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Environmental Factors Affecting Indole Metabolism under Anaerobic Conditions†

E. L. MADSEN,[1] A. J. FRANCIS,[2] AND J.-M. BOLLAG[1]*

Laboratory of Soil Microbiology, Department of Agronomy, The Pennsylvania State University, University Park,
Pennsylvania 16802,[1] and Department of Applied Science, Brookhaven National Laboratory, Upton, New York 11973[2]

The influence of physiological and environmental factors on the accumulation of oxindole during anaerobic indole metabolism was investigated by high-performance liquid chromatography. Under methanogenic conditions, indole was temporarily converted to oxindole in stoichiometric amounts in media inoculated with three freshwater sediments and an organic soil. In media inoculated with methanogenic sewage sludge, the modest amounts of oxindole detected at 35°C reached higher concentrations and persisted longer when the incubation temperature was decreased from 35 to 15°C. Also, decreasing the concentration of sewage sludge used as an inoculum from 50 to 1% caused an increase in the accumulation of oxindole from 10 to 75% of the indole added. Under denitrifying conditions, regardless of the concentration or source of the inoculum, oxindole appeared in trace amounts but did not accumulate during indole metabolism. In addition, denitrifying consortia which previously metabolized indole degraded oxindole with no lag period. Our data suggest that oxindole accumulation under methanogenic, but not under denitrifying conditions is caused by differences between relative rates of oxindole production and destruction.

Because the metabolic activities of microorganisms often influence the fate of organic pollutants, prudent management of surface water and groundwater may best be achieved through an understanding of the prevalence of a particular microbiological transformation and its response to physiological influences. Conclusions about the biodegradability of a chemical may be misleading if based on results from only one site or one type of environment (10). For example, Wang et al. (22) found that sediments from two lakes, but not a third, mineralized the acaricide chlorobenzilate. Moreover, in anaerobic studies, the prevailing type of microbial metabolism, as defined by final electron acceptor, has been found to govern the occurrence of specific metabolic transformations (2, 25). For example, aryl dehalogenation was demonstrated in subsurface sediments under methanogenic but not sulfate-reducing conditions (9).

Despite the potential for the contamination of drinking waters by N-heterocyclic compounds (12, 17) and although aspects of their aerobic metabolism are understood (6), little is known about the fate of these compounds in anaerobic environments. Most studies describing anaerobic metabolism of N-heterocyclic compounds have involved pure cultures of fermentative organisms (7, 8, 16, 19). However, Balba and Evans (1) and Wang et al. (23) using methanogenic consortia have reported transformations of tryptophan and indole, respectively. More recently, Berry et al. (3) found that indole is hydroxylated to an intermediate compound, oxindole, before being mineralized by sewage sludge under methanogenic conditions.

This report addresses the concept that anaerobic biodegradation of organic compounds may be governed by the prevailing metabolic regimen (i.e., methanogenesis or denitrification) and is qualitatively independent of the source of the active microbial community. Accordingly, we assessed the influence of inoculum source, inoculum concentration,

temperature, and pH on methanogenic indole transformation. In addition, indole metabolism under denitrifying conditions was examined.

MATERIALS AND METHODS

Culture conditions. A mineral-salts medium was prepared as described by Berry et al. (3). After being autoclaved for 15 min to remove O_2, the medium was maintained under a positive pressure of N_2 gas which was previously passed through copper filings at 300°C to remove traces of O_2. To facilitate its dissolution, we added crystalline indole when the medium temperature had cooled to 50°C; the final indole concentration was 32 to 50 mg (0.27 to 0.43 mmol) per liter. When the medium reached room temperature, 1.2 g of $NaHCO_3$ and 0.12 g of $Na_2S \cdot 9H_2O$ were added and the pH was adjusted with a solution of HCl. Unless otherwise stated, the pH of the medium was 7.0. To inhibit methanogenesis and favor denitrification, we altered the medium by omitting $Na_2S \cdot 9H_2O$ and adding KNO_3 (3.1 g/liter). Oxindole (95 mg/10 ml of boiled, partially cooled distilled water) was added to serum bottles through the rubber stoppers with a syringe.

