APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1985, p. 402–407
0099-2240/85/020402-06$02.00/0

Vol. 49, No. 2

Influence of Naturally Occurring Humic Acids on Biodegradation of Monosubstituted Phenols by Aquatic Bacteria

ROBERT SHIMP† AND FREDERIC K. PFAENDER*

Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill, North Carolina 27514

Received 6 August 1984/Accepted 15 November 1984

Samples of the microbial community from Lake Michie, a mesotrophic reservoir in central North Carolina, were adapted to various levels (100 to 1,000 μg/liter) of natural humic acids in chemostats. The humic acids were extracted from water samples from Black Lake, a highly colored lake in the coastal plain of North Carolina. After adaptation, the microbial community was tested for its ability to degrade the monosubstituted phenols *m*-cresol, *m*-aminophenol, and *p*-chlorophenol. Adaptation to increasing levels of humic acids significantly reduced the ability of the microbial communities to degrade all three phenols. The decline in biodegradation was accompanied by a decrease in the number of specific compound degraders in the adapted communities. Short-term exposure of the community to increasing levels of humic acids had no significant effect on the ability of the community to degrade *m*-cresol. Thus the suppressive effect of humic acids on monosubstituted phenol metabolism was the result of long-term exposure to the humic materials. Increasing the levels of inorganic nutrients fed to the chemostats during the humic acid adaptation had little effect on the suppressive influence of the humic acids, indicating that nutrient limitation was probably not responsible for the metabolic suppression. The results of the study suggest that long-term exposure to humic acids can reduce the ability of microbial communities to respond to monosubstituted phenols.

Biodegradation rates of organic pollutants are influenced by a wide variety of factors (3). Among these factors is the organic exposure history of the microbial community in a given environment. The types of organic materials the community has encountered in the past may influence the ability of the community to respond to new pollutants.

Except in highly polluted environments, the organic exposure history of most aquatic microbial communities is dominated by naturally occurring substrates. In a previous paper (21), we described the influence of labile, naturally occurring substrates on the biodegradation of the monosubstituted phenols *m*-cresol, *m*-aminophenol, and *p*-chlorophenol. Many aquatic environments also contain significant quantities of humic materials. These materials are believed to result from the complexation of the "leftovers" of microbial activities on lignins and other compounds (5). They are biologically quite stable (2).

In the present study, we have examined how the adaptation of a natural microbial community to true aquatic humic acids influences the ability of the community to degrade the monosubstituted phenols mentioned above. For the purposes of this work, the term "adaptation" refers to the process of long-term exposure of the microbial community to specific carbon substrates. This definition does not imply that adaptation to a new substrate will necessarily enhance any metabolic function within the community, such as biodegradation of phenols. It simply refers to the process of exposure and subsequent response.

MATERIALS AND METHODS

Details of the adaptation and metabolism methods used in this study are described elsewhere (21). The following description provides only an overview of the methods used.

The humic acids used were extracted from samples of Black Lake, a highly colored lake on the coastal plain of North Carolina. These humic acids were isolated by precipitation at pH 2 to separate the bulk humic acids fraction from the fulvic acids fraction. The humics were redissolved in water and purified by being passed through an XAD-8 resin column. The humics were eluted from the column with 0.1 N NaOH. The humic acids fraction was separated from the elutant by precipitation in acid. After centrifugation, the humics were thoroughly rinsed at pH 2, dialyzed, and freeze-dried. The resulting material consisted of humic acids with a trace of fulvic acids (13). The exact structure of these materials is not known. However, chemical oxidation or hydrolysis of the materials or both results in the formation of primarily carboxylated aromatics and short-chain aliphatics (13).

Samples of the microbial community from Lake Michie, a relatively unpolluted, mesotrophic reservoir near Durham, N.C. were adapted to the humic acids in completely mixed chemostats. The chemostats had residence times of 3 days. The humics were fed to the chemostats in a distilled, deionized water solution which was supplemented with inorganic nutrients (see reference 21 for composition).

To assess the impact of the humic acids on the ability of the adapted microbial community to degrade the monosubstituted phenols, the concentration of the humics fed to the chemostats was varied from 100 to 1,000 μg/liter. This method compensated for internal factors which might influence the community, such as containment in the chemostats and changes in temperature.

