Anaerobic Capacities of Leaf Litter

KIRSTEN KÜSEL AND HAROLD L. DRAKE*

*Lehrstuhl für Ökologische Mikrobiologie, BITÖK, Universität Bayreuth,
95440 Bayreuth, Germany*

Received 19 March 1996/Accepted 14 August 1996

Leaf litter displayed a capacity to spontaneously form organic acids, alcohols, phenolic compounds, H₂, and CO₂ when incubated anaerobically at 20°C either as buffered suspensions or in a moistened condition in microcosms. Acetate was the predominant organic product formed regardless of the degree of litter decomposition. Initial rates of acetate formation in litter suspensions and microcosms approximated 2.6 and 0.53 μ mol of acetate per g (dry weight) of litter per h, respectively. Supplemental H₂ was directed towards the apparent acetogenic synthesis of acetate. Acetoclastic methanogenesis was induced by partially decomposed litter after extended lag phases; freshly fallen litter did not display this capacity.

There is considerable evidence confirming the association of anaerobic processes (16, 21, 24, 26, 32, 36) and anaerobic bacteria (2, 7, 12, 16, 22, 38) with terrestrial soils. Anaerobic zones are present in soil aggregates, and anaerobic processes in soils are stimulated by rainfall (21, 24, 25). The net emission of methane from a forest soil has recently been demonstrated; in that study, the methanogenic activity of the litter was greater than that of the mineral soil (36). This observation supports the concept that the formation of anaerobic microsites in terrestrial soils is not restricted to aggregated mineral soil but is dependent on the localized concentrations of readily available microbial substrates (26). Because litter has a large amount of labile organic matter (28), this upper-soil material might harbor anaerobic processes in situ under certain conditions. In particular, low-molecular-weight organic acids indicative of anaerobic processes (11) are present in the organic horizons of forest soils (9, 27, 29). The objective of the present study was to evaluate the anaerobic capacities of the litter layers of a beech forest site.

Preparation and analysis of litter suspensions and microcosms. Litter was obtained in November 1993 after foliage litter fall and in August 1994 from a beech (*Fagus sylvatica* L.) forest on limestone located at Geisberger Forst in east-central Germany. The top leaf litter layer (L horizon) had a thickness of 1 to 2 cm, and the underlying weakly decomposed litter layer (Of horizon) varied in thickness over the year (0 to 1.5 cm). The mean annual precipitation approximated 850 mm/year (37), and maximum summer temperatures approximated 23°C. Pooled samples from each horizon were collected in plastic bags, stored at 2°C, and evaluated within 24 h. The pHs of the L and Of horizons (measured in 1:2.5 [vol/vol] suspensions in 0.02 N CaCl₂) were 6.8 and 6.5, respectively, for litter collected in November; the pH of leaf litter collected in August was 5.6. The organic carbon and nitrogen contents of oven-dried (60°C) homogenized litter were quantitated with an element analyzer (CHN-O-Rapid; Foss-Heraeus, Hanau, Germany). The organic carbon contents of the L and Of horizons approximated 470 and 390 g/kg (dry weight) of litter, respectively; the nitrogen contents of the L and Of horizons approximated 9 and 17 g/kg (dry weight) of litter, respectively. Dry weights of organic mat-

ter were obtained by weighing before and after drying for 4 days at 60°C.

For litter suspensions, twigs and large pieces of roots were removed aseptically and 30 g (fresh weight) of litter was placed into sterile 500-ml infusion bottles (Merck ABS, Dietikon, Switzerland). After anaerobic conditions were established by repeatedly (three times) evacuating the bottles and flushing them with N₂, 270 ml of a sterile reduced bicarbonate-buffered mineral solution (15) was added. Bottles were closed with rubber stoppers and aluminum seals and put on an end-over-end shaker for 20 min. Bottles were then incubated horizontally (without shaking) in the dark at 20°C under an atmosphere of either N₂-CO₂ or H₂-CO₂ (80:20, vol/vol, with an initial overpressure of 25 to 30 kPa at room temperature). The initial pH of the litter suspensions approximated 6.8 and did not decrease below 6.5 during incubation.