After collection, freshwater sediments from Buffalo Run stream (Bellefonte, Pa.) and an organic soil (Carlisle muck from State College, Pa.) were diluted at a ratio of 1:3 (vol/vol) with media, stirred vigorously, and dispensed as slurries to N_2-flushed serum bottles (160 ml). The media were added to the serum bottles in 100-ml portions so that the final concentration of the soil and sediments was 0.5%. Municipal sewage sludge was collected from primary anaerobic digesters (State College, Pa.), filtered through cheesecloth, and mixed with the media in ratios ranging from 1:100 to 1:1. When mixing was 1:1, the amount of indole added to the medium was doubled. While the inoculated media were vigorously stirred, 100-ml portions were dispensed to the N_2-flushed serum bottles. All serum bottles were subsequently sealed with butyl rubber stoppers and aluminum crimp seals and incubated stationary in the dark. Unless otherwise indicated, incubation temperatures for bottles
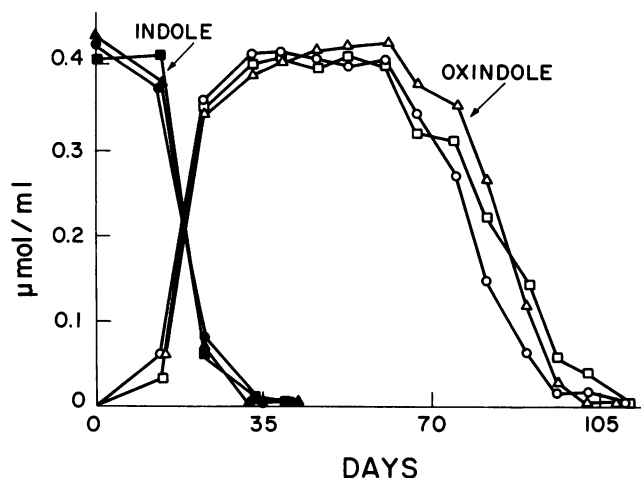
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* Corresponding author.

FIG. 1. Methanogenic transformation of indole by suspensions of sediment (0.5%) derived from the shore (■, □), edge (●, O), and bottom (▲, △) of Buffalo Run stream.

containing sewage sludge, freshwater sediments, and organic soil were 35, 22, and 22°C, respectively. All treatments were prepared in triplicate. Control bottles were sterilized by autoclaving on three successive days. The initial concentration of both indole and oxindole in sterile treatments remained unchanged throughout the incubation periods.

Efficacy of medium components in permitting methanogenesis or denitrification was confirmed by analyzing liquids in the serum bottles and headspace gases. The methanogenic treatments always produced methane. However, methanogenesis was eliminated completely when the reducing agent ($Na_2S \cdot 9H_2O$) was omitted and a high concentration of nitrate was added to the medium. Also, traces of nitrite and nitrous oxide always appeared, although transiently, under denitrifying conditions.

Analyses. The medium (2 ml) was withdrawn periodically from serum bottles, frozen immediately, and stored frozen. Subsequently, samples were thawed, mixed with methanol (1:1, vol/vol), and centrifuged ($8,000 \times g$). The supernatant was then filtered through a nylon filter (0.2-μm pore size) and injected onto a high-performance liquid chromatograph. The concentrations of indole and oxindole were then monitored with a high-performance liquid chromatography system (Waters Associates, Inc., Milford, Mass.) which consisted of a 6000A pump, a U6K injector, and a 480 Lambda-Max variable-wavelength spectrophotometer. Compound separation was achieved through a Radial-Pak cartridge (Nova C_{18}, 5 μm; Waters Associates) with a radial compression module (RCM-100). The mobile phase consisted of water and methanol (1:1, vol/vol) at a flow rate of 1.5 ml/min.