After an adaptation period of approximately five to seven residence times (2 to 3 weeks), the microbial communities were removed from the chemostats and tested for the following parameters: total size of the community, using the acridine orange direct count method (10); the general metabolic activity of the community by amino acids turnover time; the number of specific degraders of the pollutants *m*-cresol, *m*-aminophenol, and *p*-chlorophenol, most-proba-

* Corresponding author.
† Present address: Environmental Safety Department, Ivorydale Technical Center, Procter and Gamble Co., Cincinnati, OH 45217.

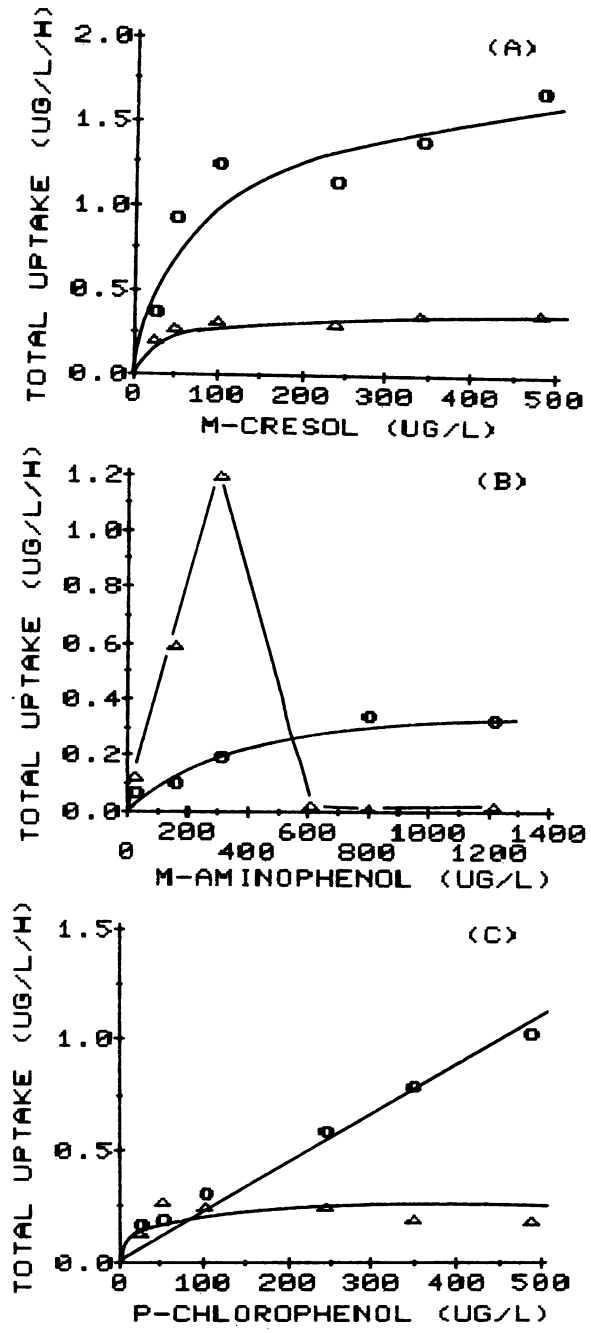

FIG. 1. Total uptake of (A) *m*-cresol, (B) *m*-aminophenol, and (C) *p*-chlorophenol by microbial communities adapted to humic acids in the screening study. Symbols: ○, 100 μg of humic acids per liter; △, 1,000 μg of humic acids per liter.

ble-number method (12); and the biodegradation rates of the test pollutants (16).

Rate data from the biodegradation assay were processed by using the Hanes-Wolff linearization of the Michaelis-Menten enzyme kinetics model. This method estimates the kinetic parameters V_{max} and K_m from the model. If saturation kinetics were not obtained and the Hanes-Wolff line had an r^2 of <0.85, a linear model was fitted to the data. K_1 represents the first-order rate constants from this model.

Estimates of the kinetic parameters were statistically compared by using the small-sample *t*-test for the difference between the slopes of two straight lines. When the Michaelis-Menten model was used, the slopes of two Hanes-Wolff lines ($1/V_{max}$) were compared. When the first-order model was used, the values of K_1 were compared. There were never cross comparisons between the two models.