For litter microcosms, summer-dried leaf litter (L horizon) (5 to 6 g [fresh weight] of litter) was placed into sterile 500-ml infusion bottles which were closed with rubber stoppers and aluminum seals. After anaerobic conditions were established by repeatedly (three times) evacuating the bottles, and flushing them with N₂, 10 ml of anaerobic, degassed, sterile, deionized water was applied evenly over the leaf litter by syringe; this water was completely absorbed. Bottles were then incubated upright (without shaking) in the dark at 20°C. Following the incubation period, a 10-ml aliquot of water was added. Bottles were then placed on an end-over-end shaker for 10 min and subsequently placed on the seals (i.e., upended) to allow the excess water to accumulate in the neck of the bottle. After the leachate was removed with a sterile syringe, its volume and pH were determined prior to the analysis of organic compounds. These irrigations were repeated at 3- to 8-h intervals. During initial irrigations, the supplemented water was partially retained; however, after four to five supplementations of water, a balance between input and output of water was reached.

Headspace gases were measured with Hewlett-Packard Co. (Palo Alto, Calif.) 5980 series II gas chromatographs as previously described (16). Negative pressures that developed concomitantly with H₂ uptake were corrected with sterile argon. Gas solubilities were calculated from standard solubility tables (31); gas values included the total amounts in both the liquid and gas phases. Aliphatic acids, alcohols, and aromatic compounds were identified with Hewlett-Packard 1090 series II high-performance liquid chromatographs as previously described (16). Aqueous samples were clarified by centrifugation and microfiltration prior to analyses. All data are means of

* Corresponding author. Phone: 49-(0)921-555 640. Fax: 49-(0)921-555 799. Electronic mail address: harold.drake@bitok.uni-bayreuth.de.

FIG. 1. Initial (A) and long-term (B) product profiles of anaerobic leaf litter suspensions. The summer-dried L horizon was collected in August 1994 from Geisberger Forst. The initial gas phase was N_2 - CO_2 . ▲, acetate; □, ethanol; ◆, malonate; +, formate; ●, isopropanol; +, lactate; ○, propionate; ▽, butyrate; ▽, isobutyrate; △, succinate; ■, methane.

triplicate experiments. The coefficient of variation approximated 3%; the maximum variance observed was 17%. Statistical significance was determined by the Student *t* test ($P < 0.05$).

Initial and long-term product profiles of anaerobic litter suspensions and microcosms. Summer-dried leaf litter initially formed short-chain carboxylic acids and alcohols when incubated anaerobically as buffered suspensions (Fig. 1A). Subsequent to carboxylic acid and alcohol synthesis, malonate, formate, and lactate were consumed. After 20 h, acetate production increased concomitantly with the turnover of these compounds, with rates approximating $3.3 \mu\text{mol}$ of acetate per h per g (dry weight) of litter. Trace levels of propionate and butyrate were detected after 20 and 50 h, respectively. Transient amounts of H_2 (approximately $30 \mu\text{mol}$ of H_2 per g [dry weight] of litter) were formed. No methane was detected.

When summer-dried leaf litter was incubated at 20°C in microcosms and periodically wetted with small amounts of water under anaerobic conditions, the initial spectrum of organic acids and alcohols detected in the water phase was sim-

ilar to that in buffered litter suspensions (Fig. 2A). Small transient amounts of citrate, tartrate, and pyruvate were also detected (data not shown). The pH of the water removed between wettings decreased from 5.9 to 5.6 during the incubation period. During the initial 6 h, the compounds detected were below the concentration limits required for integration and thus could not be quantified. After 8 h of incubation, acetate was identified as the predominant product; ethanol, isopropanol, propionate, succinate, and butyrate also accumulated. Small amounts ($0.1 \mu\text{mol/g}$ [dry weight] of litter) of protocatechuate and 4-hydroxybenzoate were also detected. CO_2 and H_2 were produced in significant quantities (Fig. 2B). No methane was detected.