Quantification of indole and oxindole was done by the external standards method at a wavelength of 271 nm. Peak areas were measured with a 3390A Integrator (Hewlett-Packard Co., Palo Alto, Calif.). Calibration curves for indole and oxindole were linear in the concentration range of 3 to 50 μg/ml (0.026 to 0.43 μmol/ml).

Production of methane and nitrous oxide in the serum bottles was determined by injecting 100 μl of headspace gas into a series 1800 Varian Aerograph gas chromatograph. The gas chromatograph was equipped with a thermal conductivity detector and a Porapak Q column (600 cm, 50/80 mesh) maintained at 50°C. The detector temperature was 200°C. Helium was the carrier gas at a flow rate of 40 ml/min.

The transient appearance of nitrite in denitrifying media was determined with an ion chromatograph (Dionex, 2010i; Sunnyvale, Calif.) fitted with an Anion Fiber Suppressor and Anion Separator columns (3 by 250 mm). The eluent was 0.003 M $NaHCO_3$–0.0024 M Na_2CO_3. The flow rate was 180 ml/h, and the injection volume was 100 μl.

RESULTS

Freshwater sediments. Complete metabolism of indole by methanogenic sediments from the shore, edge, and bottom of Buffalo Run stream occurred within 33 days (Fig. 1). As indole disappeared, stoichiometric amounts of oxindole appeared in the sediment suspensions and persisted for over 4 weeks. Subsequently, oxindole was eliminated by all three inocula over 110 days. The pattern of oxindole appearance during indole metabolism by these methanogenic freshwater sediments (at 22°C with an inoculum of 0.5%) differed markedly from our previous experiments with methanogenic sewage sludge (3). In the earlier tests, whose temperature (35°C), inoculum source (sewage sludge), and inoculum concentration (9%) differed from that shown in Fig. 1, only small amounts of oxindole briefly accumulated.

Influence of temperature and concentration of sewage sludge. To identify the cause of stoichiometric production of oxindole (Fig. 1), we examined the effects of temperature and inoculum concentration on indole metabolism by sewage sludge microorganisms. As the incubation temperature declined from 35 to 15°C, the rates of indole and oxindole metabolism by 9% sewage sludge decreased (Fig. 2). Less predictably, the 20°C decrease in temperature had two additional effects: a nearly threefold increase in the maximum concentration of oxindole, and a delay in the onset of oxindole disappearance relative to that of indole.
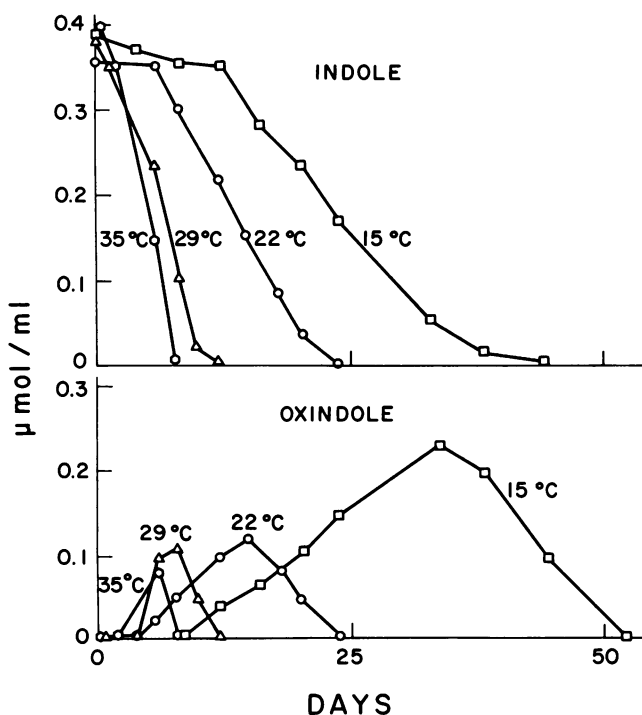

FIG. 2. Disappearance of indole and appearance of oxindole at different temperatures in 9% digested sludge under methanogenic conditions.

