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Degradation of Humic Acids in a Microbial Film Consortium from Landfill Compost

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Bacterial biofilms are ubiquitous in nature and industrial settings. In this study, a biofilm consortium was enriched in a continuous-flow-cell system using humic acid as the sole carbon and energy source. The degradation of the humic acids by the consortium under two supplementary carbon sources was investigated by ultraviolet (UV) absorbency, Fourier transform infrared (FTIR) spectroscopy, and electrospray mass spectrometry (ES-MS). The morphological characteristics of the biofilm consortium and the isolated cultures from the biofilm were observed under an epifluorescence microscope. The metabolic diversity of the selected cultures from the degradative consortium, based on substrate usage pattern, was examined using Biolog EcoPlates. Microscopic analysis revealed that the biofilm was formed by various morphotypes of bacteria, fungi, and yeasts, as well as amoebas. The substrate usage profiles of the bacteria confirmed that, in addition to yeasts and fungi, two groups of bacterial consortia were developed in the biofilm to degrade the humic acids. The degradation of humic acid in the biofilm was mostly carried out in a secondary or a cometabolic path. Addition of the readily digestible external carbon source enhanced the growth of the biofilm consortium. The FTIR and ES-MS spectra confirmed the changes in chemical structure of the humic acid by the biofilm community.

1. Introduction

Composting is a widely used strategy for the stabilization and mineralization of organic matter. During composting, readily degradable organic matter is used by microorganisms as a carbon and nitrogen source. When lignocellulosic wastes are added in composting, the end products usually contain the transformed or intermediately degraded products that are classed as humic substances. The benefits of humic substances to soil ecology, fertility, and structure and plant growth have led to an increase in the application of compost as an amendment to soils.[1] However, humic acids, as the water-soluble compounds of humic substances, often cause environmental problems once released into the ecosystem, owing to the solubility and high absorptive reactivity of these acids with heavy metals and xenobiotic compounds in aqueous environments. For example, humic acids can carry heavy metal ions, insoluble organic materials, and xenobiotics and increase their solubility and motility in soil and water.[2,3] In addition, they can facilitate the formation of trihalomethanes and other carcinogenic and mutagenic activity by reaction with chlorine dosed in water purification processes.[4,5] Therefore, it is important to promote the degradation of humic acids in the natural ecosystem.

Interactions of humic substances with microorganisms have been studied by various researchers. For example, the discoloration of soil humic acid extracts by basidiomycetic and ascomycetic fungi that could also degrade lignin was reported in the early 1960s.[6] Fakoussa first demonstrated that microbes, especially filamentous fungi, could solubilize solid particles of low-rank hard coal,[7] which has a structure similar to that of humic acids. Recently, extracellular oxidization and transformation of solubilized low-rank coal by wood-rot fungi and the depolymerization of low-rank coal by extracellular fungal enzyme systems were reported.[8] Gramss et al.[9] found that fungi, some of which were propagated in contaminated soils to control xenobiotics, metabolized humic extract compounds enzymatically.

In natural ecosystems, microbial degradation of humic acids is rarely carried out by pure cultures, since decomposition in these ecosystems, which is usually effected by diverse microbial communities,[10] may lead to the transformation of organic and inorganic substances into essential elements, such as extracellular products, including extracellular proteins, as well as carbon dioxide. Such transformation also includes complete mineralization of these compounds into nutrients, such as C, N, P, and K, as well as other mineral elements for sustaining the cell viability and the ecosystem. Moreover, in natural habitats there may be numerous microbial species that affect the activity of any particular degradative strain.[11] Bacterial biofilms are ubiquitous in nature and industrial settings. For example, they are common on the liquid−solid interface of rivers, lakes, and wetlands, and they accumulate on rocks in aquatic environments. This attached (sessile) mode of growth usually dominates in environments with low organic and nutrient concentrations.[12] For the successful introduction of microorganisms and the manipulation of consortia to degrade humic acids, it is important to develop a method that could simulate the in situ degradation behavior of heterogeneous microbial consortia on humic acids.

Therefore, the first objective of this study was to develop a biofilm consortium capable of degrading humic acids that could simulate the in situ degradation

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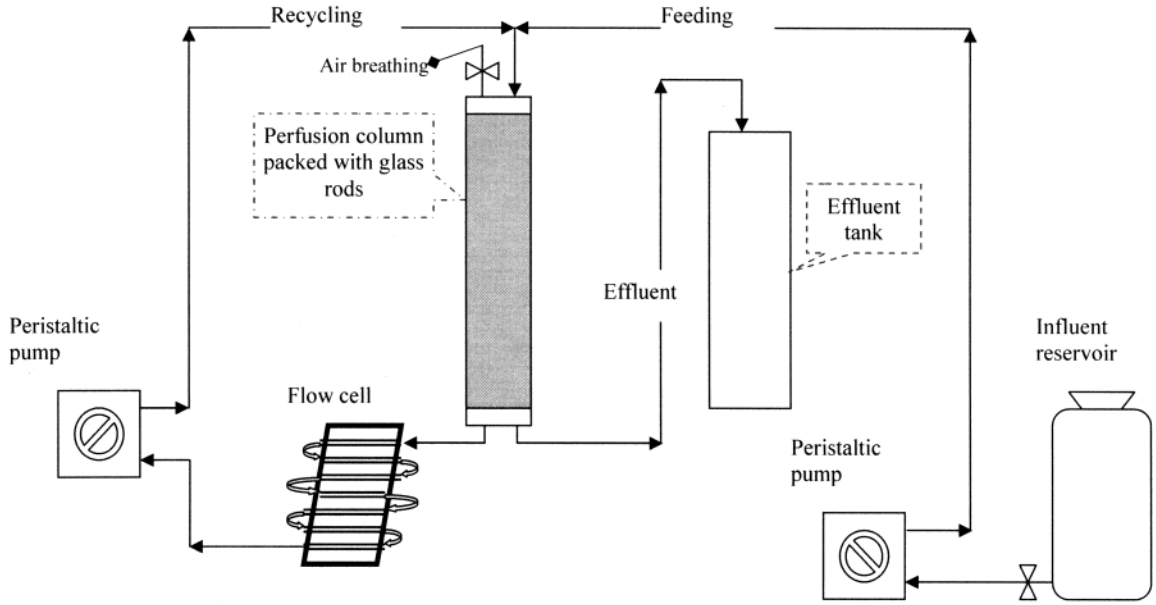