RESULTS

The initial investigation into the effects of humic acids on the biodegradation of substituted phenols was conducted in conjunction with the study of the effects of labile substrates previously described (21). These initial humic acids chemostats were fed either 100 or 1,000 μg of humic acid per liter. The results of this study are shown in Fig. 1. Adaptation to increasing levels of humic acids significantly decreased ($P < 0.001$) the ability of the Lake Michie bacteria to degrade *m*-cresol (Fig. 1A). The influence of humic acids on *m*-aminophenol metabolism was uninterpretable (Fig. 1B). It appears that increasing levels of humics stimulated biodegradation at low concentrations of *m*-aminophenol while inhibiting uptake at higher levels. It is difficult to dismiss data; however, this effect was not observed in any of the subsequent experiments and was possibly due to some unidentified experimental error. Like *m*-cresol, *p*-chlorophenol metabolism (Fig. 1C) declined as the level of humic acids fed to the chemostats increased. Saturation kinetics were not obtained from the community receiving 100 μg of humic acids per liter, so statistical comparisons could not be performed. However, it is clear from the rate plots that, overall, *p*-chlorophenol metabolism decreased substantially as the level of humics was increased.

In the second study, the Lake Michie microbial community was adapted to 100, 300, or 600 μg of humic acids per liter to further describe the effects of humic acid concentration on pollutant biodegradation. The results are shown in Fig. 2. Adaptation to increasing levels of humic acids decreased the ability of the microbial community to degrade all three of the monosubstituted phenols. The decrease in pollutant metabolism was significant in virtually all cases ($P \leq 0.05$). Unlike the previous study, *m*-aminophenol metabolism behaved as predicted by Michaelis-Menten kinetics and mimicked the behavior of the other two pollutants.

The biological data from the detailed concentration study are shown in Table 1. Acridine orange direct counts, amino acids turnover times, and plate counts on a general medium did not correlate with the response of the community to the three pollutants. Estimates of the number of specific degraders did, for the most part, correlate with pollutant metabolism. As the level of humic acid fed to the chemostats was increased, the number of most probable numbers decreased, suggesting that the reduction in biodegradation rates was due to a decline in the number of pollutant degraders.

On the basis of the two initial studies, it was clear that adaptation of the Lake Michie microbial community to Black Lake humic acids substantially decreased the ability of the community to degrade *m*-cresol, *m*-aminophenol, and *p*-chlorophenol. However, the mechanism for this effect was not evident from the data collected.

In prior work (21), we had determined that adaptation to labile substrates, such as amino acids, enhanced the ability of the Lake Michie microbial community to degrade the test pollutants. Since humic acids suppressed pollutant metabolism, we were interested in determining if the humics could influence the enhancing effects of adaptation to amino acids. Such an experiment would determine if the suppression of pollutant metabolism by humics would occur in the presence of an easily degradable substrate such as amino acids.