Dilution of sewage sludge from a concentration of 50 to 1% caused a slight increase in the lag time and total time required for indole disappearance at 35°C (Fig. 3). Also, the rate of indole metabolism appeared to be diminished slightly. More significantly, in bottles containing 50% sewage sludge, the maximum concentration of oxindole (which occurred after 3 days) corresponded to only 10% of the original substrate (Fig. 3). As the concentration of sewage sludge decreased to 5 and 1%, the maximum oxindole concentration increased in amounts representing 58% (day 6) and 75% (day 8), respectively, of the initial indole. Thus, decreasing either the temperature of incubation (Fig. 2) or the concentration of sewage sludge (Fig. 3) shifted indole metabolism toward oxindole accumulation.

Influence of pH. The experimental design which led to the data shown in Fig. 2 was modified to investigate the influence of pH on oxindole accumulation. With the temperature held constant at 35°C and with a 5% sewage sludge inoculum, the maximum observed concentrations of oxindole were found to be 63, 67, 66, and 58% of the 0.43 μmol of indole per ml initially added at pH 5, 6, 7, and 8, respectively. Although these maxima occurred at different times (days 18, 10, 7, and 8 for pH 5, 6, 7, and 8, respectively), they were not significantly different, as judged by an F test at the 95% confidence level. Thus, oxindole accumulation during methanogenic indole metabolism was unchanged by this range of pH values.

Organic soil and sewage sludge under methanogenic and denitrifying conditions. The results of indole metabolism by suspensions of an organic soil (Carlisle muck) under denitrifying and methanogenic conditions are shown in Fig. 4. Conditions which favored methanogenesis resulted in a pattern of indole degradation and oxindole accumulation very similar to that seen for freshwater sediments (Fig. 1). The concentration of indole fell below the detection limit by
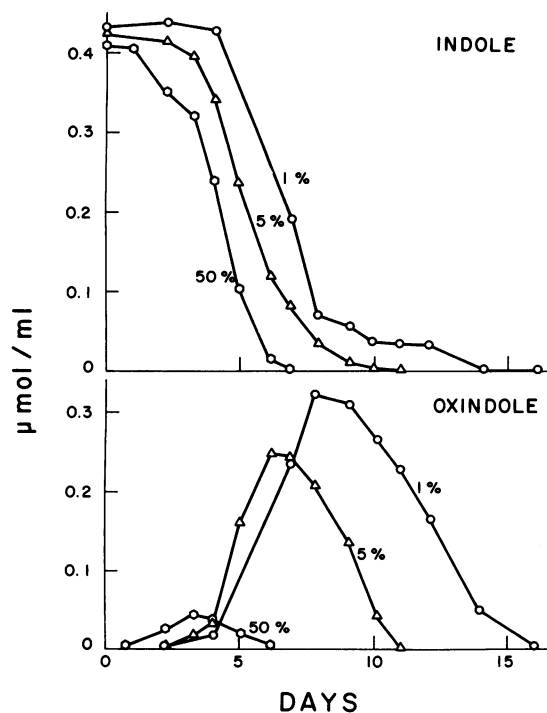

FIG. 4. Transformation of indole by suspensions of Carlisle muck (0.5%) under methanogenic and denitrifying conditions.

FIG. 3. Disappearance of indole and appearance of oxindole in a medium containing 50, 5, or 1% methanogenic digested sludge.

