"Humic Coverage Index" as a Determining Factor Governing Strain-Specific Hydrocarbon Availability to Contaminant-Degrading Bacteria in Soils

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We report development of a novel parameter for quantifying the amount of humic and fulvic acids per unit surface area in a particular soil. This quantity, the "humic coverage index" (HCI), provides a measurement of the relative spatial extents and/or thicknesses of the humic/fulvic overlayers in different soils, and, therefore, can be used in modeling various soils' behavior in sequestration processes in which humic materials are involved. HCI is herein applied to modeling biodegradation of aromatic and aliphatic hydrocarbons (phenanthrene, pyrene, and hexadecane) by several bacterial strains. Results indicate that, for the cases studies here, contaminant biodegradation is highest at a particular HCI and decreases if the coverage density of humic material is lower or higher than this optimum value. The HCI value at which maximal degradation was observed varied across different strains (indicating strain-specific differences in ability to degrade contaminants sorbed to humic materials) and, to a lesser extent, across different contaminants. The HCI concept is also demonstrated to be useful in explaining soil-, strain-, and contaminant-specific variations in the ability of fulvic acid supplementation to enhance contaminant biodegradation. Finally, we show that, in general, strains which are comparatively better at degrading contaminants in high-HCI soils also show enhanced contaminant mineralization in vitro in the presence of humic acids, such as when hydrocarbons are adsorbed onto these materials.

Introduction

There has recently been considerable interest in ascertaining how soil physicochemical properties affect contaminant bioavailability and biodegradability. An understanding of sequestration processes and mechanisms is important for two reasons. First, a better grasp of these processes would help to understand why the success rates of bioremediation applications are often highly variable in different soils and should contribute to more successful site-remediation applications. Conversely, substantial interest in sequestration derives from its anticipated impact on the risk-assessment paradigms and strategies which are used to trigger cleanup of contaminated sites. With respect to the latter of these, the argument which has been advanced (*1*−*3*) is that sequestered contaminants, by virtue of their reduced bioavailability, pose a diminished risk to human health or to ecoreceptors. If true, this may mean that remediation of these particular sites is unnecessary and may provide minimal ecological, health, or safety benefits.

Soil-dependent reductions in bioavailability of organic chemicals were first reported in the late 1950s; most published reports at this time dealt with decreased efficacy of soil-applied herbicides. For instance, simazine was found to be much less effective in soils with high organic matter (OM) contents (*4*−*6*). Other researchers observed similar trends with 2,4-D, diuron, and several carbamates (*7*) and showed that the magnitude of this effect was highly correlated with soil OM content. Thus, most early models of soil-dependent bioavailability reduction focused on the role of soil OM and proposed sorption on (*8*) and dissolution in (*9*) SOM as the primary salient mechanisms. Later models then proposed as a second mechanism diffusion into three-dimensional micropores of soil particles themselves (*10*).

The most recent sequestration models recognize the likely involvement of both mechanisms. Foremost among these models are those of Weber (*11*, *12*) and Pignatello (*13*). Each of these authors has proposed a two-site model to describe the suite of interactions between hydrophobic contaminants and soil particles; in each of these models, an important distinction is drawn between two types of OM. The first of these, comprised of humic and fulvic acids (HA, FA) bound or complexed at the surface of soil particles, has been described as a porous, flexible, lipophilic "rubbery" (*13*) or "soft" (*11*) state. Some authors, in fact, suggest that the gel-like, colloidal nature of this phase may confer an "indistinct" surface to soil particles on which it is prevalent (*13*). This is in contrast to the second type of OM—the rigid, inflexible, "hard" (*11*), or "glassy" (*13*) humin, which predominates in the cores of soil particles. This has been described as providing the "internal surface" of a soil particle (*14*), rendered partially inaccessible to the bulk solution phase by overlayers of HA and FA. According to the two-site models, the overall process of sequestration consists both of dissolution of contaminant-(s) into the "rubbery" HA/FA phase which coats the outer surface of soil particles and entrapment within the "glassy" humin phase's micropores. It seems logical to hypothesize that the temporal progression of the competing processes probably depends on the relative dominances of the two OM types. In other words, soils which contain almost all of their OM as "hard" humin will probably be dominated almost immediately by porosity-mediated sequestration mechanisms, while these may be greatly delayed in soils with high relative contents of "soft" HA and FA. This hypothesis was supported by our recent results with regard to Fenton's reagent-based chemical oxidation of polycyclic aromatic hydrocarbons (PAH) in various model soils (*15*); these experiments showed that porosity-mediated sequestration was particularly important in low-OM soils and less so as OM content increased.

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We also studied bacterial PAH mineralization in these same soils and showed (*16*) that sequestration processes were primarily dependent upon soil OM content. However, for short soil/contaminant contact times (40 days), we found very little evidence for any effect of soil physicochemical differences on contaminant biodegradation. For example, despite examining soils which spanned a 10-fold OM range (2.4%−24.3%), we found PAH mineralization totals which varied at most by a factor of 2 and rarely by that much. In both of these regards, our results corroborated those of another recently published study (*17*). These results did, however, contradict other studies which implicated nanoporosity (*15*, *18*) and humin content (*19*) as being important in the sequestration process. One possible explanation for this apparent contradiction is that our earlier work focused on *Mycobacterium austroafricanum* GTI-23 (*20*) as a test strain. It may be that Mycobacteria, by virtue of their extremely lipophilicity, are atypical in their abilities to degrade sorbed contaminants when compared to other soil microbes. We therefore extended our biodegradation experiments to encompass a range of other isolates. On one hand, we hoped that this approach might provide a more-sensitive assay to better discern the effect of different soil properties on the degradation process. Second, we wanted to assess the extent to which *M. austroafricanum* might or might not represent the behavior of the broader range of hydrocarbon-degrading bacteria.