Figure 1. Schematic diagram of the continuous-flow system used to develop a humic acid degradative biofilm consortium.

behavior of heterogeneous consortia on humic acids in natural ecosystems. Another objective was to investigate the effects of nutrient amendments on the humic acid degrading efficiency of the biofilm consortium.

2. Materials and Methods

2.1. Materials. The humic acids were extracted from humus from an anaerobic laboratory digester fed with lignocellulosic residues (tobacco dust). The humus was suspended with 0.5 M NaOH under N_2 at a solid-to-liquid ratio of 3:20 (w/v). After the treated slurry was left for 24 h in the dark at room temperature, the supernatant was separated by centrifugation. The filtrate was precipitated at a pH of 1.17−1.50 with 1.0 M HCl, and the precipitate was washed with 0.1 M HCl, followed by distilled water, then freeze-dried, and stored in the dark at −16 °C.

2.2. Development of a Biofilm Consortium Capable of Degrading Humic Acids: Inoculate, Culture Conditions, and Experimental Setup. A 1 g sample of 1 year compost from a simulated landfill reactor, fed with grass, was used as the inoculate. A minimum salt solution supplied with humic acids was used as the culture medium to develop a humic acid degrading consortium, with the concentration of humic acids increasing from 0.005 to 0.01, 0.02, 0.04, and 0.05 g/L during the first 5 months. The minimal salt solution consisted of equal volumes of solution A and solution B,[13] which were autoclaved separately and then mixed together with the humic acid solution. Solution A consisted of 1.0 g/L NH_4Cl, 0.12 g/L $MgSO_4$, and 1.0 mL of a trace element solution containing 4.0 g/L EDTA, 1.5 g/L $CaCl_2$, 1.0 g/L $FeSO_4 \cdot 7H_2O$, 0.35 g/L $MnSO_4 \cdot 2H_2O$, and 0.5 g/L $NaMoO_4 \cdot 2H_2O$. Solution B consisted of 4.24 g/L Na_2HPO_4 and 2.7 g/L KH_2PO_4.

The humic acid solution was prepared by dissolving freeze-dried humic acids in distilled water by initially adjusting the pH above 11 with 2.0 N NaOH solution and then neutralizing to pH ≈ 7 with concentrated HCl. The final humic acid concentration of the solution was 1.0 g/L. The humic acid solution was used without autoclaving.

In this study, a continuous-flow system consisting of a packed column and flow cell was used to allow microscopic observations of the growth of a biofilm consortium able to degrade humic acids. The experimental system consisted of a perfusion glass column (25 mm i.d., 300 mm length), packed with hollow glass rods (5−8 mm length and 1−3 mm i.d.), to facilitate attached growth. The column was continuously irrigated from the top with the humic acid−minimum salt solution using a peristaltic pump at a constant rate of 0.8 L/day. The effluent was recycled at a constant rate of 0.6 L/day. A continuous-flow cell[13] was connected in line in the recirculation loop to facilitate observation of biofilm development over time using optical microscopy. Biofilms growing on the glass rod surfaces and in the flow cells were also stained with Acridine Orange and viewed with a Nikon Eclipse E400 epifluorescence microscope using 60×, 1.4 numerical aperture oil immersion lenses. The system was maintained at room temperature (23−25 °C). A schematic diagram of the experimental setup is shown in Figure 1.

2.3. Characterization of the Degradative Consortium. Microbial numbers in the consortium were determined after 5 months of acclimation. Tryptic soy agar (TSA) and Rose Bengal chloramphenicol agar were used to enumerate the total viable aerobic microbial and fungal colonies, respectively, using the spread plate technique. The total number of anaerobic viable colonies was determined on a TYEG (Trypticase yeast extract glucose) medium in an anaerobic incubation chamber.

Individual microbial members of the consortium were isolated with 10% TSA plates and subjected to the Gram staining procedure. The staining results and colony morphology of the isolated bacteria were observed with a Nikon Eclipse E800 microscope using 100×, 1.3 numerical aperture oil immersion lenses. The morphologies of the isolated fungi and yeast cultures were observed with a Nikon Eclipse E400 microscope using 60×, 1.4 numerical aperture oil immersion lenses.

