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January 2007

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Anaerobic oxalate consumption by microorganisms in forest soils

Steven L. Daniel, Christine Pils, Harold L. Drake

Research in Microbiology 158 (2007) pp. 303-309

Abstract

The microbial consumption of oxalate was examined under anaerobic conditions in soil suspensions at 15°C. With soil (horizon Ah, pH 6.4) from a beech forest, microbial consumption of added oxalate (15 mM) began after 10 days, and oxalate was totally consumed by day 20. The presence of supplemental electron donors (acetate, glucose, vanillate, or hydrogen) or electron acceptors (nitrate or sulfate) did not significantly influence anaerobic oxalate consumption, whereas supplementation of soil suspensions with CO₂/bicarbonate totally repressed oxalate consumption. Thus, CO₂-, nitrate- or sulfate-respiring bacteria were apparently not active in the anaerobic consumption of oxalate in these soil suspensions. With soil (horizon Bt, pH 7) from a beech forest, oxalate consumption began after an approximate lag of 14 days, and oxalate was totally consumed by day 41. With both soils, acetate was the major aliphatic organic acid detected during oxalate consumption. Near pH-neutral soils from two additional forest field sites were also competent in anaerobic oxalate consumption. In contrast, anaerobic oxalate consumption was negligible in suspensions prepared with acidic soils (<pH 4.2) collected from three different forest field sites. These results suggest that forest soils and their resident microbial populations have different capacities relative to anaerobic oxalate consumption.

1. Introduction

Oxalate ($\text{C}_2\text{O}_4^{2-}$) is a low-molecular-weight organic acid commonly found in soils [3,26]. Sources of oxalate in soils include: exudates from plant roots; breakdown products from plant, animal, and microbial biomass; and metabolites produced and excreted by microorganisms, especially free-living and mycorrhizal fungi [6,11,26,34]. Oxalate levels in soils and soil solutions range from 10^{-3} to 10^{-6} M with concentrations being slightly greater in rhizosphere soils than in non-rhizosphere soils [7,26]. The range in oxalate levels reflects the fact that in aerobic soils oxalate is constantly being synthesized and degraded [12,13,33]. The overall fate of oxalate in anaerobic soils is less obvious.

By virtue of its capacity to chelate metals, oxalate plays an important role in the solubilization and transport of soil metals, including the weathering of rock and podzolization [6,8,9,12,19]. Through its interactions with Al and Fe in soils, oxalate impacts plant nutrition by increasing the availability of P; other poorly soluble micronutrients (e.g., Fe, Mg, and Ca) in soils are also mobilized by the presence of

oxalate [9,12,28,29]. Moreover, given its ability to complex and remove (via immobilization or precipitation) excess metal cations, oxalate has the potential to detoxify such metals as Al, Ca, Ni, and Cu in soils [6,8e10]. Oxalate is not limited to metal interactions in soils. Recent studies have shown that oxalate can enhance the bioavailability of the persistent organic pollutant dichlorodiphenyltrichloroethane (DDT) in soils [20]. Thus, aerobic and anaerobic microbial processes in soils that affect oxalate availability or oxalate-metal interactions may ultimately influence the nutritional-toxicological status, including the pH [2,4,14], of terrestrial environments.

Information exists on the aerobic mineralization of soil oxalate by microorganisms [18,21,27], and, a large number of aerobic oxalate-degrading bacteria have been isolated from soils [1,24]. In contrast, little, if anything, is known about the turnover (e.g., synthesis or degradation) of oxalate in soils under anaerobic conditions or the types of soil microorganisms that engage in the anaerobic metabolism of oxalate. Therefore, the goals of this study were to determine the capacity of forest soil microorganisms to consume oxalate under anaerobic conditions and to assess the potential influence of environmental parameters (e.g., electron acceptors and donors) on the anaerobic consumption of oxalate.

