro- and nanopore systems, where the contaminant became unavailable and protected from weathering and bacterial degradation. What remained after years of degradation was contaminant trapped between aggregated particles and within micro- and nanopore networks, which was therefore, unavailable to the degrader community.

This progression was supported by changes in the pyrene fluorescence intensity for specific entities and entire images. When pyrene was freshly spiked the result was a diffuse blue fluorescence covering entire particles with a few bright points of blue to indicate sorption within macropore openings, cracks, and between aggregated particles. Therefore, the diffuse blue color yielded a majority of low fluorescence intensity values. As the contamination aged pyrene progressed through the inter-particle pore network via diffusion and/or capillary action and degraded via biological activity. As a result, the blue pyrene fluorescence concentrated into brighter points within the image, and thus, increased the fluorescence intensity accordingly. Therefore, the gradual transition from freshly spiked to aged PAH sorption could be documented by the increase in fluorescence intensity.

Significant findings for this chapter centered on the ability of fluorescence microscopy: (i) as a rapid, alternative method to identify PAH contamination presence and total concentration; and (ii) to monitor HOC transport and availability with age. In summary, there is great potential in using fluorescence microscopy as a non-invasive, rapid, and straightforward tool to determine in situ contaminant content, fate, and transport.
7.1 SUMMARY OF RESULTS

CHAPTER 3:

- Synthetic clay CEC, N₂ BET surface area, pH, and percent organic matter fell within the range given by two pristine natural soils, PMN and SF.
- PSA values for synthetic clays were within the range given for PMN and SF.
- Pyrene sorption to HA+PG synthetic clay was statistically similar to that within natural soils (P = 0.05 and 0.06 for SF and PMN, respectively).
- When SOM was added to clay, the “synthetic” matrix deviated significantly from the characteristics of blank clay and instead, behaved more like a natural soil in regard to chemical and physical properties and pyrene sorption.
- The synthetic clay had many properties similar to two natural soils and, therefore, was considered a defined, reproducible, and realistic model for studies investigating soil interactions with pyrene.

CHAPTER 4:

- Montmorillonite clay retained 1.7 and 3.5 times more HA and PG, respectively, than hematite.
- Adding 20-30% hematite into the clay system yielded 1.2 times more HA sorption.
- PG sorption was 10.1 and 21.0 times greater than HA for hematite and clay, respectively.
• Freundlich $n$ values for HA or PG sorption to hematite or clay, save for HA on clay, yielded nonlinear sorption isotherms.

• Increasing PG by 50 mg L$^{-1}$ in the HA+PG clays yielded a greater total SOM loading concentration compared to increasing HA by the same amount, where addition of 20% hematite increased the total loaded concentration over that for clay.

• The total SOM content loaded onto clay and hematite was independent of the sequence in which HA and PG were added.

• The amount and type of SOM sorbed to clay influenced soil aggregation, where PG yielded the greatest increase in PSA.

• Generally, PSAs increased as follows: blank mineral phases < HA clay < HA on 80% clay : 20% hematite < PG clay < HA+PG clay.

• Fluorescence intensity linearly increased with greater SOM content as follows: PG clay < HA clay < HA+PG clay.

• Blue HA fluorescence encompassed whole clay particles and suggested that sorption generally occurred along the surface.

• The majority of green PG fluorescence was within pore spaces, cracks, edges, and between aggregated particles.

• There was considerable overlap and interconnection of green PG and blue HA fluorescence on the HA+PG clays, where the greatest density resided within pore spaces, edges, cracks, and between aggregated particles.

CHAPTER 5:

• Pyrene sorption within molecular sieve pores formed bright blue fluorescent dots.
• Pyrene sorption on PG clay was 9.4 times greater than that for HA clay.

• Sorbed pyrene amounts increased as follows: HA clay < HA+PG clay < HA+PG on 80% clay : 20% hematite < PG clay.

• Freundlich \( n \) values for pyrene sorption to HA clay, PG clay, HA+PG clay, and HA+PG on 80% clay : 20% hematite yielded nonlinear sorption isotherms.

• Pyrene sorption to PMN and SF soils was statistically similar to each other (P = 0.587) and that on HA+PG clay, suggesting that pyrene behavior in various soils was relatively standard.

• For HA clays, blue pyrene fluorescence encompassed whole particles and suggested that sorption was primarily surficial.

• For PG clay, HA+PG clay, and natural soil, the majority of pyrene fluorescence emanated from between aggregated particles and within pore spaces, cracks, and edges, with some diffuse surficial blue as well.

• Pyrene fluorescence intensities follow the sorbed HPLC concentrations, where a large sorbed concentration (as determined by HPLC) yielded a large fluorescence intensity.