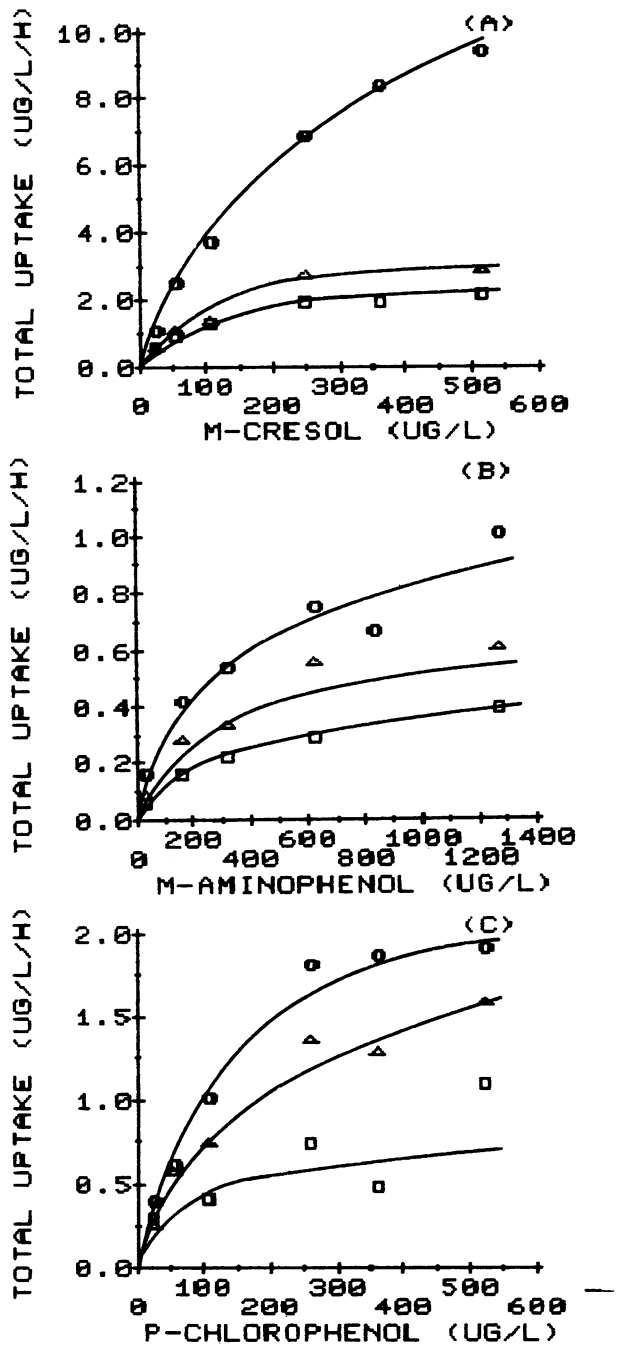

FIG. 2. Total uptake of (A) *m*-cresol, (B) *m*-aminophenol, and (C) *p*-chlorophenol by microbial communities adapted to humic acids in the detailed concentration study. Symbols: humic acids at ○, 100 μg/liter; △, 300 μg/liter; □, 600 μg/liter.

Studies were conducted involving two concentrations of amino acids (100 and 1,000 μg/liter). One set of chemostats received the amino acids alone. Another set received each concentration of amino acids plus 100 μg of humic acids per liter. A third set received the amino acids plus 1,000 μg of humics per liter.

The results of this study are shown in Fig. 3. Only the data for the metabolism of *m*-cresol are shown. The results for the other two pollutants were very similar to those for *m*-cresol. As in the prior study (21), adaptation to increasing levels of amino acids alone enhanced the ability of the microbial community to degrade *m*-cresol (Fig. 3A). However, the presence of humic acids in the amino acids feed solutions had a significant effect on this enhancement. As the level of humics present was increased from 0 to 100 to 1,000 μg/liter, the difference between the rates of *m*-cresol metabolism at the two levels of amino acids declined. At 1,000 μg of humic acids per liter, there was virtually no difference between *m*-cresol metabolism at the two levels of amino acids (Fig. 3C). This suggests that the suppressive effect of humic acids was active even in the presence of labile substrates such as amino acids.

The next experiment conducted addressed the question of whether the effect of humic acids on pollutant metabolism was due to long-term adaptive effects or some short-term effect. Adsorption of the test pollutants by the humics during the biodegradation assay would be an example of such a short-term process. Four levels of humic acids, 100, 300, 600, and 1,000 μg/liter were added to unadapted, fresh samples of the microbial community from Lake Michie and *m*-cresol metabolism was measured immediately. In one experiment, the humics were added to the community suspended in Lake Michie water. In a second, the humics were added to the community suspended in the basic feed solution used in the chemostat studies. This should detect any effects which result from the background levels of humics present in the water from Lake Michie.

The addition of increasing levels of humics did not appreciably affect the ability of the unadapted Lake Michie microbial community to degrade *m*-cresol (Fig. 4). *m*-Cresol uptake was relatively unchanged at all levels of humic acids. Indeed, in the study on the microbial community suspended in feed water, the addition of increasing levels of humics led to a small but steady increase in *m*-cresol uptake. Thus, the suppressive effect of humic acids observed in the earlier experiments was the result of processes which occurred over the long-term adaptation period in the chemostats.

The final experiment of this study examined the interactions of humic acids with inorganic nutrients and their effect on the biodegradation of *m*-cresol. We thought it was possible that the humics might deplete metal concentrations in the nutrient solutions fed to the chemostats. This could lead to the suppression of monosubstituted phenol metabolism in the microbial communities from the chemostats.

TABLE 1. Microbiological parameters for the detailed concentration study of humic acids

Chemostat (humic acids, μg/liter)	Total cells (× 10^6 cells per ml)	AAT (h)[a]	MPNs (× 10^3 cells per ml)[b]			CFU (× 10^5 cells per ml)
			CH_3	NH_2	Cl	
100	2.09	3.02	>16.4	>4.1	11.5	2.16
300	1.85	4.85	15.9	3.97	15.6	1.39
600	1.84	0.80	5.33	0.72	3.58	1.83

[a] AAT, Amino acids turnover times.
[b] MPNs, Most probable numbers.