2. Materials and methods

2.1. Soils

Soils were obtained from hardwood and coniferous forests in east-central Germany (Table 1); this region has a mean annual air temperature of 6e 8 _ C [16]. Unless noted otherwise, most of the experiments in this study were conducted with soil (Ah horizon) collected from different locations within a beech forest located at the Geisberg field site (Table 1). The Ah-horizon soil (0e 10 cm) is a well-drained, silty loam and contains approximately 95 and 6.7 g kg₋₁ (dry weight) of organic carbon and total nitrogen (C/N ratio of 14), respectively [15,16].

Field site (horizon)	Geology	Vegetation	pH (CaCl ₂)
<i>Near pH-neutral soils</i>			
Geisberg (Ah)	Limestone	Beech	6.4
Geisberg (Bt)	Limestone	Beech	7.0
Wendelingrund (Ah)	Clay	Mixed hardwood	6.0
Oschenberg (Ah)	Limestone	Mixed hardwood	7.3
<i>Acidic soils</i>			
Hohe Warte (Ah)	Sand	Pine	4.2
Waldstein (Oh)	Granite	Spruce	2.6
Eremitage (Ah)	Sand	Beech, oak	3.1

Table 1: Forest soils from the region around Bayreuth, Germany

2.2. *Preparation and incubation of soil suspensions*

Soil samples were collected in sterile containers, transported to the laboratory, and processed immediately or stored overnight at 5 °C and processed the next day. Gravel and large root pieces were aseptically removed from soil samples. In a Mecaplex (Grenchen, Switzerland) anaerobic chamber (100% N₂ atmosphere), 25 g (fresh weight) of unsieved soil was added to 500-ml infusion bottles (Merck ABS, Dietikon, Switzerland) containing 200 ml of sterile anaerobic mineral solution at pH 7 (for Geisberg and other near pH-neutral soils) or at pH 3 (for acidic soils) (Table 1). Soil suspensions were flushed with sterile argon, mixed on an end-over-end shaker at room temperature for 1 h, supplemented with oxalate and other substrates (as indicated), and incubated horizontally at 15 ± 2 °C (without shaking) in the dark. Aerobic studies were performed in the mineral solution prepared aerobically (without resazurin and cysteine), and bottles were incubated aerobically (with shaking) in the dark. The anaerobic mineral solution contained (mg l⁻¹): K₂ HPO₄, 225; KH₂ PO₄, 225; (NH₄)₂ SO₄, 450; NaCl, 450; MgSO₄ 7H₂ O, 45; cysteine-HCl-H₂ O (reducer), 500; and resazurin (redox indicator), 1. The anaerobic mineral solution was prepared by adjusting the pH of the solution to 7 or 3, by boiling and cooling the solution under 100% argon, and by dispensing the solution under 100% argon into infusion bottles. The bottles were closed with rubber stoppers and aluminum seals and autoclaved. In some experiments, the anaerobic mineral solution was modified to contain a 100% CO₂ gas phase (instead of argon) and NaHCO₃ (7.5 g l⁻¹); the final pH approximated 6.6. Stock solutions of potassium oxalate, antibacterial (penicillin, streptomycin, and chloramphenicol) and antifungal (cycloheximide) agents, electron acceptors (potassium nitrate and sodium sulfate), and electron donors (glucose, sodium acetate, and potassium vanillate) were prepared in deionized water (when necessary, free acids were neutralized with 6 N KOH), filter sterilized into sterile serum bottles, aseptically sealed with sterile stoppers and aluminum crimp seals, and degassed by sparging and flushing the headspace gas with sterile argon. Substrates were added from sterile anoxic stock solutions via sterile, argon-degassed needles and syringes to soil suspensions at final concentrations indicated; final oxalate concentrations approximated 15 mM. H₂ (as an electron donor) was added as a sterile gas to soil suspensions at the final concentration indicated.