When suspensions of summer-dried leaf litter were incubated for longer periods, ethanol, formate, and isopropanol were consumed and the volatile fatty acids acetate, propionate, and butyrate accumulated (Fig. 1B). Acetate was the predominant organic product detected. Isobutyrate and isovalerate were detected sporadically in concentrations up to $20 \mu\text{mol/g}$

FIG. 2. Product profiles of leaf litter subjected to periodic wetting with water in anaerobic microcosms. The summer-dried L horizon was collected in August 1994 from Geisberger Forst. The initial gas phase was N_2 . Every acetate datum point in panel A corresponds to one cycle of sampling and rewetting. (A) Products in the aqueous phase (sum of the water absorbed in the bottle and the leachate). ▲, acetate; □, ethanol; ◆, malonate; +, formate; ●, isopropanol; +, lactate; ○, propionate; ▽, butyrate; △, succinate. (B) Products in the gas phase. ●, CO_2 ; +, H_2 ; ○, CO_2 obtained from dry litter (without wetting) under aerobic or anaerobic conditions.

FIG. 3. Consumption of supplemental H_2 by anaerobic suspensions of freshly fallen leaves (A) and decomposed leaf litter (B). The L and Of horizons (A and B, respectively) was collected in November 1993 from Geisberger Forst; the initial gas phase was either $\text{N}_2\text{-CO}_2$ or $\text{H}_2\text{-CO}_2$. $\times$, H_2 ($\text{H}_2\text{-CO}_2$); Δ , acetate ($\text{H}_2\text{-CO}_2$); $\blacktriangle$, acetate ($\text{N}_2\text{-CO}_2$); $\circ$, formate ($\text{H}_2\text{-CO}_2$); $\bullet$, formate ($\text{N}_2\text{-CO}_2$); $\square$, methane ($\text{H}_2\text{-CO}_2$); $\blacksquare$, methane ($\text{N}_2\text{-CO}_2$).

(dry weight) of litter; longer fatty acids were not detected. Catechol, protocatechuic acid, 4-hydroxybenzoate, 4-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, and benzoate in concentrations up to $2 \mu\text{mol/g}$ (dry weight) of litter were detected. Except for 3,4-dihydroxybenzaldehyde, all aromatics were consumed. Unidentified compounds with retention times indicative of aromatic compounds were also periodically detected, but on the basis of their A_{210} , their concentrations were low. Acetate, butyrate, and propionate were consumed after extensive incubation periods, and methane was produced concomitantly with their consumption (Fig. 1B). The extended lag phase observed prior to the onset of CH_4 production by partially decomposed litter in the present study was consistent with other results demonstrating that organic surface layers of forest soils have negligible methanogenic activity (19, 23).

Capacities of freshly fallen and decomposed litter to consume supplemental H_2 . Leaf litter from the top L horizon and from the underlying Of horizon consumed supplemental H_2 under anaerobic conditions (Fig. 3). For L-horizon suspensions, consumption of supplemental H_2 approximated $13 \mu\text{mol}$ of H_2 consumed per g (dry weight) of litter per day and yielded formate as a transient product (Fig. 3A). The final amounts of acetate formed were significantly larger for H_2 -supplemented

litter suspensions than for suspensions lacking H_2 (Fig. 3A). H_2 -supplemented Of-horizon litter did not form additional amounts of formate and consumed supplemental H_2 at rates approximating $22 \mu\text{mol}$ of H_2 consumed per g (dry weight) of litter per day (Fig. 3B). The increased amount of acetate formed by H_2 -supplemented L-horizon and Of-horizon suspensions yielded stoichiometries that approximated 6.4 and 4.8 mol of H_2 consumed per mol of acetate formed, respectively. These stoichiometries were indicative of H_2 -dependent acetogenesis (5, 6, 35): $4\text{H}_2 + 2\text{CO}_2 \rightarrow \text{CH}_3\text{COOH} + 2\text{H}_2\text{O}$. The initial formation and subsequent consumption of formate by L-horizon suspensions are suggestive of a nontightly coupled interspecies transfer of formate (30). H_2 and CO_2 can be converted to formate by a formate-hydrogen lyase (38). The subsequent acetogenic consumption of H_2 -derived formate (6) would also yield the 4:1 H_2 -to-acetate stoichiometry approximated in the present findings. After an extended incubation period, acetoclastic methanogenesis was engaged in by Of-horizon suspensions (Fig. 3B) but not by suspensions of freshly fallen litter (Fig. 3A).