Materials and Methods

Chemicals. Phenanthrene (98%) and pyrene were obtained from Aldrich Chemical Co. (Milwaukee, WI). *n*-Hexadecane (99%+) was from Sigma Chemical (St. Louis, MO), as were 9-^{14}C-phenanthrene (46.9 mCi/mmol), 4,5,9,10-^{14}C-pyrene (58.7 mCi/mmol), and 1-^{14}C-hexadecane (2.2 mCi/mmol). Minnesota peat fulvic acid and humic acid standards were purchased from the International Humic Substances Society (St. Paul, MN).

Bacteria. Strains GTI-1 and GTI-2 (*Burkholderia* spp.) and GTI-7 and GTI-9 (*Sphingomonas* spp.) have been previously reported (*21*), as has *Mycobacterium austroafricanum* GTI-23 (*20*). Strains GTI-6 and GTI-22 were similarly isolated from manufactured gas plant (MGP) site soils and were identified, based on 16s rRNA gene sequencing (MIDI Labs, Newark, DE) as *Pseudomonas veronii* (100% sequence identity) and *Pseudomonas stutzeri* (99+%), respectively. *Mycobacterium vanbaalenii* PYR-1 (*22*, *23*) was kindly provided by Dr. Carl Cerniglia. A hexadecane- and dodecane-degrading strain of *Acinetobacter* (MVC Dod3) was isolated from soils collected at a second location at the same chronically crude oil-contaminated site described previously (*24*). This isolate, based on 16s rDNA sequences in the GenBank database, is most closely related (96% identity) to *A. calcoaceticus*.

Soils − Collection and Characterization. Six model soils were collected from noncontaminated grassy or wooded areas in the Northwestern Greater Chicago area. Total organic carbon (TOC) contents of the soils were measured as per ASTM Method D2974-87. Soil OM was fractionated into humic acids (HA), fulvic acids (FA), and humin as previously reported (*16*). All solid samples (whole soils, HA, humin) were measured using a Shimadzu TOC-V/SSM-5000A analyzer; aqueous FA was determined using a Shimadzu TOC-500 analyzer.

N_2 Adsorption Characterization of Soils. High-resolution nitrogen adsorption−desorption isotherms were measured at 77.4 K using a Quantachrome Instruments Autosorb-1C. Before the measurements, the soil samples were outgassed at 120 °C for 24 h. Determination of the void volume was performed with helium using standard procedures. Low-pressure data were corrected on thermal transpiration effect according to standard procedures. Specific surface areas of soils were assessed by the BET method applied in the region of relative pressures from $p/p_0 = 0.05-0.3$ (*25*).

Mineralization of Contaminants in Soil. Mineralization experiments were conducted as previously described (*26*). Soil (9 g dry wt), in 30-mL crimp-top bottles, received 1.8 mg per bottle ($\therefore$200 ppm) of hydrocarbon (phenanthrene, pyrene, or hexadecane), containing 50 000−80 000 dpm ^{14}C, in 4 mL of methylene chloride; solvent was allowed to evaporate overnight in a fume hood. Soils were then moistened (2 mL of water), and bottles were sealed and sterilized by exposure to a gamma-emitting ^{60}Co source, with an exposure time of approximately 4 h (resulting in a total applied dose of 30−40 kGy). Spiked, sterile bottles were inoculated upon return of the soils from sterilization; hence, approximately 72 h elapsed between spiking/watering of soils and the actual inoculation. To inoculate bottles, bacteria were collected from R2A agar plates (which had been subcultured 1 week earlier from plates which were actively degrading the hydrocarbon of interest) in a small volume (2−3 mL) of mineral media (*21*, *26*). This was adjusted to an A_{600} of 1.2 (± 0.1); one mL of this suspension was then used to inoculate each bottle of soil.

A second series of biodegradation experiments was conducted with soils that were amended with fulvic acid prior to hydrocarbon spiking. The soils used in these experiments, soils #1, 5, and 2, were chosen because they had the lowest TOC levels of the six; it was thus reasoned that they would be most likely to show effects of exogenously added FA. Two-gram quantities of sterile soil were weighed into 30-mL serum bottles; fulvic acid was dissolved in deionized water (1 mL/bottle), filter-sterilized (0.22-μm filter), and added to give a final concentration of either 10 or 20 mg of FA per 2 g of soil. The bottles containing FA-amended soils were allowed to equilibrate for approximately 72 h in a sterile transfer hood, at which time hydrocarbon (1000 ppm total concentration, ≈90 000 dpm ^{14}C per bottle) was added in 1 mL of methylene chloride, which was allowed to evaporate overnight. Spiked hydrocarbons were allowed to age (in the dark) in the soil for 14 days; inoculation and tracking of $^{14}CO_2$ release were done as described above. Soil bottles were kept in the dark to minimize the potential for photochemical hydrocarbon degradation.