2.3. *Sampling of soil suspensions*

At designated times during incubation, soil suspensions were sampled and analyzed for total oxalate (i.e., the sum of both soluble and insoluble oxalate) and soluble carbonaceous compounds. For the analysis of total oxalate, samples (0.5 ml) were aseptically removed from soil suspensions, acidified with 1 ml of 1 N HCl, and mixed for 30 min at 50 °C to extract oxalate. Sample acidification was necessary, given the high oxalate-binding capacity of the soils used in this study and allowed the solubilization and extraction of bound oxalates in soil suspensions. For the

analysis of soluble carbonaceous compounds, 1-ml samples were aseptically taken directly from soil suspensions and not subjected to acidification or heating. Acid extracts and 1-ml samples of soil suspensions were clarified by microcentrifugation and microfiltration prior to quantitative analysis.

2.4. Analytical methods

Concentrations of total oxalate in clarified acid extracts and soluble carbonaceous compounds (oxalate, acetate, glucose, and vanillate) in clarified (non-acidified, non-heated) samples were determined with a Hewlett-Packard 1090 series high-performance liquid chromatograph (HPLC). The HPLC was equipped with a Hewlett-Packard 1050 UV detector (210 nm), a Hewlett-Packard 1047A refractive index detector, a Hewlett-Packard 3396 series II integrator, and an Aminex ion exclusion HPX-87H column (300 by 7.8 mm). Analysis conditions included: column temperature (60 °C); mobile phase (0.01 N H₂SO₄) at a flow rate of 0.8 ml min⁻¹; and injection size (20 µl). Headspace gases (H₂ and methane) were measured by a Hewlett-Packard 5980 series II gas chromatograph as previously described [16]. Soil pH was measured with a standard pH meter/combination electrode in 1:2.5 suspensions of soil in 0.02 N CaCl₂. The dry weights of soils were obtained by weighing before and after drying at 105 °C for 16 h. Substrate and product values for soil suspensions are expressed in millimolar units; all values represent the means of duplicate experiments.

3. Results and discussion

3.1. Anaerobic oxalate consumption by Geisberg soils

3.1.1. Nature of oxalate-consuming activities

In well-drained, aerated soils, like the forest soils examined in this study (Table 1), aerobic processes are central to the microbial turnover of organic matter. However, O₂-free zones also exist in soil aggregates and in soils subjected to increased moisture and organic matter (i.e., conditions which promote rapid O₂ consumption and depletion) [25,31]. Indeed, anaerobic bacteria as well as anaerobic processes do occur in anoxic soil microsites [23,25,31]. Furthermore, some of these processes result in the anaerobic synthesis of a variety of lowmolecular-weight aliphatic organic acids [16,35].

In the present study, oxalate was consumed under anaerobic conditions by Ah-horizon soils from a beech forest at the Geisberg field site (Fig. 1; Table 1). Following a 10-day lag in activity, oxalate (15 mM) was completely consumed within 20 days. In the absence of supplemental oxalate, soil suspensions did not contain detectable levels (<0.1 mM) of endogenous oxalate, and oxalate synthesis in these soil suspensions was not detected during incubation. Autoclaved soil suspensions also failed to consume oxalate, indicating that the observed anaerobic oxalate-consuming activities were biotic in nature and thus due to microorganisms (Fig. 1). This report is the first to document the microbial consumption of oxalate by terrestrial soils under anaerobic conditions and, as such, extends the number of

habitats known to have anaerobic oxalate-consuming capabilities, the others being aquatic sediments and the mammalian gut [1]. In comparison, aerobic oxalate consumption by Ah-horizon soil began after a 2-day lag period, and oxalate was totally consumed by day 4 (Fig. 1). Given the immediate and rapid consumption of oxalate under aerobic conditions and the lag phase observed before the onset of anaerobic activities, aerobic oxalate consumption plays a more dominant role in oxalate consumption. This is not unexpected given the oxic nature of these soils and the fact that free low-molecular-weight organic acids such as oxalate and acetate have a short half-life (e.g., 1e5 h) in oxic soils [13]. In short-term microcosm studies, aerobic rates of oxalate degradation (based on mineralization of ^{14}C -oxalate to $^{14}\text{CO}_2$) can be as high as $32 \text{ mmol d}^{-1} \text{ g}^{-1}$ (dry weight) of soil [32,33]. Factors such as soil type, depth, vegetation, resident microbial communities, and climate can impact the capacity of soils to aerobically consume organic acids [32].