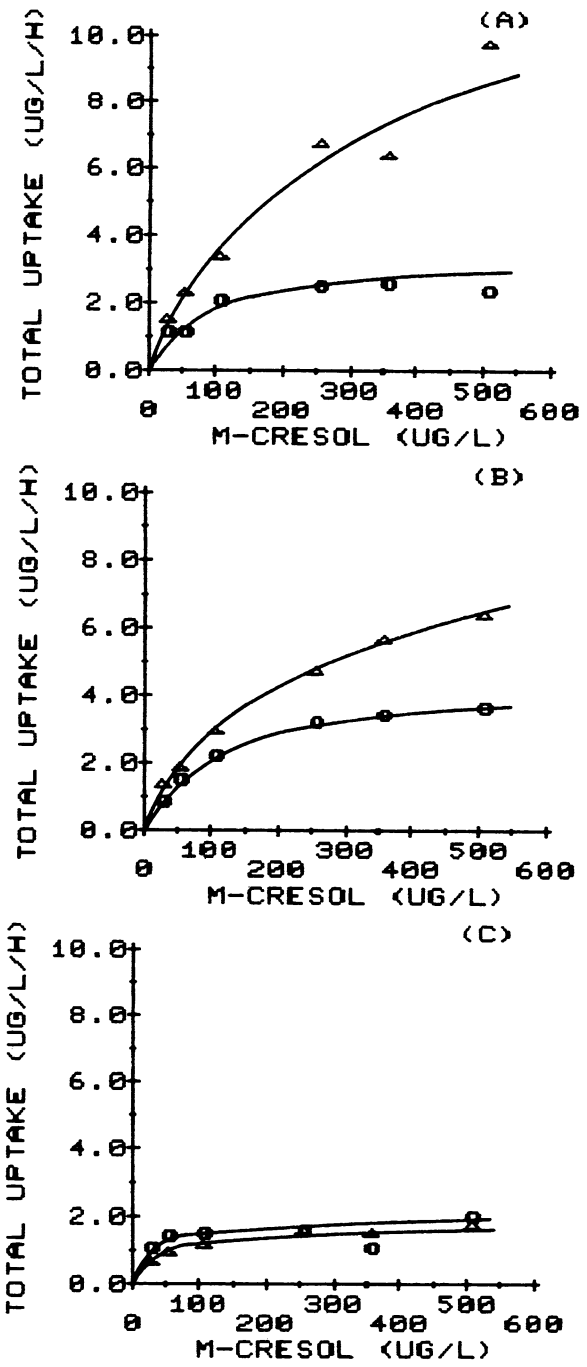

FIG. 3. Total uptake of *m*-cresol by microbial communities adapted to (A) amino acids, (B) amino acids + 100 μg of humic acids per liter, and (C) amino acids + 1,000 μg of humic acids per liter. Symbols: ○, 100 μg of amino acids per liter; △, 1,000 μg of amino acids per liter.

Three concentration studies were conducted, using chemostats receiving 100 and 1,000 μg of humics per liter. One set of chemostats received the normal levels of inorganic nutrients (these levels approximated those found in Lake Michie; see reference 21). The second received two times the normal levels, and the third received ten times the normal amounts. After the usual adaptation period, the microbial communities were tested for their ability to degrade *m*-cresol.

The results of this experiment are shown in Fig. 5. Overall, the presence of increasing levels of inorganic nutrients had little effect on the suppressive influence of humics. *m*-Cresol uptake declined significantly ($P < 0.05$) as the humics concentration increased in the chemostats receiving normal and twofold normal inorganic nutrient levels. The data from the 10-fold normal chemostat could not be compared statistically. However, it appears from the curve that, if the 100-μg/liter curve had reached saturation, it would have had a significantly higher V_{max} than that from the 1,000-μg/liter chemostat. Note also that the uptake of *m*-cresol at any single level of humic acids decreased somewhat as the level of nutrients present increased.

DISCUSSION

The results of this study indicate that, alone or in conjunction with a readily usable carbon source, such as amino acids, Black Lake humic acids had detrimental effects on the ability of the humics-adapted Lake Michie microbial community to degrade *m*-cresol, *m*-aminophenol, and *p*-chlorophenol. Since humics have often been regarded as stimulators of the activity of both algae (18) and bacteria (6, 7, 20), this result is surprising.

Whereas the data conclusively illustrate that the Black Lake humics reduced biodegradation of the phenols, the results of these studies do not clearly identify a mechanism for this effect. The data from the second concentration study (Fig. 2, Table 1) indicate that the decrease in pollutant uptake could be correlated with diminished numbers of pollutant degraders. However, this finding does not identify the underlying mechanism which caused the reduction in biodegradation, nor how humics might inhibit the degraders.

We examined several possible explanations for the suppressive effect of humics on the biodegradation of monosubstituted phenols by the Lake Michie microbial community. First, the microbial communities in the chemostats may have been starved due to the humics being relatively unutilizable carbon and energy sources. Second, the humics could adsorb the test pollutants during the biodegradation assay, preventing metabolism by the bacteria. And third, the adsorptive capacity of the humics may have depleted vital micronutrients in the chemostats.