Effect of Humic Acid on Bacterial Mineralization of Phenanthrene and Hexadecane. Mineralization bottles were prepared as above; instead of soil, each bottle received 5 mL of R2A agar (Difco, Detroit, MI). Hydrocarbon mineralization was evaluated in the presence or absence of HA. For the no-HA bottles, 8 μg of phenanthrene (or hexadecane), along with 30 000−50 000 dpm of ^{14}C-hydrocarbon, was dissolved in 1 mL of acetone and spread evenly over the surface of the solidified agar. Once the acetone had evaporated, 200 μL of bacterial suspension ($A_{600} = 1.5 \pm 0.2$ in MSM) was added. Triplicate treatments were done for each of eight phenanthrene-degrading cultures (*Burkholderia* spp. GTI-1 and GTI-2, *Sphingomonas* spp. GTI-7 and GTI-10, *Pseudomonas* spp. GTI-6 and GTI-22, *Mycobacterium austroafricanum* GTI-23, and *M. vanbaalenii* PYR-1) and one hexadecane degrader (*Acinetobacter* sp. MVC Dod3).

Mineralization of HA-sorbed hydrocarbon by the same bacteria was assessed as follows. Aliquots of 8 mg of HA were weighed out; each of these was spiked with an amount of hydrocarbon (including ^{14}C label) equal to that used above (8 μg in 1 mL of acetone). Following spiking, HA was allowed to "age" in the dark for 14 days prior to being transferred to gel bottles and inoculated as described above. Bottles were stored in the dark throughout the experiment.

Statistical Analysis. All statistical analyses were performed using the Analysis ToolPak add-in for Microsoft Excel. The "paired two-sample for means" *t*-test (two-tail) was used to determine values of *p* for comparisons among data sets.

TABLE 1. Total Organic Carbon (TOC), Humic Acid (HA), and Fulvic Acid (FA) Contents (as Determined from SOM Fractionations) for Each Soil, as Previously Reported (Bogan and Sullivan, Submitted)[a]

soil	TOC[b] (%)	HA[b] (%)	FA[b] (%)	BET surface area (m^2/g)	total pore volume[c] (mL/g)	BET *C* constant	HCI (mg/m^2)	*h* (nm)
1	2.32	0.14	0.01	30.3	0.088	95	0.05	0.05
2	5.78	0.71	0.05	24.5	0.056	126	0.31	0.31
3	11.16	2.18	0.16	4.92	0.024	43	4.76	4.81
4	24.28	3.13	0.97	3.03	0.017	22	13.5	13.9
5	3.58	0.23	0.03	23.2	0.060	72	0.11	0.11
6	9.13	1.69	0.10	15.4	0.042	51	1.16	1.18

[a] Also included are BET specific surface areas, pore volumes, and energetic *C* constants, as measured by N_2 adsorption isotherms. HCI is the humic coverage index; *h* is the estimated equivalent thickness of the humic/fulvic overlayer (assuming uniform coverage). [b] Expressed as a mass percentage of the whole soil. [c] Estimated from the amount adsorbed at the relative pressure $p/p_0 = 0.99$.

Results

Soil Characteristics. Table 1 presents various characteristics of the six soils used in this work. The soils spanned a wide range of OM contents, from 2.32% (soil #1) to 24.28% (soil #4). Other significant differences became evident when the organic matter in the soils was fractionated. Soils #1 and #5 were markedly lower in summed HA+FA (6.5% and 7.1% of OM, respectively) than the other four soils, which contained between 13.2% (soil #2) and 20.9% (soil #3) of these constituents. The humic material in soil #4 was highly enriched in FA (4.0% of OM, or approximately 1% of the total soil mass). In contrast, none of the other soils contained > 0.16% FA by mass.

Soil pore volumes and surface areas, measured by the N_2-adsorption BET method, also spanned a wide range of values; upon closer inspection, these quantities were found to both be highly inversely correlated (increasing pore volume and surface area with decreasing OM) with OM content for five of the soils (data not shown). The exception to the linear correlation was soil #4, which displayed higher-than-expected surface area and pore volume.

Surface-Area Determinations and Derivation of "Humic Coverage Index". The specific surface areas of the different soils, determined by the BET method of N_2 adsorption, can be considered an estimate of the surface area contributed by the overlayer of HA and FA, plus any exposed surface of humin/mineral core particles. This assumption is justified because N_2 at 77 K cannot penetrate into narrow micropores, especially those which are "buried" by the HA/FA overlayer (*14*). Microporosity in the low-OM samples was evaluated from the comparison plots (not shown) using the standard isotherm on nonporous silica surface (*27*). No appreciable amount of micropores was found; the surface areas determined from the comparison plot method were close to the BET surface areas.