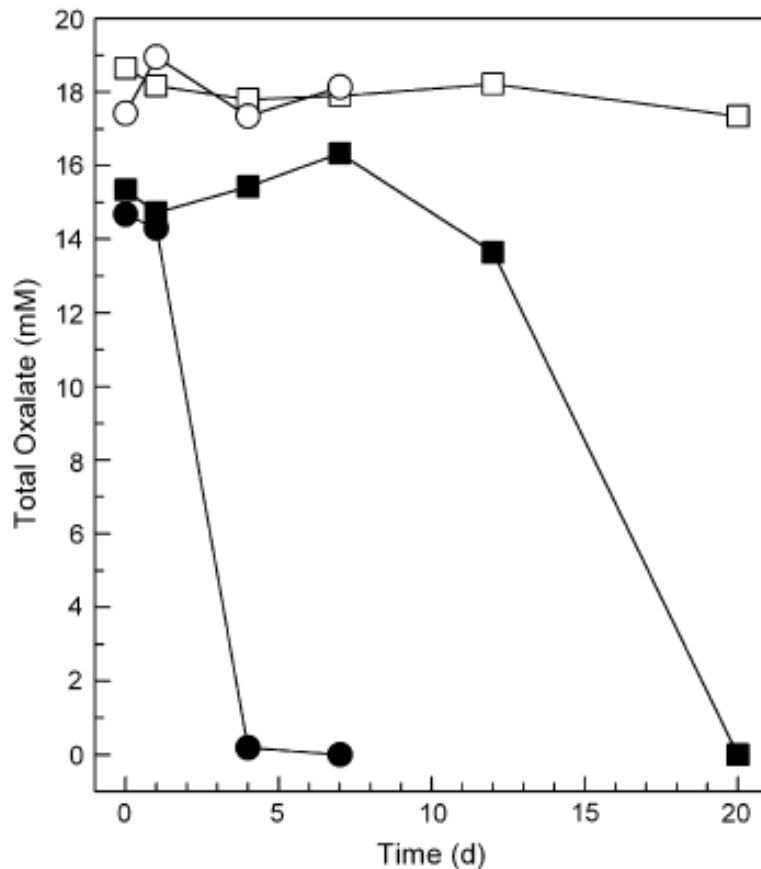


Fig. 1. Anaerobic and aerobic consumption of oxalate by forest soil suspensions. Soil (Ah horizon) was collected from the Geisberg field site; soil dry weight was 52.9%. For autoclaved controls, soil suspensions were autoclaved for 45 min at $121 \text{ }^\circ\text{C}$ and cooled prior to the addition of oxalate. Symbols: ■, anaerobic; ●, aerobic; □, anaerobic autoclaved control; and ○, aerobic autoclaved control.

Likewise, Ah- and Bt-horizon soils displayed different capacities relative to

anaerobic oxalate consumption (Fig. 2). While both soils were competent in oxalate consumption, the time required for Bt-horizon soil (10e20 cm) to completely consume oxalate (i.e., total oxalate) was basically twice that of Ah-horizon soil (0e10 cm). With Ah-horizon soils (Fig. 2A), oxalate was actively consumed from day 8 to 20, whereas oxalate consumption by Bt-horizon soil occurred over a longer time frame, from day 8 to 41 (Fig. 2B). Most of the oxalate consumed in these soil suspensions, especially with Bt-horizon soils, was in the form of insoluble or bound oxalates (Fig. 2). Thus, differences in oxalate-consuming activities might be due to the decreasing availability of nutrients, particularly soluble oxalate, in deeper horizon soils. Interestingly, when both Ah- and Bt-horizon soil suspensions were resupplemented with oxalate, oxalate consumption began immediately with no apparent lag in oxalate-consuming activities (Fig. 2). Such adaptations to oxalate input have been documented for microbial populations in sagebrush steppe soil [21] and the mammalian gut [1]. In these systems, exposure to increasing amounts of oxalate increases oxalate-degrading activities as well as concentrations of indigenous oxalate-degrading microorganisms.