Theoretical aspects of the anaerobic capacities of leaf litter for the turnover of organic carbon. Litter harbored a capacity to spontaneously form organic acids, alcohols, H_2 , and CO_2 when subjected to anaerobic conditions. These products are indicative of diverse fermentative processes. Acetate was the predominant organic product under anaerobic conditions. The averaged maximum amount of acetate formed in litter suspensions was approximately seven times greater (data not shown) on a dry-weight basis than the amount of acetate formed in mineral soil suspensions (16). The high-level initial anaerobic activities of summer-dried litter demonstrated that litter microfloras respond quickly to moist anaerobic conditions. Anaerobic processes might be initiated in situ after rainfalls or might be restricted to moist microzones between leaves. Certain obligate anaerobes and associated processes can survive extended periods of aerobic drying and are rapidly revived by anaerobiosis (8, 20, 32). The emission of methane from forest surface soil layers (36) demonstrates that obligate anaerobes can indeed achieve an active state in surface soils.

The anaerobic degradation of lignin appears to be mostly limited to the anaerobic utilization of low-molecular-weight lignin-derived compounds (3, 4). Vanillate, vanillin, ferulate, syringate, caffeate, *o*-coumarate, and *p*-coumarate are the predominant phenolic compounds extractable from beech litter (13, 14, 33, 34). Although these compounds were not detected in the incubation studies, it cannot be ruled out that they were formed and rapidly consumed. Aromatic methoxyl groups are readily utilized by many acetogens (10), and it is noteworthy that all aromatic compounds detected lacked methoxyl groups. The detection and subsequent consumption of small concentrations of catechol, protocatechuic acid, 4-hydroxybenzoate, and 4-hydroxybenzaldehyde indicated that litter-derived aromatic compounds were subject to further anaerobic breakdown.

Litter both produced and consumed H_2 under anaerobic conditions. The consumption of supplemental H_2 by L- and Of-horizon suspensions was concomitant with the apparent acetogenic synthesis of acetate. This is the first report on the potential occurrence of acetogenic bacteria in leaf litter; it is particularly surprising that acetogenic activity was so easily enriched from freshly fallen litter (Fig. 3A). The complete consumption of supplemental H_2 by suspensions of freshly fallen leaves (L horizon) was protracted in comparison with that by suspensions of Of horizons. Hydrogen enhances the capacity of certain acetogens to grow mixotrophically (1) and to use the methoxyl groups of aromatic compounds (17, 18). Hydrogen consumption under such conditions would be de-

pendent upon the availability of other substrates. Thus, the qualitative and quantitative differences in substrate availability between L and Of horizons might influence the utilization of H_2 . It is also possible that Of horizons harbored a greater number of H_2 -consuming bacteria than did freshly fallen leaves from the L horizons. In contrast to its capacity for the acetogenic uptake of H_2 , freshly fallen leaf litter did not display any methanogenic capacity. These contrasting observations may be due to the enhanced capacity of acetogens to tolerate periods of aeration (32).

We express sincere appreciation to the technical assistants of Central Analytics (BITÖK) for analyses of carbon and nitrogen and to C. Kuhnner for review of the manuscript.

Support for this study was provided by the German Ministry for Education, Research and Technology (0339476A0).