Table 1 also presents calculated "humic coverage indices," or HCIs, for each soil. In each case, this number is derived from the summed HA and FA contents of the soil (expressed on a mass basis), divided by the BET surface area. The resultant quantity, with units of mg/m^2, provides an expression of how much HA+FA coats the surfaces of the core soil particles. HCI can be conceptualized as combining the extent and thickness of the humic/fulvic acid overlayer. Assuming spherical particles and a 1 g/cm^3 density for the HA/FA overlayer, the amount of HA/FA required for monolayer coverage of all particles is estimated to be approximately 1 nm (or 1 mg/m^2). It follows, therefore, that soils #1, #2, and #5 do not have sufficient HA+FA content to fully cover their core-particle surfaces, even as a monolayer, as their HCI values are well below this threshold. In fact, soils #1 (HCI = 0.05 mg/m^2) and #5 (0.11 mg/m^2) will have, at most, approximately 15% of their total surface areas covered by HA and FA. In contrast, soils #3 (4.76 mg/m^2) and #4 (13.5 mg/m^2) are likely to be covered by an extensive humic layer, sufficient to coat the core particle surfaces to a great enough extent that N_2 access to any micropores which may be present is not possible. Soil #6 exhibits intermediate behavior; coverage is close to that which could provide a uniform monolayer. The BET energy constant *C* can be used as a one-parameter estimate of the energy of adsorption. This quantity is known to decrease as the mineral or clay surfaces are covered by organic overlayers (*28*, *29*). Our results are in agreement with this trend (Table 1). The only exception is soil #2, which exhibits somewhat higher *C* constant (*C* = 126), which may indicate an increased surface roughness.

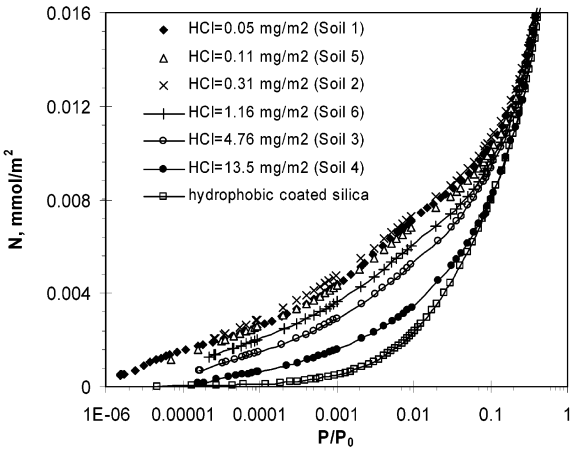

FIGURE 1. High-resolution N_2 adsorption isotherms on soils with various HCI, reduced by the BET surface areas. Adsorption isotherm on hydrophobic coated silica is shown for comparison (*29*).

High-resolution (low-pressure) adsorption (Figure 1) is a more sensitive method to study energetics of gas adsorption than the BET method, which is limited to the range of relative pressures above $p/p_0 = 0.03-0.05$. For soils with different HCI, adsorption isotherms expressed as the amount adsorbed per unit area show important differences in the low-pressure region. Adsorption per unit area decreases as the degree of humic coverage increases. For comparison we also plotted the isotherm on strongly hydrophobic surface prepared via chemical bonding of octyldimethylsilyl (ODMS) ligands to a silica surface (*28*). For each of the soils, N_2 exhibits substantially higher energy of adsorption than for ODMS. This fact may reflect a higher energy of interactions of N_2 with humic substances than with ODMS as well as nonuniformity of the humic coverage even for high-HCI soils.

Contaminant Mineralization. Relationships between the cumulative extents of mineralization of various hydrocarbons by the different isolates used in this work and HCI are shown in Figure 2. *Mycobacterium austroafricanum* GTI-23 was able to mineralize the most PAH (both pyrene and phenanthrene) and was generally less variable across the suite of soils. Some small exceptions to this overall pattern were seen. For example, GTI-23 mineralized approximately 30% less phenanthrene in the most humic-rich soil (soil #4, HCI = 13.5 mg/m^2) than in the four other soils in which it was tested; differences among these four were statistically insignificant ($p > 0.67$). In the case of pyrene degradation by GTI-23 (Figure 2D), mineralization was highest in soils with HCI values of 0.3 and 4.8 mg/m^2 (soils 2 and 3); degradation in soil #6, which was intermediate between these two (1.16 mg/m^2) with respect to HCI, was somewhat lower than expected. As HCI increased or decreased from this range, mineralization