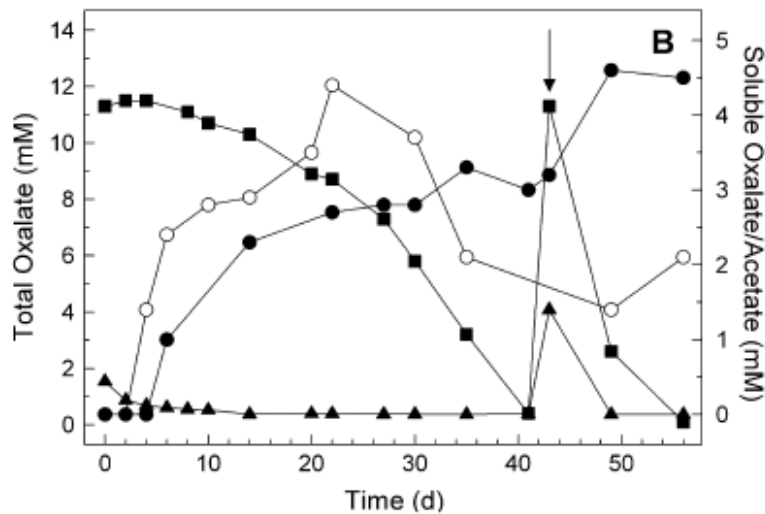
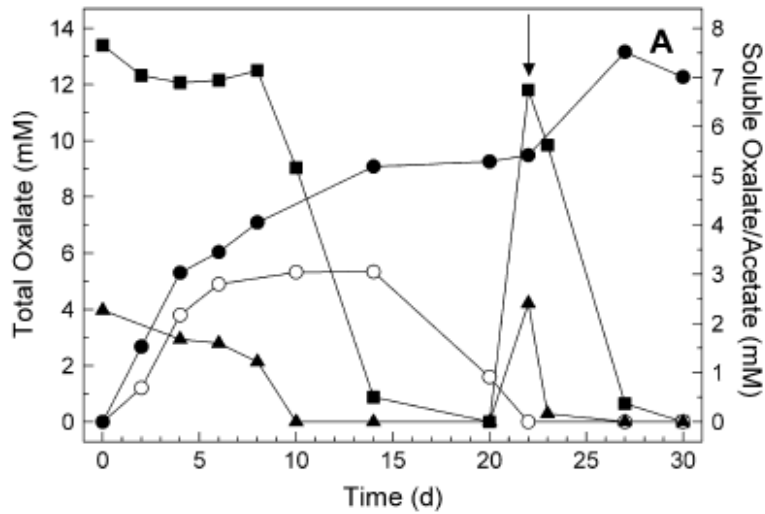


Fig. 2. Anaerobic oxalate consumption and acetate synthesis by microorganisms present in Ah-horizon (A) and Bt-horizon (B) forest soil suspensions. Soils were collected from the Geisberg field site; soil dry weights were 54.9 and 74.1% for Ah- (0-10 cm) and Bt- (10-20 cm) horizon soils, respectively. Symbols: ■, total oxalate; ▲, soluble oxalate; ●, soluble acetate; and ○, soluble acetate (acetate formed by soil suspensions which were not supplemented with oxalate). Arrows indicate when soil suspensions were re-supplemented with oxalate.