REFERENCES

- Breznak, J. A. 1994. Acetogenesis from carbon dioxide in termite guts, p. 303–330. In H. L. Drake (ed.), *Acetogenesis*. Chapman and Hall, Inc., New York.
- Chin, S., and S. Zinder. 1982. Unpublished results.
- Colberg, P. J., and L. Y. Young. 1985. Anaerobic degradation of soluble fractions of [^{14}C -lignin]lignocellulose. *Appl. Environ. Microbiol.* **49**:345–349.
- Colberg, P. J., and L. Y. Young. 1985. Aromatic and volatile acid intermediates observed during anaerobic metabolism of lignin-derived oligomers. *Appl. Environ. Microbiol.* **49**:350–358.
- Dickert, G. 1992. The acetogenic bacteria, p. 517–533. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), *The prokaryotes*, 2nd ed. Springer-Verlag, New York.
- Drake, H. L. 1994. Acetogenesis, acetogenic bacteria, and the acetyl-CoA "Wood/Ljungdahl" pathway: past and current perspectives, p. 3–60. In H. L. Drake (ed.), *Acetogenesis*. Chapman and Hall, Inc., New York.
- Dürre, P., W. Andersch, and J. R. Andreessen. 1981. Isolation and characterization of an adenine-utilizing, anaerobic sporeformer, *Clostridium purinolyticum* [sic] sp. nov. *Int. J. Syst. Bacteriol.* **31**:184–194.
- Fetzer, S., F. Bak, and R. Conrad. 1993. Sensitivity of methanogenic bacteria from paddy soil to oxygen and desiccation. *FEMS Microbiol. Ecol.* **12**:107–115.
- Fox, T. R., and N. B. Comerford. 1990. Low-molecular-weight organic acids in selected forest soils of the southeastern USA. *Soil Sci. Soc. Am. J.* **54**:1139–1144.
- Frazer, A. C. 1994. O-demethylation and other transformations of aromatic compounds by acetogenic bacteria, p. 445–483. In H. L. Drake (ed.), *Acetogenesis*. Chapman and Hall, Inc., New York.
- Gottschalk, G. 1986. Bacterial metabolism, 2nd ed., p. 208–282. Springer-Verlag, New York.
- Kuhnner, C. H., and A. Griefhammer. 1996. Characterization of a clostridial acetogen from mineral forest soil, abstr. N-71, p. 334. In Abstracts of the 96th General Meeting of the American Society for Microbiology 1996. American Society for Microbiology, Washington, D.C.
- Kuiters, A. T., and C. A. J. Denneman. 1987. Water-soluble phenolic substances in soils under several coniferous and deciduous tree species. *Soil Biol. Biochem.* **19**:765–769.
- Kuiters, A. T., and H. M. Sarink. 1986. Leaching of phenolic compounds from leaf and needle litter of several deciduous and coniferous trees. *Soil Biol. Biochem.* **18**:475–480.
- Küsel, K., and H. L. Drake. 1994. Acetate synthesis in soil from a Bavarian beech forest. *Appl. Environ. Microbiol.* **60**:1370–1373.
- Küsel, K., and H. L. Drake. 1995. Effects of environmental parameters on the formation and turnover of acetate by forest soils. *Appl. Environ. Microbiol.* **61**:3667–3675.
- Liu, S., and J. M. Suflita. 1993. H_2 - CO_2 -dependent anaerobic O-demethylation activity in subsurface sediments and by an isolated bacterium. *Appl. Environ. Microbiol.* **59**:1325–1331.
- Liu, S., and J. M. Suflita. 1995. H_2 as an energy source for mixotrophic acetogenesis from the reduction of CO_2 and syringate by *Acetobacterium woodii* and *Eubacterium limosum*. *Curr. Microbiol.* **31**:245–250.
- Magnusson, T. 1993. Carbon dioxide and methane formation in forest mineral and peat soils during aerobic and anaerobic incubations. *Soil Biol. Biochem.* **25**:877–883.