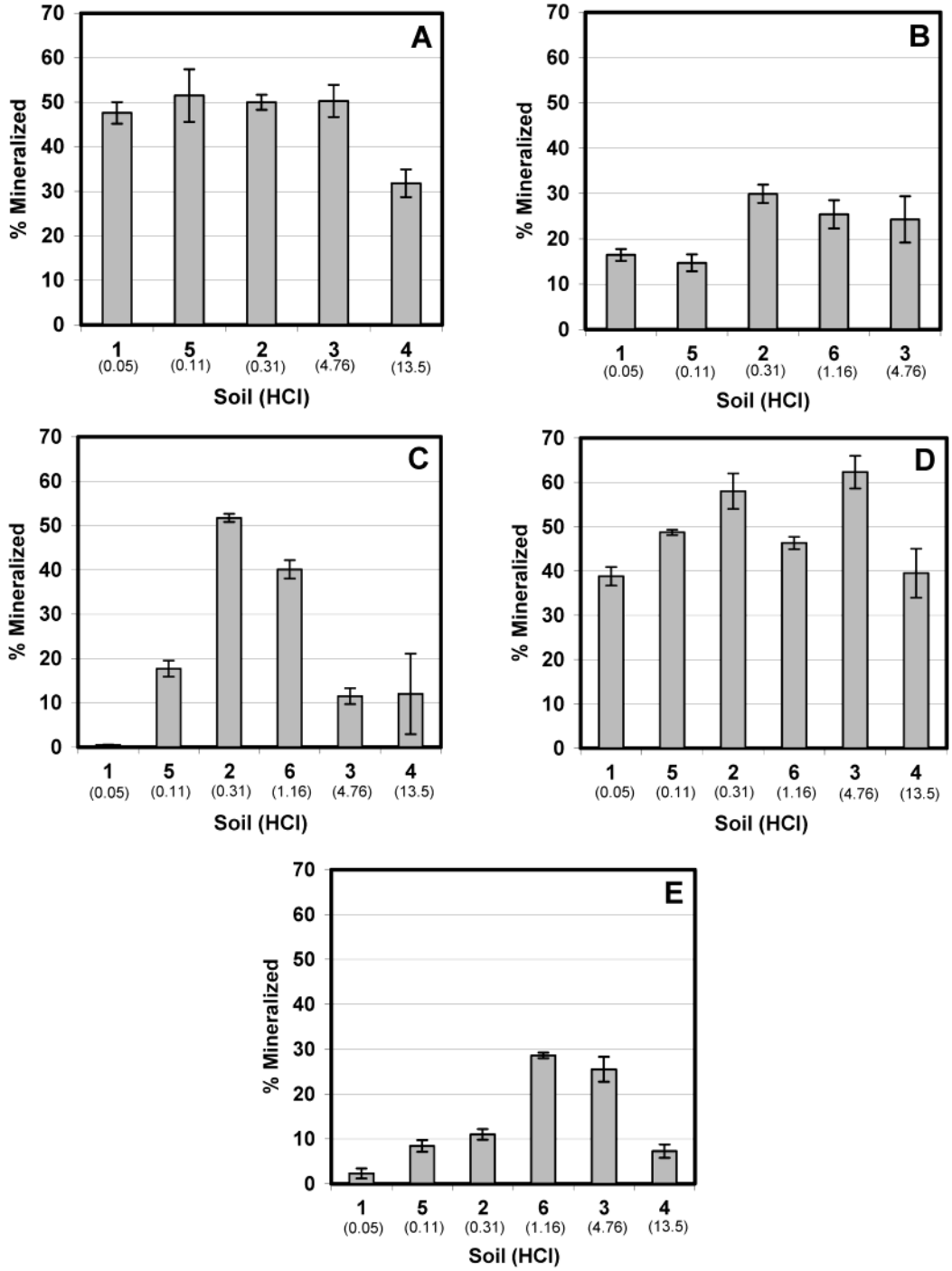

FIGURE 2. Hydrocarbon mineralization by various isolates as a function of soil HCl: (A) phenanthrene (*M. austroafricanum* GTI-23), (B) phenanthrene (*Burkholderia* sp. GTI-1), (C) phenanthrene (*Burkholderia* sp. GTI-2), (D) pyrene (*M. austroafricanum* GTI-23), and (E) hexadecane (*Acinetobacter* sp. MVC Dod3). All data reflect percentages of contaminant mineralized (initial concentration = 200 ppm) and are means and standard deviations of duplicate (A, B, C, E) or triplicate (D) cultures.

efficiency decreased somewhat, although the maximal mineralization decrease (in soil #1) was only about 37% (39% mineralized vs 62%). In comparison, most other strains showed marked preferences for a much smaller subset (1 or 2) of the soils. For example, *Burkholderia* sp. strain GTI-2 (Figure 2C) mineralized considerably more phenanthrene in soils with an HCI of 0.3−1.2 mg/m^2 (soils #2 and #6). Similarly, $^{14}CO_2$ evolution from hexadecane by *Acinetobacter* sp. MVC Dod3 (Figure 2E) was markedly higher in soils with HCI between 1.2 and 4.8 mg/m^2 (soils #3 and #6). In both of these cases, highly significant ($p < 0.07$) decreases in mineralization occurred if the soil HCI was higher or lower than this "preferred" range.

Effects of Fulvic Acid Supplementation. Supplementation of soils with fulvic acids prior to contaminant spiking was done as a means of modifying the HA/FA overlayer without changing other physicochemical aspects of the soil (e.g. humin level and composition, overall porosity). Results for these experiments (Table 2) showed that, for *M. austroafricanum*, effects of FA supplementation varied depending on the soil and contaminant, all in ways that can be explained and/or predicted using the HCI model and the data presented in Figure 2.