The results of the study in which amino acids were presented to the microbial community in conjunction with the humic acids indicate that the suppressive effect of the humics was active even in the presence of a readily utilizable substrate (Fig. 3). Indeed, the presence of increasing levels of humics nullified any enhancing effect the amino acids had on the metabolism of the phenols.

The ability of humic materials to adsorb organic compounds has been well established (15). The data from the experiment with unadapted Lake Michie communities, however, showed that addition of increasing levels of humic acids to previously unexposed bacteria had little effect on the ability of the community to degrade *m*-cresol (Fig. 4). This result is inconsistent with any sort of adsorption explanation, which would occur during the course of the biodegradation assay, regardless of any previous exposure of the microbial community to the humic acids. The negative impact of the humics on biodegradation was the result of prolonged exposure to the humics during adaptation in the chemostats.

Humic acids are able to adsorb a wide variety of inorganic ions, particularly metals (19). Seki (20) has suggested that the adsorption of such ions might immobilize vital micronu-

trients and suppress the growth and activity of microorganisms. The inorganic nutrient requirements of microorganisms have been extensively described. In general, the trace metal requirements for solid media used to culture aerobic bacteria are quite small, on the order of micrograms per liter (9). In natural environments, bacteria survive in the presence of quantities of trace inorganics which are far below those found in solid culture media (11). It seems unlikely that the adsorptive capacity of Black Lake humic acids would be substantial enough to reduce the levels of inorganic ions to suppressive levels. This conclusion is supported by the data from the nutrient study, in which increasing the level of inorganic nutrients in the chemostat feed solutions, up to 10 times the concentrations typically found in Piedmont impoundments, had relatively little effect on the suppressive influence of humic acids on *m*-cresol uptake (Fig. 5).

In addition to the possibilities tested, there are several other mechanisms which might explain the suppressive effects of the Black Lake humic acids. These were not examined during this research, but could be the focus of any future work in this area. The humic acids might release bound heavy metals into the chemostat feed solutions, producing some type of toxic or inhibitory effect on the activities of the bacteria responsible for degrading the monosubstituted phenols. There is considerable evidence in the literature that metals can affect microbial activities at very low (<1 μg/liter) levels (1, 8; R. B. Jonas, Ph.D. thesis, University of North Carolina, Chapel Hill, 1981). Thus, the desorption of even small amounts of metals into the chemostat feed solutions might have been sufficient to affect the response of the Lake Michie microbial community to the test pollutants. In this particular study highly purified humics were used, which makes this explanation unlikely.

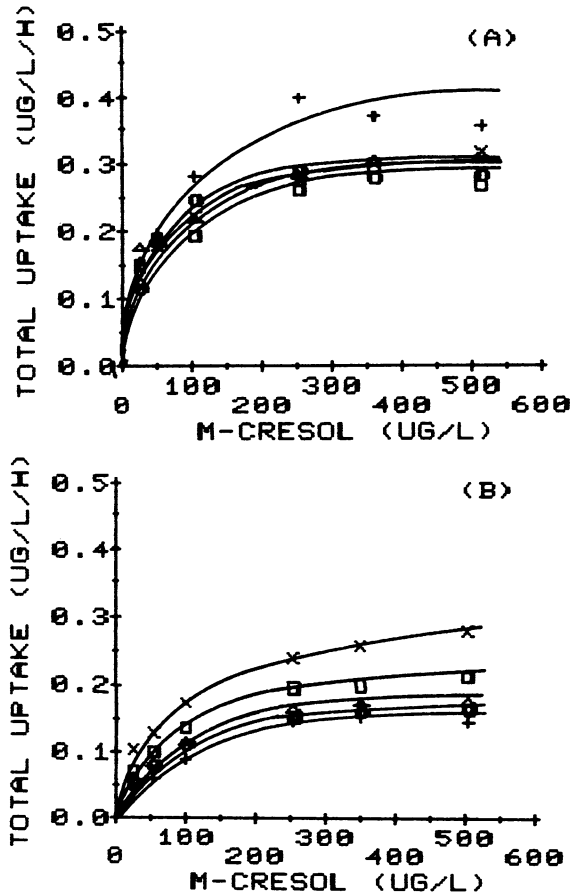

FIG. 4. Total uptake of *m*-cresol from the study of the effect of additions of various levels of humic acids on an unadapted microbial community from Lake Michie. (A) Humic acids added to community suspended in Lake Michie water; (B) humic acids added to community suspended in chemostat nutrient feed solutions. Symbols: +, no humic acids added; humic acids added at ○, 100 μg/liter; △, 300 μg/liter; □, 600 μg/liter; ×, 1,000 μg/liter.