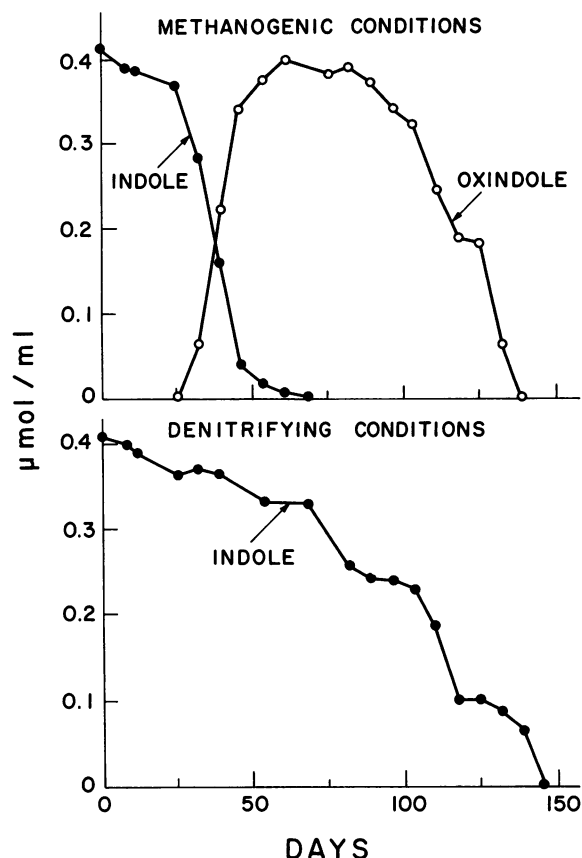day 67. Concurrently, oxindole appeared, persisted in near stoichiometric amounts for approximately 3 weeks, and subsequently fell below the limits of detection within 136 days.

In a separate experiment with a 20-fold-higher inoculum of Carlisle muck (10%), the highest oxindole concentration observed corresponded to 21% of the 0.41 μmol/ml of indole initially added (data not shown). Thus, an inverse relationship between concentration and oxindole accumulation was observed for both sewage sludge and the organic soil.

In contrast to tests under methanogenic conditions, no oxindole accumulated under denitrifying conditions (Fig. 4) during the 144 days which were required for indole to disappear. However, oxindole was detected in trace amounts on days 102, 108, and 116. On day 153, oxindole was added at a concentration of 0.37 μmol/ml to the serum bottles containing the denitrifying Carlisle muck inoculum; this oxindole was eliminated within 19 days without a lag period (data not shown).

Indole metabolism in 9% sewage sludge under methanogenic and denitrifying conditions was also examined (Fig. 5). During the 7 days required for indole elimination under methanogenic conditions, oxindole accumulated temporarily in the medium, reaching a maximum on day 4. However, under denitrifying conditions, during the 7 days required for indole disappearance, no oxindole accumulated, although a trace amount was detected at the end of day 5. On day 6, oxindole was added to the serum bottles at a concentration of 0.37 μmol/ml so that rates of oxindole disappearance could be measured simultaneously under both physiological
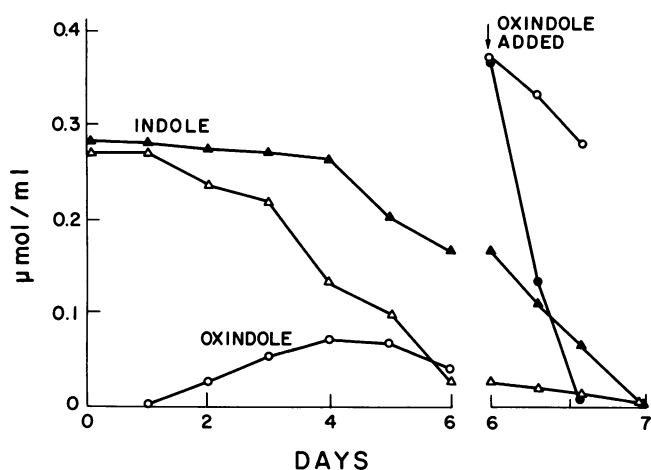
FIG. 5. Transformation of indole and oxindole by 9% digested sludge under methanogenic (○, △) and denitrifying (●, ▲) conditions.

conditions. Within 14 h after oxindole addition, the concentration of indole dropped from 0.16 to 0.07 μmol/ml and nearly all the oxindole was metabolized under denitrifying conditions. During the same period under methanogenic conditions, the oxindole concentration declined from 0.37 to 0.28 μmol/ml. Thus, under denitrifying conditions, the rate of oxindole disappearance exceeded the rate of disappearance of its likely precursor, indole. Also, oxindole was metabolized more slowly under methanogenic conditions (with oxindole accumulation) than under denitrifying conditions (with no oxindole accumulation). The tests with sewage sludge microorganisms confirm the pattern found for Carlisle muck. Under denitrifying conditions, oxindole was detected in trace quantities only during the period of most rapid indole loss. In addition, oxindole was metabolized without a lag period by cultures previously exposed to indole. This absence of a lag period contrasts with our observation (unpublished data) that 2 days pass before the onset of oxindole disappearance in 9% sewage sludge not previously exposed to indole.