We previously showed (*16*) that addition of FA (1% by mass) to soil #1 (HCI = 0.05 mg/m^2) dramatically (almost 2.5-fold) increased mineralization of pyrene spiked into this

TABLE 2. Effects of Fulvic Acid Addition on the Mineralization of Pyrene (Soils #1, #5, and #2) and Phenanthrene (Soil #1) by *Mycobacterium austroafricanum* GTI-23[a]

soil no.	0% FA	0.5% FA	1% FA
	Pyrene		
1	12.4 ± 2.1[b]	29.6 ± 3.7[b]	29.6 ± 3.0[b]
5	40.2 ± 8.0	39.1 ± 4.4	38.7 ± 1.1
2	34.1 ± 5.0	36.5 ± 3.3	34.5 ± 4.2
	Phenanthrene		
soil	**0% FA**	**0.5% FA**	**1% FA**
1	28.7 ± 3.3	30.0 ± 3.4	31.0 ± 2.9

[a] In all cases, data report cumulative $^{14}CO_2$ release over 4 weeks and represent means and standard deviations of triplicate cultures. [b] Results are as previously reported (Bogan and Sullivan, submitted).

TABLE 3. Effects of Fulvic Acid Addition on the Hexadecane Mineralization by *Acinetobacter* sp. MVC Dod3 in Soils #1, #5, and #2[a]

soil no.	0% FA	0.5% FA	1% FA
1	1.41 ± 0.16	1.70 ± 0.19	1.68 ± 0.02
5	2.45 ± 0.27	3.45 ± 0.68	4.83 ± 0.89
2	2.72 ± 0.16	3.59 ± 0.88	7.35 ± 1.20

[a] Data report cumulative $^{14}CO_2$ release over 5 weeks and represent means and standard deviations of triplicate cultures.

soil following FA addition ($p = 0.04$). In contrast, when this experiment was run with soils #5 and #2 (the second- and third-lowest OM soils, with HCI values of 0.11 and 0.31 mg/m^2, respectively), FA addition had essentially no effect (Table 2). Similarly, when phenanthrene mineralization by *M. austroafricanum* was examined in soil #1, addition of FA had no statistically significant impact (Table 2). Although seemingly contradictory, these results are, in fact, all in agreement with the HCI-based results shown in Figure 2. For example, pyrene mineralization by *M. austroafricanum* was lowest in soils with HCI < 0.1 mg/m^2 (soil #1) or > 13 mg/m^2 (soil #4). By contrast, both soil #5 and #2 already display essentially maximal mineralization, indicating that they are at or close to the "optimal" HCI value. It is therefore not surprising that addition of more humic material to these soils did not increase pyrene mineralization. Similarly, with the exception of soil #4, all of the model soils— including #1—displayed roughly the same phenanthrene mineralization (Figure 2A), regardless of HCI. This explains the failure of FA supplementation to increase mineralization of this PAH in soil #1 (Table 2), whereas a similar treatment significantly enhanced pyrene mineralization.

The HCI approach shows similar utility in explaining the effects of FA supplementation on hexadecane mineralization by *Acinetobacter* sp. MVC Dod3. As seen in Table 3, addition of 0.5% or 1% FA to soil #1 resulted in a ≈19%, statistically insignificant ($p = 0.17$) increase in hexadecane mineralization by this strain, whereas $^{14}CO_2$ evolution by the same isolate cultured in soils #5 and #2 increased by approximately 97% ($p = 0.09$) and 170%, ($p = 0.04$), respectively, when supplemented with 1% FA. The effects of these FA-supplementation levels on the HCI of these soils is as follows (all values are mg/m^2):

	0% FA	0.5% FA	1.0% FA
soil #1	0.05	0.21	0.38
soil #2	0.31	0.51	0.72
soil #5	0.11	0.33	0.54

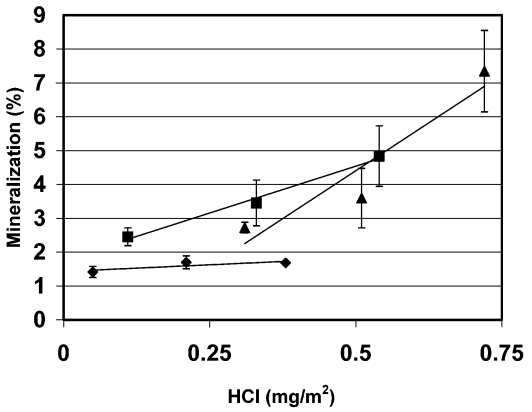

FIGURE 3. FA-dependent increases in *Acinetobacter* hexadecane mineralization as a function of HCI. Data points denoted by "◆" indicate soil #1 with 0%, 0.5%, and 1% fulvic acid supplementation levels, while "■" represents the corresponding treatments for soil #5, and "▲" stands for soil #2. All data are derived from triplicate cultures of each condition.