• “Pseudo-maps” depicting the location of individual SOM components, pyrene, and a mineral phase in relation to each other can be constructed.

• For the synthetic soil “pseudo-maps” a greater percentage of pyrene fluorescence is associated with PG (9.7% and 75.2% for clay and mixed soil, respectively) compared to HA (3.6% and 53.8% for clay and mixed soil, respectively).

• For the natural soil “pseudo-map” a greater percentage of pyrene fluorescence is associated with HS-type components (53.3%) compared to amino sugars (47.4%).
CHAPTER 6:

- Fluorescence intensity could be used as a rapid alternative method to determine PAH presence and general total concentration in field soils.
- A calibration curve could be generated by plotting the amount of sorbed pyrene versus the corresponding fluorescence intensities.
- The curve accurately predicted pyrene concentrations in well-aged, natural soils and had potential for determining other individual and total PAH concentrations therein.
- As pyrene (or PAHs) aged within the MN soils, sorption generally progressed from particle surfaces to inter- and intraparticle spaces in accordance with the dual-mode model.
- Fluorescence intensity increased with soil age, likely from pyrene condensing in pore spaces.

7.2 CONCLUSIONS

PAH sequestration and availability have been investigated for well over two decades. The majority of these studies were limited to arduous, destructive, and potentially hazardous methods (such as HPLC, FTIR, and NMR analyses, or radioisotope probing) that led to much speculation and theorizing with little direct observation. Therefore, studies visualizing the progression of PAH sorption would substantiate (or negate) hypotheses concerning contaminant availability and provide for the development of more effective remediation technology. The research presented in this dissertation highlighted the value of using fluorescence microscopy to study contaminant content, fate, and transport within soil matrices.
The first objective for this research was to determine the ability of synthetic clay to simulate natural soils, particularly the precision with which synthetic clay modeled the chemical, physical, and pyrene sorption properties of natural soils. When SOM was added to clay, the “synthetic” matrix deviated from the characteristics of blank clay and instead, behaved significantly ($P = 0.05$ and $0.06$ for SF and PMN, respectively) like a natural soil in regard to chemical and physical properties, particle aggregation, pyrene sorption and fluorescence intensity. Based on these similarities synthetic clay appeared to be an acceptable surrogate for modeling PAH sorption to natural soils.

Using the synthetic soil described in Chapter 3, the second objective for this work was to determine the impact different SOM constituents, specifically HA and PG, have on soil tertiary structure and how these constituents interacted with each other and the mineral phase. Overall, an increase in the SOM content sorbed to a given solid phase promoted greater fluorescence intensity and particle aggregation. Fluorescence microscopy allowed direct observation of individual SOM components sorbed to clay, where HA primarily yielded a surficial coating of particle grains compared to PG sorption along edges, within pore spaces, and between aggregated particles. Furthermore, when HA and PG were combined the result was a highly inter-connected matrix along the clay surface and within inter- and intraparticle spaces. The significant finding of this work was that PG exerted a major influence on the tertiary structure and fluorescence intensity of synthetic clay. Including this biological constituent with humic substances suggests that SOM properties were the function of a super-mixture composed of different constituents.

Using the synthetic soil discussed in Chapter 4, the objective for the pyrene spiking study was to examine HOC sorption, specifically the impact two different SOM constituents
(HA and PG) had on sorbed pyrene concentrations and location, and compare the results with pyrene sorption to two natural soils. Sorbed pyrene concentrations increased with a general increase in the synthetic soil complexity (such as adding multiple SOM components and/or mineral phases), culminating in statistically similar contaminant sorption upon comparison of HA+PG synthetic clay and two natural soils (P = 0.05 and 0.06 for SF and PMN, respectively). In accordance with results from Chapter 4, it appeared that the pyrene sorption capacity of a soil was contingent on two factors: (i) the type of SOM components present and their respective concentrations, and (ii) the surface area produced by particle aggregation, which was also affected by the SOM content and type. In essence, a greater SOM content increased the soil adhesive factor that promoted particle aggregation, and hence, offered more surface area and a matrix conducive to pyrene sorption. Specifically, PG had the greatest sorption to clay, produced the most particle aggregation, and resulted in the highest sorbed pyrene concentrations.