- Mayer, H. P., and R. Conrad. 1990. Factors influencing the activity of methanogenic bacteria and the initiation of methane production upon flooding of paddy soil. *FEMS Microbiol. Ecol.* **73**:103–112.
- Parkin, T. B., and J. M. Tiedje. 1984. Application of a soil core method to measure field denitrification rates. *Soil Biol. Biochem.* **16**:323–330.
- Peters, V., and R. Conrad. 1995. Methanogenic and other strictly anaerobic bacteria in desert soil and other oxic soils. *Appl. Environ. Microbiol.* **61**:1673–1676.
- Sextstone, A. J., and C. N. Mains. 1990. Production of methane and ethylene in organic horizons of spruce forest soils. *Soil Biol. Biochem.* **22**:135–139.
- Sextstone, A. J., T. B. Parkin, and J. M. Tiedje. 1984. Temporal response of soil denitrification rates to rainfall and irrigation. *Soil Sci. Soc. Am. J.* **49**:99–103.
- Sextstone, A. J., N. P. Revsbech, T. B. Parkin, and J. M. Tiedje. 1985. Direct measurement of oxygen profiles and denitrification rates in soil aggregates. *Soil Sci. Soc. Am. J.* **49**:645–651.
- Smith, K. A., and J. R. M. Arah. 1986. Anaerobic micro-environments in soil and the occurrence of anaerobic bacteria. *FEMS Symp.* **33**:247–261.
- Stevenson, F. J. 1967. Organic acids in soil, p. 119–146. In A. D. McLaren and G. H. Peterson (ed.), *Soil biochemistry*, vol. 1. Marcel Dekker, New York.
- Stevenson, F. J. 1994. Humus chemistry, 2nd ed., p. 1–58. John Wiley & Sons, New York.
- Tani, M., T. Higashi, and S. Nagatsuka. 1993. Dynamics of low-molecular-weight aliphatic carboxylic acids (LACAs) in forest soils. I. Amount and composition of LACAs in different types of forest soils in Japan. *Soil Sci. Plant Nutr.* **39**:485–495.
- Thiele, J. H., and J. G. Zeikus. 1988. Control of interspecies electron flow during anaerobic digestion: significance of formate transfer versus hydrogen transfer during syntrophic methanogenesis in flocs. *Appl. Environ. Microbiol.* **54**:20–29.
- Umbreit, W. H., R. H. Burris, and J. F. Stauffer. 1972. Manometric and biochemical techniques. Burgess Publishing Co., Minneapolis.
- Wagner, C., A. Griefhammer, and H. L. Drake. 1996. Acetogenic capacities and the anaerobic turnover of carbon in a Kansas prairie soil. *Appl. Environ. Microbiol.* **62**:494–500.
- Whitehead, D. C., H. Dibb, and R. D. Hartley. 1981. Extractant pH and the release of phenolic compounds from soils, plant roots and leaf litter. *Soil Biol. Biochem.* **13**:343–348.
- Whitehead, D. C., H. Dibb, and R. D. Hartley. 1983. Bound phenolic compounds in water extracts of soils, plant roots and leaf litter. *Soil Biol. Biochem.* **15**:133–136.
- Wood, H. G., and L. G. Ljungdahl. 1991. Autotrophic character of acetogenic bacteria, p. 201–250. In J. M. Shively and L. L. Barton (ed.), *Variations in autotrophic life*. Academic Press, San Diego, Calif.
- Yavitt, J. B., T. J. Fahey, and J. A. Simmons. 1995. Methane and carbon dioxide dynamics in a northern hardwood ecosystem. *Soil Sci. Soc. Am. J.* **59**:796–804.
- Zech, W., R. Sterzer, M. Kauperjohann, and I. Kögel-Knabner. 1991. Bodengesellschaften des Bamberger Beckens und der nördlichen Frankenalb, p. 123–151. In W. Zech (ed.), *Bayreuther Bodenkundliche Berichte*, vol. 17. Exkursionsführer (Supplementband NO-Bayern) zur Jahressagung der deutschen Bodenkundlichen Gesellschaft 1991 in Bayreuth. Universität Bayreuth, Bayreuth, Germany.
- Zinder, S. H. 1993. Physiological ecology of methanogens, p. 128–206. In J. G. Ferry (ed.), *Methanogenesis*. Chapman & Hall, New York.