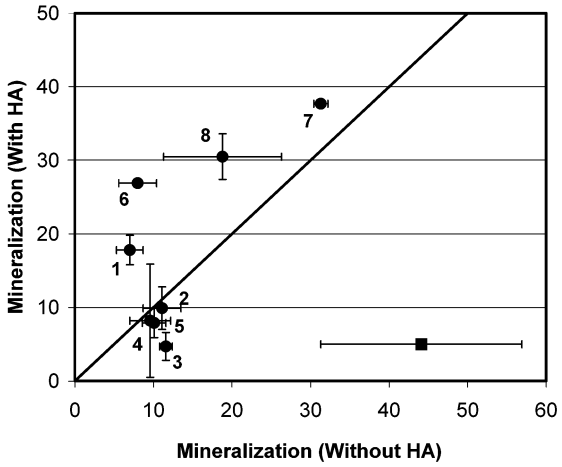

FIGURE 4. Relative abilities of various bacterial isolates to mineralize "free" hydrocarbons and those adsorbed onto Minnesota peat humic acid. Solid diagonal indicates no effect of HA (a 1:1 ratio of mineralization totals under the two conditions). Data points denoted by "●" represent phenanthrene mineralization by (1) *Burkholderia* sp. GTI-1, (2) *Burkholderia* sp. GTI-2, (3) *Pseudomonas veronii* GTI-6, (4) *Pseudomonas stutzeri* GTI-22, (5) *Sphingomonas sp.* GTI-7, (6) *Sphingomonas sp.* GTI-9, (7) *Mycobacterium austroafricanum* GTI-23, and (8) *Mycobacterium vanbaalenii* PYR-1. "■" is hexadecane mineralization by *Acinetobacter* sp. MVC Dod3.

Note that, in this experiment, the starting hexadecane concentration was 1000 ppm, 5-fold higher than that in the initial (Table 1) mineralization studies. This thus explains the apparent discrepancy in mineralization extents between the two experiments; the *masses* of hexadecane mineralized in the two rounds of experiments were similar. Figure 3 graphically depicts hexadecane mineralization versus HCI for the FA-supplemented soils and clearly shows that the closer the starting soil was to the HCI value at which *Acinetobacter* displayed maximal hexadecane mineralization (ca. 1−1.5 mg/m^2—see Figure 2E), the greater was the magnitude of the FA-dependent mineralization increase.

Mineralization of HA-Bound Hydrocarbons. Results of in vitro experiments, in which mineralization of isolated hydrocarbon was compared to that seen when the hydrocarbon had been previously sorbed onto HA, are shown in Figure 4. These results indicated that the various bacteria could be divided into three categories. The only strain in which hydrocarbon mineralization was negatively impacted

by HA sorption was *Acinetobacter* sp. MVC Dod3 (hexadecane). In contrast, *Burkholderia* GTI-1, *Sphingomonas* GTI-9, and both *Mycobacterium* isolates mineralized more phenanthrene when HA was present. The two *Pseudomonas* isolates and the other *Burkholderia* (GTI-2) and *Sphingomonas* (GTI-7) strains were unaffected by HA. Our *Mycobacterium* results corroborate those of other authors, who have reported enhanced pyrene catabolism (measured as bacterial growth and proliferation) among several *Mycobacterium* species (*30*, H.-Y. Holman, personal communication) grown in the presence of HA-complexed PAH. Furthermore, growth of the pathogenic *M. avium* in swamp water was greatly enhanced by addition of humic or fulvic acids (*31*); similar effects have been reported for at least one alkane-utilizing *Mycobacterium* strain (*32*).

Discussion

In contrast to our previous *Mycobacterium* results (*16*), the experiments described herein resulted in a range of mineralization extents, even after only short soil/contaminant contact times. This has allowed us to propose a parameter based on the relative extents to which the HA/FA overlayers present in each soil cover the mineral/humin cores of that soil's particles. We refer to this quantity as the "humic coverage index" or "HCI". Such a parameter is consistent with, and justified by, current soil-particle models; however, to the best of our knowledge, it has not been described or proposed to date.

Current conceptual models of contaminant bioavailability and sequestration (*11*−*13*) recognize the 2-fold importance of the layer of organic material (mainly HA and FA) which coats soil particles and constitutes the interface with the aqueous phase. Sequestration has been divided into two phases, which may or may not be temporally separated. In the first, contaminant adsorbs onto humic material; clearly, the speed and extent of this process will be dictated by the spatial extent of the humic layer as well as by the nature of the contaminant (e.g. hydrophobicity). The second phase of sequestration involves gradual contaminant migration into micropores in the soil particle itself. Gas-adsorption data (*13*) argue that these pores can be "buried" within and/or under a "soft" layer of OM and are likely located within the "hard" carbon of the humin/mineral soil-particle core (and/or the mineral portion itself). The solution-phase analogue of hard-carbon micropores, ordered rigid HA "pseudomicelles", have been shown to be important in sequestering hydrophobic molecules (*33*, *34*). It may be expected that this latter component of the sequestration process would depend on the thickness of the HA/FA layer, although our previously reported results (*15*) also argue for significant contaminant-specific differences. For example, we noted that smaller, less hydrophobic PAHs appeared to cross the HA/FA layer and enter buried humin micropores more quickly than higher-molecular-weight polycyclics. To our knowledge, the present paper is the first to attempt to quantify the HA/FA overlayer in this way as part of a model of contaminant bioavailability and sequestration.

Previous studies (*17*) which attempted to use bacterial hydrocarbon (phenanthrene) mineralization to identify important soil characteristics involved in sequestration concluded that soil organic content was the most important factor, with several other parameters (CEC, % silt, Hg-accessible pore volume)—some or all of which may be expected to correlate with OM—also showing good correlation. One key difference between these studies and ours was our inclusion of several different bacterial strains. Chung and Alexander (*17*, *35*) used a strain (P5-2) which, in fact, displayed relatively small differences in its ability to mineralize phenanthrene across a wide suite of soils. In soils with OM contents spanning 0.07−11%, phenanthrene mineralization (even after 200 days of aging) varied less than 2-fold (28%−50%). However, P5-2 was originally isolated (*36*) specifically for its unusually high ability to catabolize PAHs sorbed to a highly hydrophobic surface (polyacrylic beads). Thus, P5-2 is comparable to the *Mycobacterium austroafricanum* strain included herein, which frequently displayed no clear optimal HCI value. This type of strain might have some use from the standpoint of risk assessment, in that it may be taken to represent a highly conservative "worst case" for bioavailability, approximating availability to receptor organisms which can take up even strongly sorbed contaminants. However, these strains are clearly of limited use in discerning effects of individual soil parameters on bioavailability.