DISCUSSION

Using mass spectrometry and fractionation of radioactivity Berry et al. (3) showed that oxindole is an intermediate metabolite during methanogenic indole transformation in sewage sludge. The present investigation extends the earlier physiological information toward the area of environmental pollution and its control. During methanogenic transformation of indole by microorganisms derived from freshwater sediments and an organic soil, oxindole temporarily accumulated in stoichiometric amounts. Gibson and Suflita (9) reported a similar transient stoichiometric conversion of 3,4-dichlorobenzoate to monochlorinated benzoates by pond sediments and aquifer solids. In instances in which such conversions occur, unforeseen health and environmental problems may develop because the new compound is likely to differ from its precursor in both toxicological and transport properties. Knowledge of habitat characteristics such as temperature, pH, prevalent electron acceptor for microbial metabolism, and the likelihood of dilution or mixing (at stream-sediment interfaces, for example) may allow particular conversions of organic compounds to be predicted, thus facilitating the management of water quality and its use.

Results presented here contribute to the concept that anaerobic biodegradation of an organic chemical is strongly influenced by the prevailing metabolic regimen (methanogenesis or denitrification) and several environmental factors, but apparently not by the inoculum source. Under methanogenic conditions, an inverse relationship between inoculum concentration and oxindole accumulation was found with sewage sludge (Fig. 3) and Carlisle muck. Also, temporary stoichiometric conversion of indole to oxindole was effected by both Carlisle muck (Fig. 4) and freshwater sediments (Fig. 1). Moreover, by the manipulation of temperature and inoculum concentration, the trend toward conversion of indole to oxindole by sewage sludge (Fig. 2 and 3) was found to resemble that of freshwater sediments (Fig. 1). If neither temperature nor inoculum concentration influenced oxindole accumulation, unique metabolic characteristics of the two different microbial communities would be implicated.

We also observed a uniform pattern of indole metabolism under denitrifying conditions, regardless of the source of inoculum. With both Carlisle muck (Fig. 4) and sewage sludge (Fig. 5), oxindole never accumulated at high levels. However, traces of oxindole were detected during the period of rapid indole disappearance, and added oxindole was metabolized without a lag period. These two observations suggest that oxindole is an intermediate metabolite of indole during its biodegradation under denitrifying conditions. Accordingly, the absence of oxindole accumulation during indole metabolism under denitrifying conditions could be explained by a rate of oxindole disappearance that equals the rate of its appearance. Data supporting this hypothesis are shown in Fig. 5, in which oxindole loss actually exceeded the rate of disappearance of its likely precursor, indole. Tests which prove that oxindole is a metabolite of indole under denitrifying conditions have not yet been completed.

Oxindole accumulation during anaerobic indole biodegradation appears to be controlled by competing rates of oxindole formation and elimination. These rates are affected differentially by temperature, concentration of inoculum, and the prevailing type of physiological metabolism. The sequential formation and elimination of oxindole seemed to predominate in tests at low temperature and low inoculum concentration, while simultaneous formation and elimination of oxindole appeared to operate at higher temperature and inoculum concentration. We do not know if the effect of inoculum dilution resulted from reduced numbers of microorganisms or from diluted carbon, nutrients, inhibitors, predators, etc., which were present in the inocula. Regardless of temperature, inoculum source, or inoculum concentration, oxindole always accumulated under methanogenic conditions, but never under denitrifying conditions. Thus, the type of physiological metabolism (as defined by final electron acceptor) had a dominant effect on oxindole accumulation during indole transformation. The mechanism which shifted relative rates of oxindole production and elimination is uncertain, but we speculate that community structure, species diversity, and the size and activity of microbiological populations (which varied with temperature, concentration of inocula, and final electron acceptor) caused the observed patterns of oxindole accumulation.