In Ah- and Bt-horizon soil suspensions, acetate was the major organic acid formed during anaerobic incubation and, in most cases, was not readily consumed (Fig. 2); methane was not detected in oxalate-supplemented soil suspensions. Slight increases in acetate concentrations were also observed in soil suspensions following oxalate resupplementation (Fig. 2). Whether oxalate or an oxalate-derived metabolite (e.g., formate) was utilized for acetate synthesis is unknown. However, acetogenesis contributes significantly to the acetate-forming potentials of soils [15,16], and oxalate is a growth-supportive substrate for some acetogenic bacteria [5]. Overall, these findings support previous studies which have shown that most terrestrial soils have a high capacity to anaerobically form acetate from endogenous matter and that the acetate, once formed, is not subject to immediate anaerobic consumption due to the absence or low levels of acetoclastic methanogens and sulfatereducing bacteria [15,16].

Oxalate also experienced a protracted residence time in soil suspensions. This delay in the onset of anaerobic oxalate-consuming activities varied from 8 to 50 days and was independent of the time of year that Geisberg soils were collected (Figs. 1e5). One explanation might be that the numbers of anaerobic oxalate-consuming microbes in these oxic soils were very low, and the delay could simply represent the time required for the enrichment of oxalate-consuming microbes. Our experimental approach also involved the use of dilute soil suspensions supplemented with 15 mM oxalate. These conditions are not indicative of in situ conditions and were used in order to maximize anaerobic processes, minimize pH changes, and provide oxalate levels that could be quantified by HPLC analysis. Thus, the release of inhibitory substances in soil suspensions or the exposure of native microbial populations to high, potentially inhibitory, amounts of oxalate may be responsible for the delay. There is precedent for the latter in that the growth of some aerobic oxalate-degrading bacteria from soils is inhibited in culture media containing increased concentrations of oxalate [30].

With soils from coniferous forests, *Streptomyces* spp., not saprophytic fungi, appear to be a microbial group involved in the aerobic consumption of oxalate [14]. Fungi are generally thought to play a minor role in the consumption of soil oxalate, since they are less active in the degradation of insoluble oxalates [14,21]. However, the types of microorganisms actively engaged in oxalate degradation under in situ conditions are unknown. In order to determine the microbial types (prokaryotes or eukaryotes) that might be involved in the anaerobic consumption of oxalate in forest soils, Geisberg soil suspensions were supplemented with a mixture of antiprokaryotic agents (penicillin, 0.3 mg ml⁻¹; streptomycin, 0.2 mg ml⁻¹; and

chloramphenicol, 0.03 mg ml⁻¹; values represent final concentrations in soil suspensions) or an antieukaryotic agent (cycloheximide, 0.4 mg ml⁻¹; value represents final concentration in soil suspensions). The inclusion of the antiprokaryotic mixture completely inhibited the anaerobic consumption of oxalate, whereas the antieukaryotic agent had no effect on oxalate utilization (data not shown). These results suggested that prokaryotic microorganisms, rather than eukaryotic microorganisms (e.g., fungi), were responsible for the anaerobic oxalate-consuming activities in these forest soils.

To our knowledge, the only anaerobic oxalate-degrading prokaryote that has been isolated to date from soils (i.e., oxic prairie and garden soils) is the acetogenic bacterium *Moorella thermoacetica* [5]. However, this obligate anaerobe is also an obligate thermophile, requiring a temperature range of 45e65 °C for growth [5], and is therefore unlikely to be involved in the anaerobic consumption of oxalate at the incubation temperatures (15e20 °C) used in the present study. In this regard, mesophilic (20e40 °C) anaerobic oxalate-degrading bacteria (*Oxalobacter formigenes*, *Oxalobacter vibrioformis*, *Oxalophagus oxalicus*, strain Ox-8, and *Desulfovibrio vulgaris* subsp. *oxamicus*) have been isolated from anoxic aquatic sediments [1]. Whether these or oxalate-degrading bacteria similar to these participate in anaerobic oxalate consumption in soils has yet to be documented.