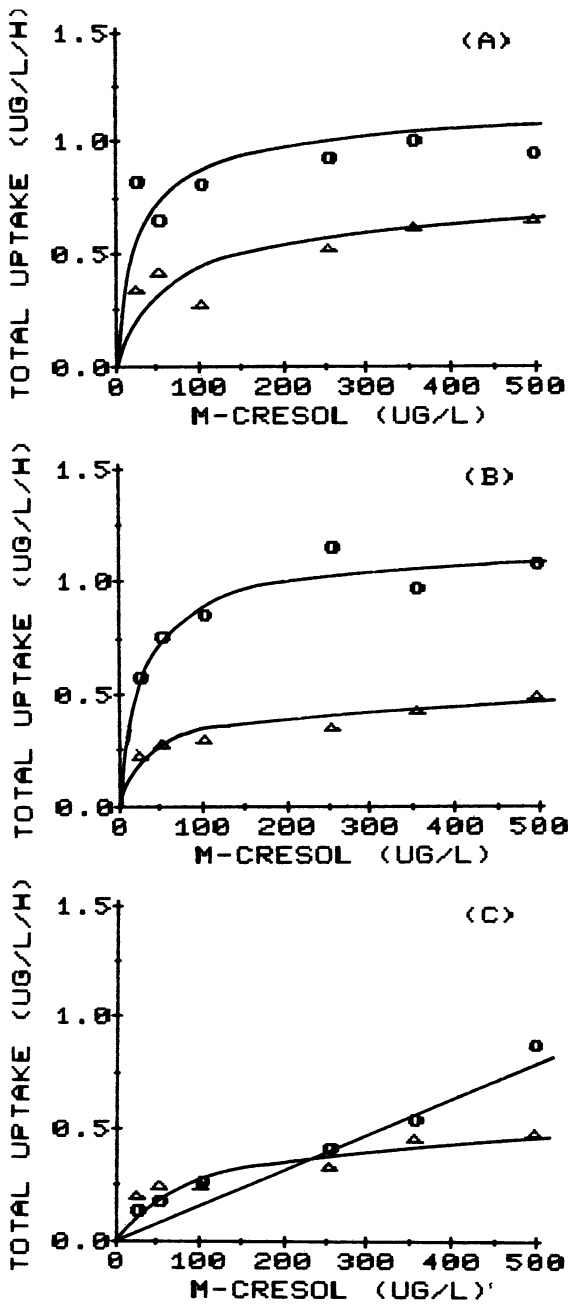

FIG. 5. Total uptake of *m*-cresol by microbial communities adapted to humic acids with nutrient feed solutions containing (A) normal levels of inorganic nutrients, (B) 2-fold normal levels, and (C) 10-fold normal levels. Symbols: ○, 100 μg of humic acids per liter; △, 1,000 μg of humic acids per liter.

There is evidence in the literature that humic acids can significantly alter the activities of a number of bacterial enzymes. Butler and Ladd (4) found that both humic acids and fulvic acids suppressed the activities of a number of proteases. The authors suggest that such inhibitions are due to the irreversible binding of the enzyme to the humic acid molecules. The activity of indoleacetic acid-oxidase can be inhibited by the high-molecular-weight fraction of soil humic acids (14). Pflug and Ziechmann (17) found that humic acids could interact with the cell wall of *Micrococcus luteus* in such a way that the action of lysozyme could be completely inhibited.

Black Lake humic acids could affect the biodegradation pathway for monosubstituted phenols. The oxidation products of these materials have been characterized as aromatic in nature (13). Thus, it is possible that the humic acids might interact with the enzymes which are responsible for transporting and metabolizing aromatic molecules (such as the test pollutants).

This research clearly demonstrates that Black Lake humic acids had a detrimental influence on the response of Lake Michie microbial communities to monosubstituted phenols. Before identifying the causes of such effects, it is important to determine if the results described here can be extended to other environmental settings and other types of humic (or fulvic) materials. It is also important to determine if humic acids can have similarly suppressive effects on the metabolism of other compounds, particularly those which, unlike monosubstituted phenols, are not structurally related to the natural materials. Studies of the mechanisms of the biological interactions of humics and microbial biodegradation capacities should be addressed only after the interactions have been shown to be pervasive.