Investigations of carbon and electron flow during organic matter decomposition in freshwater and saltwater habitats have provided abundant information describing the ecological relationships between different groups of anaerobic microorganisms. Such studies have focused on competition between methanogens and sulfate reducers (13, 24) and the interaction between denitrifying and sulfate-reducing micro-

organisms (14). A related, but distinct, area of comparative anaerobic metabolism emphasizes transformation of the electron donor rather than the electron acceptor. For example, substituted benzenes (15), halogenated organic compounds (4), and a probable indole metabolite, anthranilic acid (5), have been transformed under denitrifying conditions in sediment samples and in media inoculated with sewage or single microorganisms, respectively. Similarly, under methanogenic conditions, consortia have metabolized ferulate and benzoate (11) and hydroquinone and catechol (20). Like Gibson and Suflita (9), who monitored the appearance of dichlorophenoxyacetates under sulfate-reducing and methanogenic conditions, we found that the appearance of a transformation product can be regulated by the final electron acceptor being utilized in the reaction. However, the findings of the two studies provide different physiological reasons for the accumulation of a chemical under one but not another anaerobic regime. Gibson and Suflita (9) suggest that sulfate inhibits a dehalogenation reaction; thus, dichlorophenoxyacetates appeared during the metabolism of 2,4,5-trichlorophenoxyacetate only when sulfate had been depleted and methanogenesis had begun. Our data do not suggest that methanogenic and denitrifying metabolic pathways of indole are different but rather that differences between competing rates cause oxindole accumulation under methanogenic but not denitrifying circumstances.

The objective of the present investigation was to gain insight into several environmental and physiological factors which influence the anaerobic biodegradation of indole. We recognize that the rates of indole and oxindole metabolism implicit in our data should not be considered absolute because these rates will vary with cell density and substrate concentration. In this regard, Simkins and Alexander (18) have rigorously demonstrated that the relationship between cell density and substrate concentration controls patterns and rates of chemical mineralization by microorganisms.