3.1.2. Impact of electron acceptors on anaerobic oxalate-consuming activities

Various electron acceptors (nitrate, sulfate, and CO₂/bicarbonate) were examined for their ability to stimulate the anaerobic consumption of oxalate by Ah-horizon soil from the Geisberg field site (Fig. 3). Surprisingly, anaerobic oxalate consumption was totally repressed by the addition of CO₂ /bicarbonate to soil suspensions. While further studies are needed to resolve the nature of this repression, it is interesting to note that the parent material of this soil is limestone (calcium carbonate) and that microorganisms in Ah-horizon soils from the Geisberg field site have been shown to be metabolically active (e.g., capable of acetogenesis and denitrification) when suspended in an anaerobic CO₂ /bicarbonate-buffered mineral solution [15,16] .

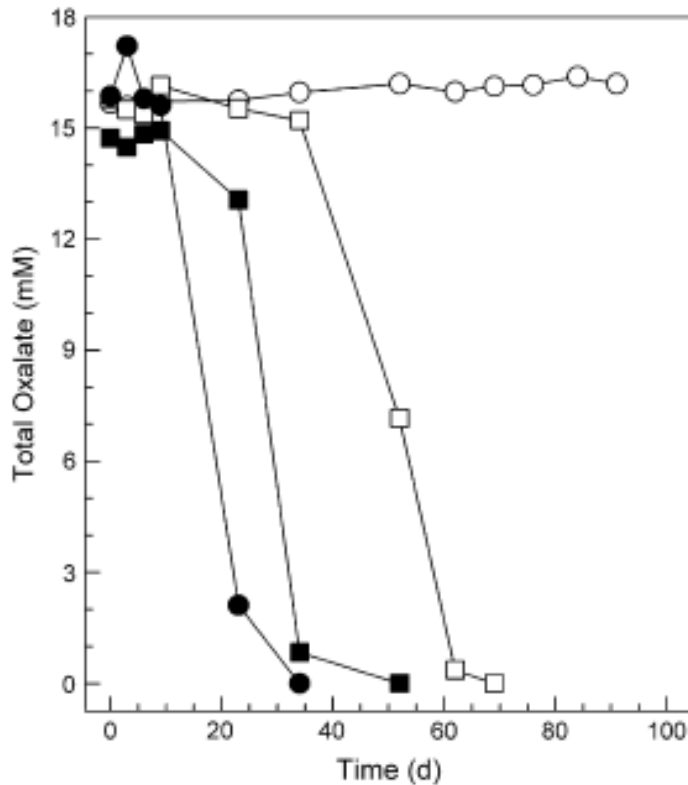


Fig. 3. The influence of supplemental electron acceptors (nitrate, sulfate, or CO₂/bicarbonate) on the anaerobic consumption of oxalate by forest soil suspensions. Soil (Ah horizon) was collected from the Geisberg field site; soil dry weight was 68.4%. Symbols: ■, control (no supplemental electron acceptor added); □, potassium nitrate (5 mM); ●, sodium sulfate (5 mM); and ○, CO₂/bicarbonate (100% CO₂ gas phase and 7.5 g l⁻¹ NaHCO₃).

In forest and grassland soils, nitrate-enriched conditions stimulate the anaerobic consumption of acetate [16,35]. Here, the addition of nitrate as a supplemental electron acceptor to soil suspensions was initially inhibitory to anaerobic oxalate consumption (Fig. 3); however, oxalate was eventually consumed following an extended lag phase of activity (compared to controls which contained no supplemental electron acceptor). In contrast, the supplementation of soil suspensions with an additional 5 mM sulfate appeared to be slightly stimulatory to the onset of oxalate consumption; this amount was considered additional since the anaerobic mineral solution normally contained 3.6 mM sulfate. A sulfate-free mineral solution was developed in order to better assess the impact of sulfate on oxalate consumption and the possibility that oxalate-utilizing, sulfate-respiring bacteria, like *D. vulgaris* subsp. *oxamicus*, were involved in oxalate consumption. Sulfate additions to soil suspensions prepared in the sulfatefree solution had essentially no effect on oxalate consumption (data not shown). A slight inhibition in oxalate consumption was observed when soil solutions were amended with sodium molybdate (a known inhibitor of sulfate-reducing bacteria); however, this inhibition was reversed upon continued incubation. Thus, from these results, it would appear

that CO₂-, nitrate- and sulfate-respiring bacteria were not actively involved in anaerobic oxalate consumption in suspensions of Geisberg soils.