ACKNOWLEDGMENT

This work was supported by the Environmental Protection Agency through assistance agreement CR-809235-02-4.

LITERATURE CITED

1. **Albright, L. J., J. W. Wentworth, and E. M. Wilson.** 1972. Technique for measuring metallic salt effects upon the indigenous heterotrophic microflora of the natural water. Water Res. **6**:1589–1596.
2. **Alexander, M.** 1965. Biodegradation: problems of molecular recalcitrance and microbial fallibility. Adv. Appl. Microbiol. **7**:35–80.
3. **Anonymous.** 1967. Required characteristics and measurement of biodegradability. J. Water Pollut. Control Fed. **39**:1232–1235.
4. **Butler, J. H. A., and J. N. Ladd.** 1971. Importance of the molecular weight of humic and fulvic acids in determining their effects on protease activity. Soil Biol. Biochem. **3**:249–257.
5. **Christman, R. F., and R. T. Oglesby.** 1971. Microbiological degradation and the formation of humus, p. 769–796. *In* K. V. Sarkanen and C. H. Ludwig (ed.), Lignins: occurrence, formation, structure and reactions. Wiley-Interscience, New York.
6. **DeHaan, H.** 1974. Effect of a fulvic acid fraction on the growth of a *Pseudomonas* from Tjeukemeer, the Netherlands. Freshwater Biol. **4**:301–310.
7. **DeHaan, H.** 1976. Evidence for the induction of catechol −1,2-oxygenase by fulvic acid. Plant Soil **45**:129–136.
8. **Gillespie, R. A., and R. F. Vaccaro.** 1978. A bacterial bioassay for measuring the copper chelation capacity of seawater. Limnol. Oceanogr. **23**:543–548.
9. **Guirard, B. M., and E. E. Snell.** 1981. Biochemical factors in growth, p. 79–112. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
10. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nuclepore filters for counting bacteria for fluorescence microscopy. Appl. Environ. Microbiol. **33**:1225–1228.
11. **Ishida, Y., and H. Kadota.** 1981. Growth patterns and substrate requirements for naturally occurring obligate oligotrophs. Microb. Ecol. **7**:123–130.
12. **Lehmicke, L. G., R. T. Williams, and R. L. Crawford.** 1979. ^{14}C-most-probable-number method for enumeration of active heterotrophic microorganisms in natural waters. Appl. Environ. Microbiol. **38**:644–649.
13. **Liao, W., R. F. Christman, J. D. Johnson, D. S. Millington, and J. R. Hass.** 1982. Structural characterization of aquatic humic material. Environ. Sci. Technol. **16**:403–410.
14. **Mato, M. C., M. G. Olmedo, and J. Mendez.** 1972. Inhibition of indoleacetic acid oxidase by soil humic acids fractionated on Sephadex. Soil Biol. Biochem. **4**:469–473.
15. **Perdue, E. M.** 1983. Association of organic pollutants with humic substances: partitioning equilibria and hydrolysis kinetics, p. 441–460. *In* R. F. Christman and E. T. Gjessing (ed.), Aquatic and terrestrial humic materials. Ann Arbor Science Publishers, Ann Arbor, Mich.
16. **Pfaender, F. K., and G. W. Bartholomew.** 1982. Measurement of aquatic biodegradation rates by determining heterotrophic uptake of radiolabeled pollutants. Appl. Environ. Microbiol. **44**:159–164.
17. **Pflug, W., and W. Ziechmann.** 1982. Humic acids and the disruption of bacterial cell walls by lysozyme. Soil Biol. Biochem. **14**:165–166.
18. **Prakash, A., and D. J. MacGregor.** 1983. Environmental and human health significance of humic materials: an overview, *In* R. F. Christman and E. T. Gjessing (ed.), Aquatic and terrestrial humic materials. Ann Arbor Science Publishers, Ann Arbor, Mich.
19. **Reuter, J. H., and E. M. Perdue.** 1977. Importance of heavy metal organic matter interactions in natural waters. Geochim. Cosmochim. Acta **41**:325–334.
20. **Seki, H.** 1982. Organic materials in aquatic ecosystems, p. 95–171. CRC Press Inc., Boca Raton, Fla.
21. **Shimp, R. J., and F. K. Pfaender.** 1984. Influence of easily degradable naturally occurring carbon substrates on biodegradation of monosubstituted phenols by aquatic bacteria. Appl. Environ. Microbiol. **49**:394–401.