The relatively low toxicities of indole and oxindole (21) and consistent biodegradability in our tests indicate that these compounds pose little, if any, environmental hazard. However, our results emphasize that relative rates of competing processes may vary with environmental conditions and cause the accumulation of intermediate compounds in one situation but not another.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Balba, M. T., and W. C. Evans. 1980. Methanogenic fermentation of the naturally occurring aromatic amino acids by a microbial consortium. Biochem. Soc. Trans. 8:625–627.
2. Berry, D. L., A. J. Francis, and J.-M. Bollag. 1987. Microbial metabolism of homocyclic and heterocyclic aromatic compounds under anaerobic conditions. Microbiol. Rev. 51:43–59.
3. Berry, D. F., E. L. Madsen, and J.-M. Bollag. 1987. Conversion of indole to oxindole under methanogenic conditions. Appl. Environ. Microbiol. 53:180–182.
4. Bouwer, E. J., and P. L. McCarty. 1983. Transformations of halogenated organic compounds under denitrifying conditions. Appl. Environ. Microbiol. 45:1295–1299.
5. Braun, K., and D. T. Gibson. 1984. Anaerobic degradation of 2-aminobenzoate (anthranilic acid) by denitrifying bacteria. Appl. Environ. Microbiol. 48:102–107.
6. Callely, A. G. 1978. The microbial degradation of heterocyclic compounds. Prog. Ind. Microbiol. 14:205–281.
7. Campbell, L. L. 1957. Reductive degradation of pyrimidines. I. The isolation and characterization of a uracil-fermenting bacterium, Clostridium uracilicum nov. spec. J. Bacteriol. 73:220–224.
8. Durre, P., W. Andersch, and J. R. Andreesen. 1981. Isolation and characterization of an adenine-utilizing, anaerobic spore-former, Clostridium purinolyticum sp. nov. Int. J. Syst. Bacteriol. 31:184–194.
9. Gibson, S. A., and J. M. Suflita. 1986. Extrapolation of biodegradation results to groundwater aquifers: reductive dehalogenation of aromatic compounds. Appl. Environ. Microbiol. 52:681–688.
10. Gledhill, W. E., B. T. Johnson, M. Kitano, and C. M. Lee. 1980. Synopsis of discussion session: biodegradation methodology, p. 46–52. In A. W. Maki, K. L. Dickson, and J. Cairns, Jr. (ed.), Biotransformation and fate of chemicals in the aquatic environment. American Society for Microbiology, Washington, D.C.
11. Grbic-Galic, D., and L. Y. Young. 1985. Methane fermentation of ferulate and benzoate: anaerobic degradation pathways. Appl. Environ. Microbiol. 50:292–297.
12. Hawthorne, S. B., and R. E. Sievers. 1984. Emission of organic air pollutants from shale oil wastewaters. Environ. Sci. Technol. 18:483–490.
13. Isa, Z., S. Grusenmeyer, and W. Verstraete. 1986. Sulfate reduction relative to methane production in high-rate anaerobic digestion: microbiological aspects. Appl. Environ. Microbiol. 51:580–587.
14. Jenneman, G. E., M. J. McInerney, and R. M. Knapp. 1986. Effect of nitrate on biogenic sulfide production. Appl. Environ. Microbiol. 51:1205–1211.
15. Kuhn, E. P., P. J. Colberg, J. L. Schnoor, O. Wanner, A. J. B. Zehnder, and R. P. Schwarzenbach. Microbial transformations of substituted benzenes during infiltration of river water to groundwater: laboratory column studies. Environ. Sci. Technol. 19:961–968.
16. Pastan, I., L. Tsai, and E. R. Stadtman. 1964. Nicotinic acid metabolism. J. Biol. Chem. 239:902–906.
17. Robbins, W. K., and S. C. Blum. 1981. Analysis of shale oil liquids for polynuclear aromatic hydrocarbons and their N-heterocyclic analogs, p. 45–71. In W. H. Griest, M. R. Guerin, and D. L. Coffin (ed.), Health effects investigation of oil shale development. Ann Arbor Science Publishers, Inc., Ann Arbor, Mich.
18. Simkins, S., and M. Alexander. 1984. Models for mineralization kinetics with variables of substrate concentration and population density. Appl. Environ. Microbiol. 47:1299–1306.
19. Stadtman, E. R., T. C. Stadtman, I. Pastan, and L. D. Smith. 1972. Clostridium barkeri sp. n. J. Bacteriol. 110:758–760.
20. Szewzyk, U., R. Szewzyk, and B. Schink. 1985. Methanogenic degradation of hydroquinone and catechol via reductive dehydroxylation to phenol. FEMS Microbiol. Ecol. 31:79–87.
21. Tatken, R. L., and R. J. Lewis, Sr. (ed.). 1983. Registry of toxic effects of chemical substances. U.S. Department of Health and Human Services, Cincinnati.
22. Wang, Y.-S., E. L. Madsen, and M. Alexander. 1985. Microbial degradation by mineralization or cometabolism determined by chemical concentration and environment. J. Agric. Food Chem. 33:495–499.
23. Wang, Y.-T., M. T. Suidan, and J. T. Pfeffer. 1984. Anaerobic biodegradation of indole to methane. Appl. Environ. Microbiol. 48:1058–1060.
24. Ward, D. M., and M. R. Winfrey. 1985. Interactions between methanogenic and sulfate-reducing bacteria in sediments. Adv. Aquat. Microbiol. 3:141–179.
25. Young, L. Y. 1984. Anaerobic degradation of aromatic compounds, p. 487–523. In D. T. Gibson (ed.), Microbial degradation of organic compounds. Marcel Dekker, Inc., New York.