In addition to Gram staining and morphological characterization, Biolog EcoPlates (Biolog Inc., Hayward, CA), which contained 31 different substrates,

were used as a measure of the bacterial functionality, to group the isolates of the bacterial consortium on the basis of the substrate usage pattern. The microwell plates were inoculated with each suspension of the isolated bacteria. For each isolate (18 isolates in total), duplicate inoculation was performed. The microplates were incubated in the dark at 26 °C for 3 weeks. Carbon source usage patterns were obtained by observation of the color changes in the inoculated well every 2−3 days during the first week of incubation and every other day during the last 2 weeks of incubation. The results from the color changes were record as "0" (no change in color) or "1" (some change in color) over a 3 week period of incubation. The results of these experiments are summarized in Figure 3, by means of a dendrogram. The dendrogram was derived via agglomerative hierarchical cluster analysis of the Biolog EcoPlate data, which consisted of 18 samples (microbial isolates) taken over 31 variables (substrate usage patterns). The similarity measure was based on the squared Euclidean distance between the 18 microbial isolates, and complete linkage between clusters was used. This normalized linkage distance in the graph gives an indication of the similarity of substrate usage patterns of the different isolates. Except for the incubation period, no other external factors were investigated.

2.4. UV and FTIR Spectroscopy Measurements of Humic Acid Degradation. Aliquots (10 mL) of influent and effluent samples from the biofilm system were filtered through a 0.22 μm membrane. The spectrophotometric characteristics of the filtrate were then determined at wavelengths between 190 and 800 nm on a Cary 1E UV−vis spectrophotometer. On the basis of the peak characteristics of the spectra, the absorbencies at 194, 226, and 340 nm were used to evaluate the degradation of humic acids in the biofilm system. The E_4/E_6 ratios (the ratio of optical absorbencies at 465 and 665 nm) were calculated from absorbency values measured at 465 and 665 nm.[14] Fast Blue B salt (FBB) (tetrazotized *O*-dianisidine, Sigma) reactivity of the humic acids was also determined to examine the degradation of hydroxylated aromatic compounds of humic acids in the biofilm system.[9] The FBB reactivity was determined as follows: A 1 mL sample of a humic acid solution was mixed with 0.1 mL of a 4.21 mM FBB solution. The increase in the absorbency at 530 nm (UV530) was recorded twice from 0 to 30 s, and the FBB reactivity was indicated by the average rate of the increase in UV530 over the first 12 s.

Chemical changes in the humic acids, owing to degradation by the biofilm consortium, were detected with FTIR spectra using a Perkin-Elmer infrared spectrophotometer (model 1600). The influent and effluent of the biofilm system fed with a 0.05 g/L humic acid solution as the sole carbon and energy source were sampled and filtered through a 0.22 μm membrane. A drop of the filtrated liquid was evenly spread on a CaF_2 crystal cell (11101, 38 × 19 × 4 mm) and analyzed using a single scan in the wavelength range of 1000−4000 cm^{-1}.

2.5. ES-MS Measurements of the Humic Acid Degradation. Breakdown of the large molecular fractions of humic acids by the biofilm consortium was examined with ES-MS spectra. Aliquots (4 mL) of influent and effluent were separately dialyzed with distilled water (six times, over a period of 3 days) under constant stirring at 4 °C. The cutoff value of the dialyzing membranes was 10 000 Da. The retentate after dialysis was subsequently freeze-dried. The freeze-dried material was suspended in an aqueous solution of acetonitrile (1:1, v/v). The samples were then subjected to ES-MS analysis using a Micromass Quattro triple-quadropole mass spectrometer with an electrospray ionization source. Ionization was in the negative mode with the source temperature at 80 °C and the cone potential at 40 V.

2.6. Effect of Nutrient Amendments on the Degradation of Humic Acids in the Biofilm System. Degradation of humic acids in the presence of glucose or tryptic soy broth (TSB) by the biofilm was determined after 5 months of growth on the humic acid/minimal salt solution by substituting the minimum salt solution with the same volumes of glucose medium or TSB. The degradation of humic acids under conditions with a supplementary carbon source was evaluated in terms of changes in UV absorbency at 194, 226, and 340 nm, as well as the E_4/E_6 ratio and FBB reactivity.

3. Results and Discussion

3.1. Development and Characterization of the Humic Acid Degrading Consortium. Biofilm formation is an important mode of growth for humic acid degradation. In this system, continuous cultivation allowed microbial selection and the development of cooperative associations over time that resulted in a high degradation capacity and process stability. These interactions enable complete metabolism and can prevent metabolite accumulation and substrate inhibition. This property has particular significance in the microbial usage of humic acids, the chemically complex organic compounds widely present in natural aqueous systems.

A heterogeneous biofilm community was developed with the humic acids being the only carbon and energy source, as indicated in Figure 2A−D. A variety of morphological cell types and arrangements were observed both in the flow cell and on the surface of the glass rods (Figure 2A,C and Table 1). Protista, primarily amoebas, were also observed (Figure 2C). Pure cultures isolated from the consortium representing bacteria (D), yeasts (E), and fungi (F, G) are also shown in Figure 2. The biofilm that was formed in the flow cells typically consisted of a few layers of cells. These observations suggest that biofilm formation is an important mode of growth with humic acids as the sole source of carbon and energy.