Our results indicate that individual bacterial strains differ with regard to which HCI values will support maximal contaminant biodegradation. At suboptimal HCI values (soils with minimal HA and FA overlying their mineral/humin cores), migration of contaminants into narrow micropores is most likely the primary determinant of bioavailability and occurs more rapidly (*15*). In our model, this occurs because these soils' sparse, thin HA/FA layers are unable to appreciably retard entrance of contaminant into micropores. Above the "optimal" HCI value for a given bacteria, degradation becomes hindered by the increasing strength of contaminant adsorption and/or dissolution into the "soft" flexible HA/FA gel layer.

Our data strongly support at least one conclusion which may, at first, seem counterintuitive, namely, that a given soil can have an HCI which is "optimal" for degradation of one contaminant by a particular strain of bacteria, but in which degradation of other compounds by the same bacterium is less-than-maximal. Such is apparently the case with *M. austroafricanum* in the very low-HCI soils, in which phenanthrene degradation is, statistically speaking, as high as in any of the soils, but in which pyrene degradation is considerably inhibited. Mechanistically, the reason for this is not completely clear. It is known that the strengths of interactions between contaminants and HAs are not all the same, for example, more-hydrophobic compounds will display a higher affinity for a given HA (*37*). Second, the kinetics of sequestration (and of release of sequestered contaminants) vary from one contaminant to another within a particular soil (*15*); for example, smaller molecules may more rapidly enter soil micropores and may be less restricted from diffusing back out of these pores. These factors, either alone or in combination, could lead to any given soil providing an "optimal" environment for degradation of one contaminant by a particular bacterium, while being considerably less so for another contaminant with that same organism.

We also show that the HCI concept helps to explain seemingly contradictory results which we and others have observed for the ability of humic and/or fulvic acid supplementation to enhance bacterial hydrocarbon mineralization in different soils. Addition of HA stimulated PCB biodegradation in artificially spiked soil treated in slurry mode (*38*) and pyrene mineralization in solid-phase systems (*39*). These latter authors (*39*) found that, while supplementation with low levels of HA had a stimulatory effect on pyrene mineralization, high doses of HA inhibited the biodegradation process. Although they speculated that this might be due to adverse pH effects of high HA-addition rates, their data does not support this, as pH decreases did not become evident until considerably higher levels of HA supplementation than those which impeded pyrene mineralization. Our results validate and begin to explain these apparent contradictions, as we were able to observe each of these outcomes in our experiments and fit them into the HCI model. For example, FA supplementation only increased pyrene mineralization by *M. austroafricanum* in soil #1, which, among the low-HCI

soils, most inhibited pyrene mineralization; other soils (#5 and #2) were much closer to (or within) the HCI range which already supported maximal degradation. In addition, because this soil was already within the broad "optimal" range of HCIs for phenanthrene degradation by this strain, FA addition did not stimulate this process at all. Similarly, the HCI concept was also useful in predicting the relative magnitudes of the effects which FA addition to three low-OC soils would have on hexadecane mineralization by a strain of *Acinetobacter*.

On the whole, we conclude that HA or FA supplementation into soils which are already above the "optimal" HCI for the relevant microorganism/contaminant combinations will necessarily fail to enhance contaminant removal. In fact, depending on the ability of the microorganism(s) in question to degrade contaminant at HA/FA levels above their "preferred" HCI, addition of humic material may impede the biodegradation process. For example, it is clear based on the data in Figure 2C that addition of humic materials to a soil with HCI $\geq$ 1.2 mg/m^2 would almost definitely reduce *Burkholderia* GTI-2's phenanthrene mineralization. In contrast, bacteria in soils with suboptimal HCIs may be much more amenable to stimulation by humic supplementation. The magnitude of stimulatory effects may vary significantly from soil to soil, as in the case of *Acinetobacter* hexadecane degradation, depending on the exact slope of the relationship between HCI and contaminant degradability.

Hydrocarbon degradation in the nine strains which we have included in this work is influenced in several different ways by HA (enhanced in some strains, unaffected or inhibited in others). Strains with broad optimal HCI values and/or reduced effects of high HCI (e.g. *M. austroafricanum* GTI-23 and *Burkholderia* GTI-1) are also those in which HA enhanced PAH degradation in vitro (Figure 4). Conversely, strains which do not show in vitro benefits from HA addition are also those which show steeper decreases in phenanthrene mineralization with increasing soil HCI. This is true for *Burkholderia* GTI-2 on phenanthrene (Figure 2C) as well as *Acinetobacter* MVC Dod3 on hexadecane (Figure 2E). It will be of interest to use this suite of strains in further studies to model the actual biochemical effects of HA on different bacteria. Testing of this nature is currently in progress.

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