A second goal for the pyrene spiking study was to visually demonstrate via fluorescence microscopy the location of sorbed pyrene in relation to individual SOM constituents and a given mineral phase. When sorbed to HA clay, pyrene fluorescence was largely diffuse and surficial compared to sorption in PG and HA+PG clays, where bright blue points signify sorption along edges, within pore spaces, and between aggregated particles. Soil “pseudo-maps” for the synthetic soils, generated by overlapping the mineral phase brightfield images and the individual fluorescence images for each SOM component, indicated primary pyrene association with PG (9.7% and 75.2% for clay and mixed soil, respectively), compared with HS (53.3%) for the natural soils. Although fluorescence microscopy was used to observe PAH progression through live plant leaves [99, 100, 101], it
was not attempted previously with soils. Therefore, use of fluorescence microscopy as a novel technique to document *in situ* PAH location and matrix preference was a valuable tool to further knowledge of pollutant content, fate, and transport in soils and could potentially promote more effective remediation technology targeting specific soil constituents, such as PG, or regions, such as pore spaces.

The fourth objective for this research was to demonstrate that fluorescence intensity (combined with a calibration curve) could be used as a quick technique to determine PAH presence and total concentration in a contaminated soil and to preliminarily observe and measure the fate and transport of pyrene over time within a given natural soil. Although only the pyrene concentrations in two aged, creosote-contaminated soils were accurately calculated by a calibration curve (fluorescence intensity versus sorbed pyrene concentration), contaminant analysis via fluorescence microscopy showed promise as a non-destructive and rapid alternative (or supplemental) method to traditional protocols. The combination of fluorescence intensity measurements and a calibration curve has the capability to measure individual and combined contaminant concentrations *in situ* and regardless of whether the compound is free or sorbed, a significant advantage when rapid, on-site evaluations are necessary. Furthermore, the preliminary examination of PAH fate and transport in soil over time demonstrated that PAHs progressed from primarily diffuse surface coverage when freshly spiked, to bright blue points residing within inter- and intraparticle spaces after decades of aging. This finding was significant because it supported the dual-mode model and highlighted the importance of pore spaces as contaminants aged within soil. Therefore, hazardous sites that are years to decades old may require a more stringent remediation technique that targets soil inter- and intraparticle spaces.
Chapters 4 through 6 emphasized the importance of appraising pollutant age and SOM type and concentration present within a contaminated soil matrix during site characterization and prior to developing a remediation strategy. Moreover, fluorescence microscopy has great potential as a technique to evaluate in situ the fate, transport, and concentrations of autofluorescent contaminants within vegetation, soil, and water, compared to traditional extraction and analysis techniques that are arduous, costly, potentially hazardous, and destructive.
8.1 FUTURE RESEARCH

The results of this research demonstrate a need to expound on the use of fluorescence microscopy in fate and transport studies and the influence of individual SOM components within soil matrices. Below is a bulleted list of my suggested experiments.

- The contaminated synthetic and natural soils should be observed under confocal laser scanning microscopy (CLSM). This would potentially provide the researcher with a three-dimensional view of the soil particle surface coverage compared to what a simple fluorescence microscope could illustrate. CLSM may also allow examination of pores and intra-particle spaces, and the HS and PAH sorption phenomena therein.

- Another experiment would involve use of Q-dots, which are small, bright, fluorescent probes with specific excitation and emission wavelengths primarily used for immunohistochemistry stains. Attaching Q-dots to various SOM components and PAHs would allow a greater number of constituents to be visualized concurrently and, thus, generate a more complete soil map. In addition, use of Q-dots in a static equilibration system would permit the researcher to view contaminant progression more clearly, as autofluorescence is only bright at medium to high concentrations.
• Glomalin, a ubiquitous glycoprotein coating hyphae of arbuscular mycorrhizal fungi, is rapidly becoming known for its profound impact on particle aggregation. Therefore, perhaps this biological constituent should be evaluated for its impact on contaminant sorption and compare the results to those found for PG.

8.2 IMPROVEMENTS

As with any project, reflection is a prime means of improvement. Therefore, the following is a list of improvements that may greatly assist those embarking on the heels of my research.

• The entire scale of the flow cell should be increased, as this would decrease the amount of clogging within the system.

• The flow cell should be made using a square-welled microscope slide. This would yield a greater surface area to observe flow, make sample preparation easier, and eliminate fluorescence spreading, caused by the curvature of the capillary tube. The accompanying coverslip could either be super-glued or wax-sealed to the slide well; therefore, it can be removed, and the slide re-used.

• To prevent clogging of the flow cell exit tubing and formation of buffer eddies within the entry tubing, TEM screens could be inserted. This would also keep the fluorescence detector from becoming clogged by particles dislodging from the flow cell.

• Remove as many junctions in the flow cell system as possible as these are a common source of leaks.

• Install a factory-built camera shutter or amend the current version to operate more accurately with the Microfire fluorescence microscope software.
REFERENCES