Table 2 indicates that the total aerobic count was 30−40 times higher than the anaerobic count, while the fungal and yeast counts were notably lower than the bacterial counts. The addition of an external carbon source (glucose and TSB) promoted the growth of the consortium in general as shown by the numbers of plankton (microbial cells not attached to surfaces, including those that grow and multiply in suspension and those that have detached from surfaces). The humic acid degrading consortium consisted of a variety of microorganisms, including anaerobic microorganisms.

Different methods have to be used in the analysis of the microbial community, as the methods are generally population specific. For example, assessment of colony morphology focuses mainly on cultivable bacteria, which only represent a minor fraction of the total number of bacteria.[15,16] Therefore, other complementary methods

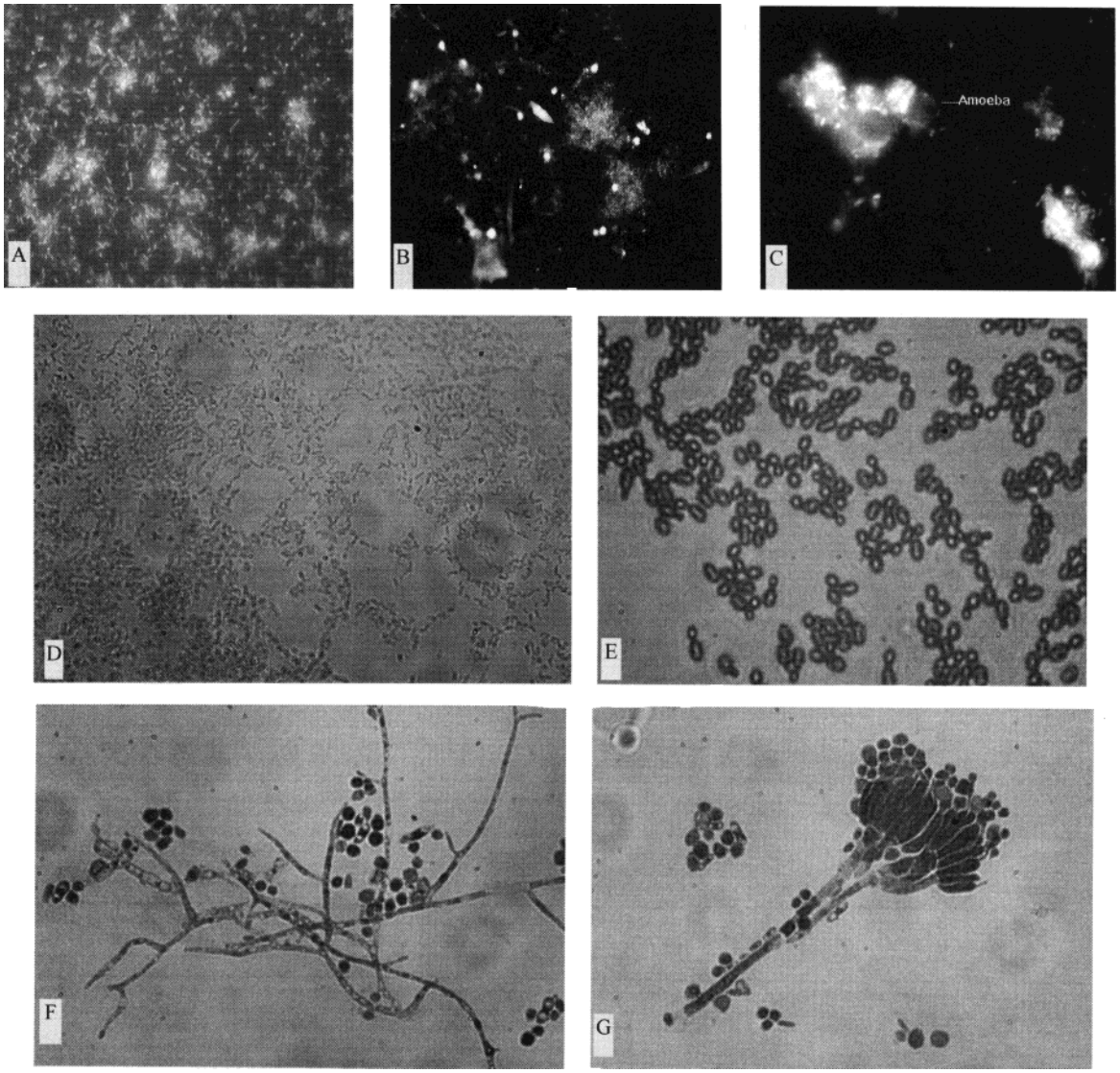

Figure 2. Microscopic images of the biofilm consortium involved in the degradation of humic acids (A−C) and the isolated pure cultures (D−G), showing (A) a free-swimming bacterial consortium, (B) a flocculate microbial consortium, (C) a flocculate microbial consortium and amoebas, (D) a bacterial culture, (E) a yeast culture, and (F and G) fungi.

Table 1. Characterization of Pure Cultures from the Biofilm Consortium

isolate no.	Gram reaction	morphology	isolate	Gram reaction	morphology
17, 21	+	very small cocci	16B, 20	−	bipolar coccobacilli
22, 16	+	small cocci	24B	+	bipolar coccobacilli
18	+	chains of cocci	9	−	short rods
26	−	chains of cocci	10, 6	+	short rods
5	+	diplobacilli	4	+	palisades and *X*, *Y* arrangement
24, 25	+	diplococci	23	−	large cocci

Table 2. Colony Count of the Biofilm Consortium[a]

	ratio	aerobic microorganisms	anaerobic microorganisms	yeasts	fungi
HA/glucose	50:0	35×10^5	1.2×10^5	52×10^2	33×10^2
	3:10	1352×10^5	219×10^5	10.2×10^3	9.4×10^3
HA/TSB	50:0	48×10^5	1.8×10^5	61×10^2	43×10^2
	1:10	1536×10^6	541×10^5	8.4×10^4	4.7×10^4

[a] HA = humic acid; TSB = tryptic soy broth.

should be considered to provide more complete information about the bacterial community.

Biolog microtiter plates have been used widely to detect physiological profiles of microbial communities under different conditions and to describe their functional structures. Characterization of microbial communities with Biolog microtiter plates was first performed by Garland and Mills.[17] Haack et al.[18] concluded from model microbial communities producing unique Biolog carbon usage profiles that the method is capable

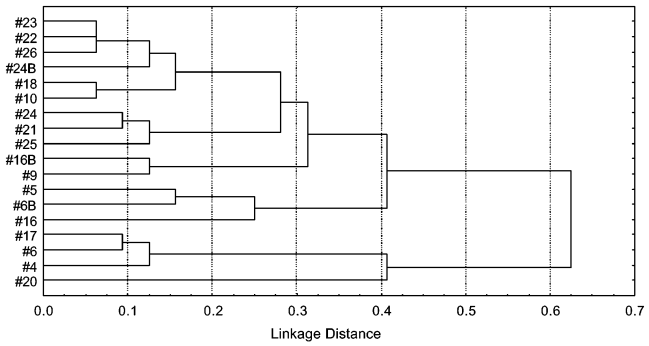

Figure 3. Dendrogram showing the similarities in the substrate usage patterns of the microbial isolates in the humic acid degradation biofilm system. The diagram was generated by agglomerative hierarchical clustering of the 18 microbial isolates (inocula), each of which was represented by 31 different substrate usage levels.

of discerning real differences in the community. Biolog plates have thus proven useful as metabolic fingerprints of bacterial communities and were also used to describe the metabolic diversity of the bacteria in biofilm communities by culturing the bacterial isolates on the Biolog microtiter plates.[19]

The dendrogram in Figure 3 indicates that the substrate usage pattern of the community obtained by EcoPlates in general showed little difference in the number of substrates used among the morphological groups classified in Table 1. For example, taking the morphological and Gram reactive properties into account, the bacterial groups, such as nos. 10 and 6 and nos. 17 and 21 should have the same metabolic structure. However, the substrate usage patterns between nos. 10 and 6, as well as between nos. 17 and 21, appear noticeably different from each other. Likewise, different morphological and Gram reactive groups, such as nos. 17, 6, 4, and 20, were assigned to the same group on the basis of their substrate usage patterns. This shows that, for the purpose of humic acid degradation, the functional structure of the bacterial community based on substrate usage is more relevant than the morphological structure of the community.

Finally, a variety of morphological bacterial species populating the humic acid degrading system can be classified into two groups, on the basis of the carbon usage profile of the community. The difference of these two groups could probably be ascribed to their different metabolic pathways and efficiencies in using the humic acids as a carbon and energy source.

Furthermore, the results obtained by the EcoPlates also represent the portion of the bacterial community amenable to cultivation. The coloration depends on bacterial growth, and therefore, it describes only the potential activity of the fraction of the microbial community able to grow in the wells.[20] In addition, Smalla et al.[21] concluded that the utilization pattern does not necessarily reflect the potential of the numerically dominant bacteria in an inoculum. This suggests that the functional structures of this microbial community were numerically limited to only two dominant bacterial groups.

It is clear that there was no significant difference in substrate usage patterns among the bacterial groups in the biofilm community, which appeared morphologically heterogeneous. Instead, various morphological structures of the bacterial consortia were frequently observed. This suggests that a heterogeneous aggregate structure of the bacteria was necessary for the biofilm

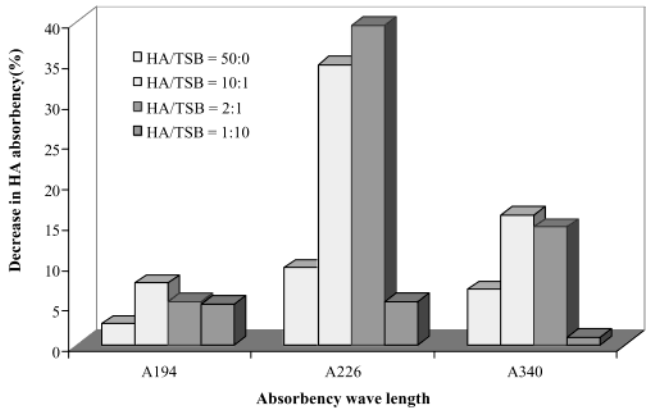

Figure 4. Effect of the humic acid (HA)/glucose ratio on the humic acid degradative efficiency of the biofilm consortia.

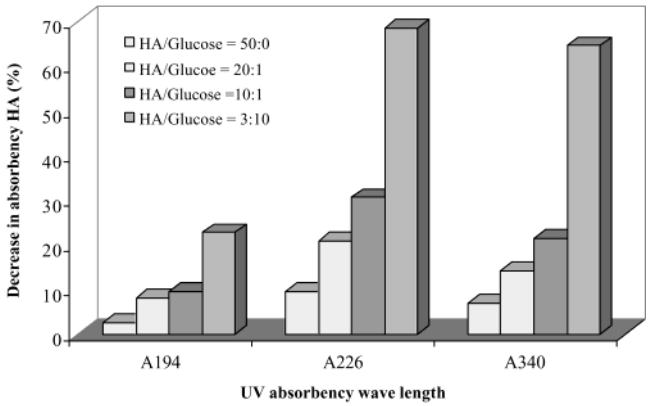

Figure 5. Effect of the HA/TSB ratio on the humic acid degradative efficiency of the biofilm consortia.

to effect the degradation of the humic acids. More heterogeneous degradative pathways accessible to microbial communities could allow enhanced degradative efficiency. Using biofilm reactors to degrade coal- and tar-related compounds, Guieysse and Mattiasson[22] also found improved degradation capacities resulting from symbiotic interactions in mixed microbial populations.

3.2. Humic Acid Degradation Efficiency of the Biofilm Consortium. 3.2.1. UV Absorbency. Addition of glucose to the biofilm consortium resulted in a decrease in UV absorbency at 230, 226, and 340 nm of the effluent (Figure 4). This effect was concentration dependent, with a larger decrease in the UV absorbency being detected at a higher glucose concentration. This suggests that the presence of glucose promoted the degradation of humic acids. In contrast, although decreases in the UV absorbency resulting from the addition of TSB were observed at humic acid/TSB ratios of 10:1 and 2:1, no further decrease was observed when the humic acid/TSB ratio was lowered to 0.1:1 (Figure 5).

From these observations, it can be postulated that the degradation of humic acids was carried out mainly via a secondary or cometabolic process, likely carried out by fungi. Some fungi, when induced by the deprivation of nitrogen, sulfur, and easily digestible carbon, may produce nonspecific enzymes that break down the large molecular structure of humic acids. The degradation of humic acids may not necessarily provide these fungi with sustainable carbon and energy sources to grow. To overcome this limitation, microbial populations in nature generally form complex communities consisting of mutually benefiting populations of different metabolic

Table 3. Changes in the E_4/E_6 Ratio and FBB Reactivity with Different Ratios of Concentrations of Humic Acids to Concentrations of Other Supplementary Carbon Sources[a]

	concn ratio	change in E_4/E_6 ratio (%)	change in FBB reactivity (%)
HA/glucose	50:0	−14.67	−2.1
	20:1	−5.88	−17
	10:1	−8.99	−22.61
	3:10	−10.34	−54.64
HA/TSB	50:0	−14.67	−2.1
	10:1	−8.16	−15.78
	2:1	−10.21	−27.64
	1:10	−2.8	193

[a] HA = humic acid; TSB = tryptic soy broth; FBB = Fast Blue B salt (tetrazotized *O*-dianisidine).

capabilities. Therefore, for continuous usage of humic acids as an energy source in a biofilm reactor, the development and growth of a diversity of functional groups of microorganisms, including bacteria or fungi, are required.

Addition of an external, easily digestible carbon source, such as glucose and TSB, facilitated the growth of these microbial populations in the consortia (Table 2). As a complex nutrient conforming to the growth requirements of the microorganism, high concentrations of TSB possibly induced genetic changes in the microorganisms, which did not favor the degradation of humic acids, as indicated in Figure 5. This would explain the decrease in humic acid degradation at high concentrations of TSB, assuming that the humic acid degradation was carried out primarily by fungi. In this case, high concentrations of TSB would probably cause overgrowth of the bacterial and yeast populations in the biofilm consortia, which would lead to suppressed fungal growth. This also suggests that the degradative organisms enriched in the biofilm with humic acids as the sole carbon and energy source may fail to function when transferred to natural environments, where other easily digestible carbon sources are present in abundance. To enrich the growth of the biofilm consortium for humic acid degradation in natural habitats via nutrient addition would require complete knowledge of the degradative behavior of the microbial community, not only on the target substrate, but also on the auxiliary carbon sources.

3.2.2. Changes in the E_4/E_6 Ratio and FBB Reactivity of the Humic Acid Solution. Table 3 demonstrates that, without exception, the E_4/E_6 ratios of the humic acid solution were reduced, owing to the microbial activities of the biofilm consortium. According to Kononova (1966),[23] the E_4/E_6 ratio is inversely related to the degree of condensation of the aromatic networks in humic acids. A low E_4/E_6 ratio is indicative of a relatively high degree of condensation of aromatic constituents in humic acids, whereas a high E_4/E_6 ratio reflects a low degree of aromatic condensation and the presence of relatively large proportions of aliphatic structures. Schnitzer and Khan[24] suggested that light absorption of aqueous humic acid solutions in the visible region of the electromagnetic spectrum increases with (1) the ratio of carbon in aromatic nuclei to carbon in aliphatic side chains, (2) the total carbon content, and (3) the molecular weight. Chen et al.[14] suggested that (1) much of the observed visible absorption by humic acids may be attributed to light scattering, which may thus contribute to the lowering of the E_4/E_6 ratio in the high-molecular-weight fraction of humic acids, (2) the E_4/E_6 ratio of humic acids is primarily governed by particle sizes and weights, and is apparently not related to the amount of aromatic condensed rings in the structure, and (3) the effect of pH on absorption and E_4/E_6 ratios can be ascribed to changes in particle size, possibly caused by folding or unfolding, or aggregation and/or dispersion of the humic acid macromolecules.

In this study, the pH values of the effluent and influent were both found to be around 7, and the particle sizes of the compounds in the effluent and influent can be considered very similar, because both of them were filtered through a 0.22 μm membrane before the analysis was performed. Therefore, the changes in the E_4/E_6 ratios of the influent and the effluent most probably resulted from the variations in the ratio of aromatic carbon and aliphatic carbon content of the compounds in the effluent and influent. The decrease in the E_4/E_6 ratio of the effluent could be due to the consumption of external aliphatic carbon sources, which were previously added into the influent in the form of glucose or TSB, and the breakdown or usage of the aliphatic carbon chain of the humic acid molecules by the biofilm consortium. Consequently, the decrease in the E_4/E_6 ratio indicated that the aliphatic carbon either in the molecular structure of the humic acid or in the external carbon source was preferentially utilized by the biofilm consortium.

FBB reacts with 1-naphthol and other hydroxylated aromatic compounds to form colored products which absorb in the range of 530−618 nm.[25] Table 3 shows that the FBB reactivity of the effluent consistently declined, except when the humic acid/TSB ratio of the influent was as low as 1:10. The decrease was more evident when the influent was supplied with an external carbon source, such as glucose and TSB. This suggests that some 1-naphthnol or other hydroxylated aromatic-like compounds in the humic acid were degraded by the biofilm consortium. The exceptional increase of FBB reactivity in the effluent when the humic acid/TSB ratio of the influent was as low as 1:10 was possibly the result of the highly vigorous metabolic activity of the biofilm microconsortium in the presence of abundant, easily digestible nutrients.

The FTIR spectra of the humic acid solution before and after biofilm degradation are shown in Figure 6. The FTIR spectrum of pure water is also given in Figure 6 as a control. Figure 6 indicates that the absorbency peak at 1770 cm^{-1} in the FTIR spectra of the influent completely disappeared in the spectra of the effluent. The FTIR absorbency peak at 1770 cm^{-1} can be attributed to the vibrations of bands such as −C═OOH, C═O of ketonic carbon, aromatic C═C, COO−, hydrogen bond, or C═O stretching. This indicates that these functional groups in the molecular structures of the humic acids were removed, altered, or reduced by the microorganisms in the biofilm. The FTIR spectra of the effluent were similar to the spectrum of pure water. This implied that the humic acid content in the effluent was reduced to a level which cannot be detected by FTIR spectrometry.

The ES-MS spectra of the humic acid solution before and after biofilm degradation are shown in Figure 7. Some peaks in the influent spectrum are obviously not present in the effluent spectrum. This indicates that some compounds, such as some polymers with repeating units of 14, such as $-CH_2-$ groups, were removed from the influent compounds as a result of the activity of microorganisms. This further confirmed that the humic acids were broken down by the biofilm consortium.

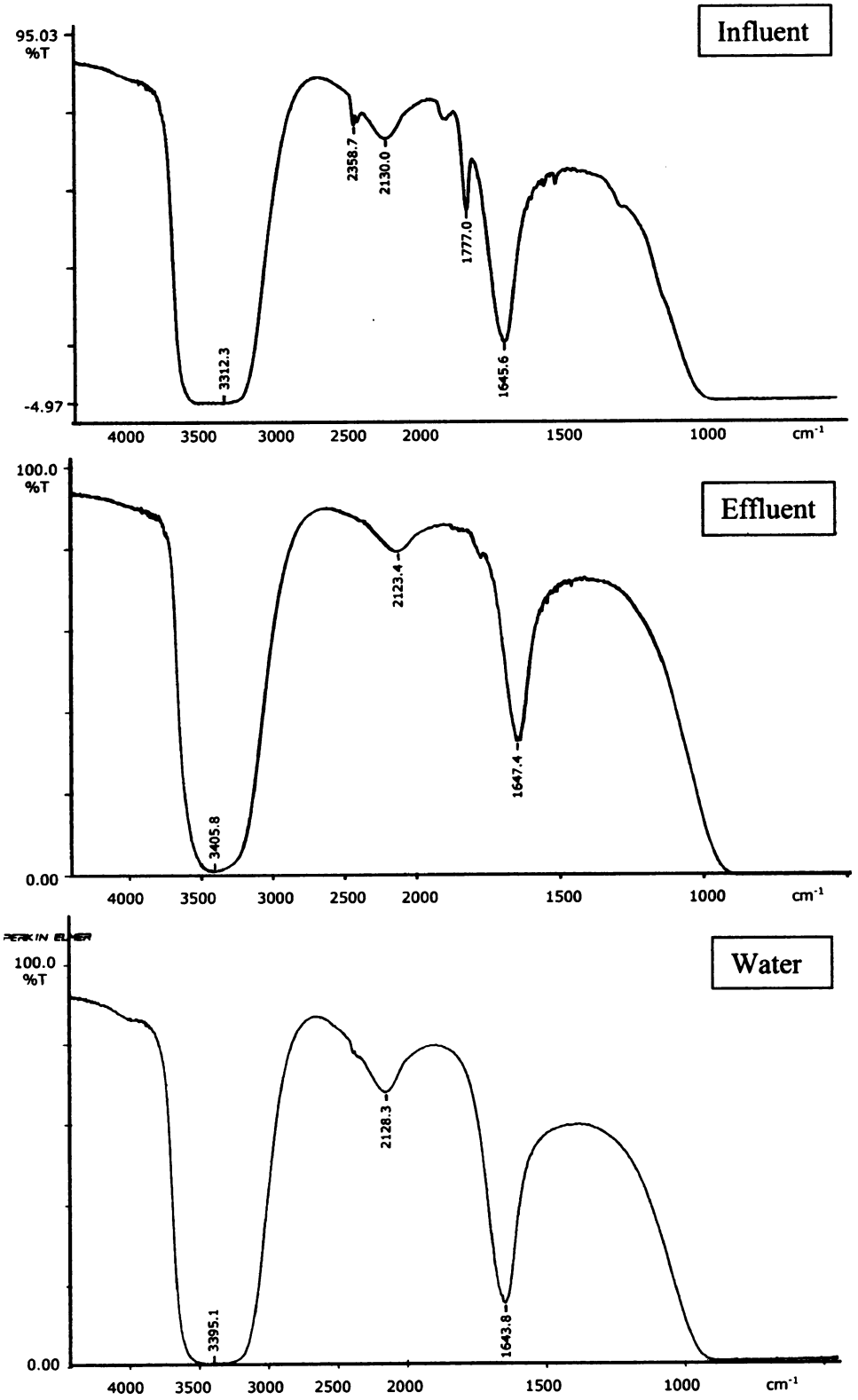

Figure 6. FTIR spectra of the influent, effluent, and control (water) samples from the biofilm system.

4. Conclusions

(1) Biolog EcoPlates and microscopic analysis revealed that a biofilm microbial community, which consisted of heterogeneous functional microbial groups, including fungi, yeasts, bacteria, as both aerobic and anaerobic microorganisms, and protista, primarily amoebas, had developed to degrade humic acids in natural aquatic environments. The bacterial species populating the biofilm could be grouped into two functional consortia, on the basis of the carbon usage profile of the community.

(2) The presence or addition of an external carbon source such as glucose and TSB promoted the growth of the biofilm consortium and improved the degradation efficiency of the humic acids, with the addition of glucose having the better effect.

(3) The degradation of the humic acids in the biofilm system occurred via a secondary or cometabolic process.

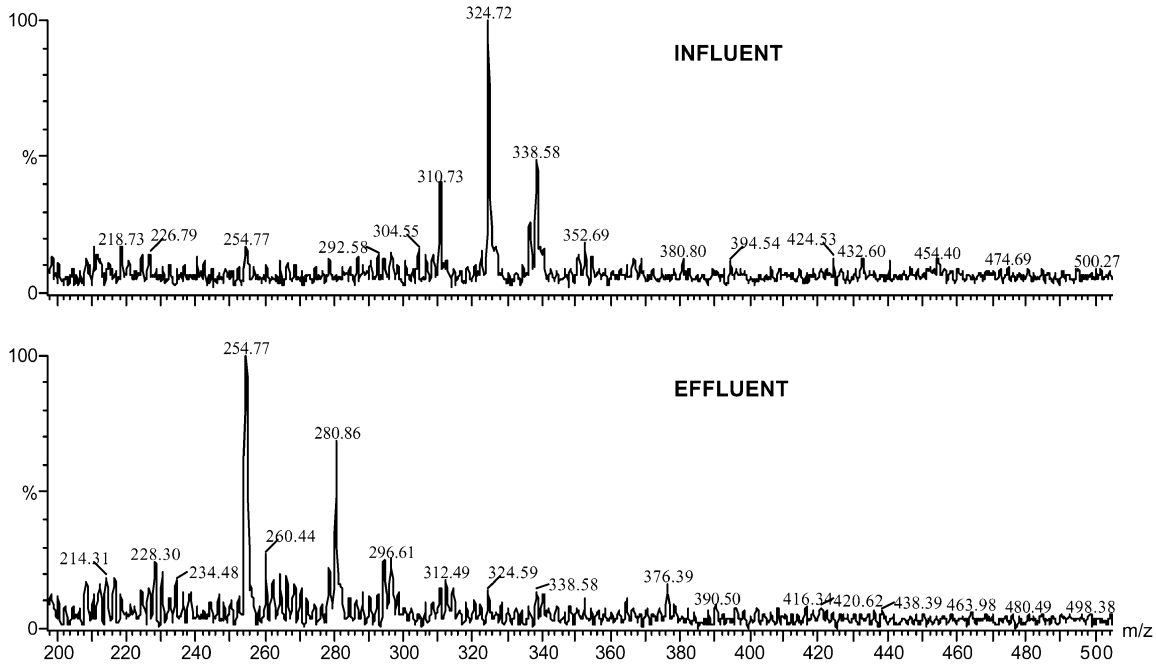

Figure 7. ES-MS spectra of the influent and effluent of the biofilm system.

(4) The chemical alterations in the structures of the humic acids detected by FTIR and ES-MS spectra confirmed the capacity of the biofilm consortium to degrade humic acids.

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