3.1.3. Impact of electron donors on anaerobic oxalate-consuming activities

Various electron donors (glucose, vanillate, hydrogen, and acetate) were examined for their ability to stimulate (e.g., via co-metabolism) the anaerobic consumption of oxalate by Ah-horizon soil from the Geisberg field site (Fig. 4). In general, supplemental electron donors did not have any influence on the ability of Ah-horizon soil from the Geisberg field site to anaerobically consume oxalate. The onset of oxalate consumption was slightly delayed (compared to controls which contained no supplemental electron donor) when soil suspensions were supplemented with glucose or vanillate. Nevertheless, once engaged, oxalate consumption occurred simultaneously with vanillate consumption; likewise, hydrogen and oxalate were consumed simultaneously in H₂-supplemented soil suspensions (Fig. 4). Only in glucose-supplemented soil suspensions were different phases observed for the consumption of glucose and oxalate.

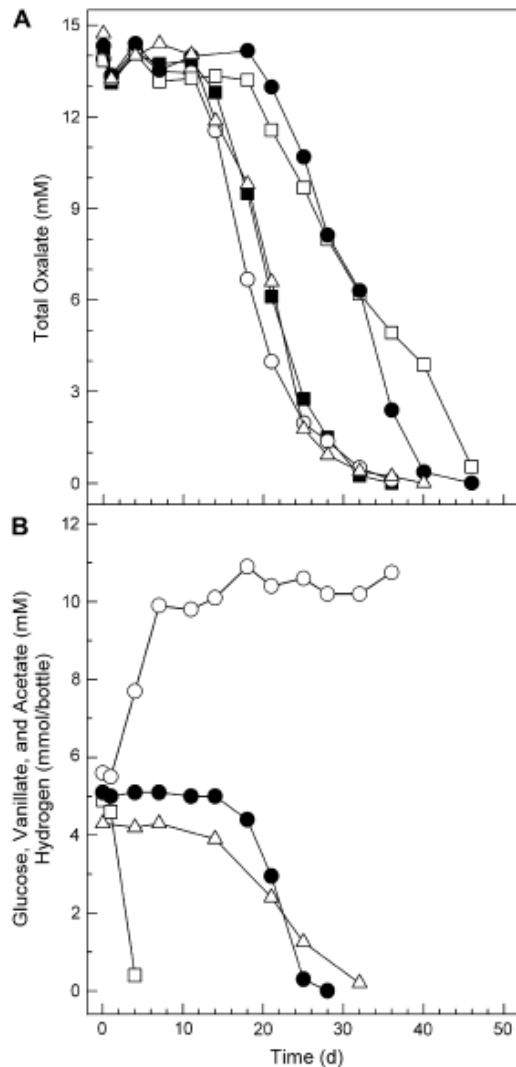


Fig. 4. Anaerobic oxalate consumption (A) in the presence of supplemental electron donors (acetate, glucose, vanillate, or H₂) (B) by forest soil suspensions. Soil (Ah horizon) was collected from the Geisberg field site; soil dry weight was 59.4%. Symbols: ■, control (no supplemental electron donor added); ○, acetate (5 mM); □, glucose (5 mM); ●, vanillate (5 mM); and △, H₂ (20% of headspace gas or 5.4 mmol H₂ bottle⁻¹).

That anaerobic oxalate consumption was not significantly stimulated by alternative electron donors or by alternative electron acceptors (see Section 3.1.2) suggests that oxalate-degrading bacteria similar to *O. formigenes*, *O. vibrioformis*, or *O. oxalicus* might be involved in the anaerobic consumption of oxalate in Geisberg soils. These non-respiring, obligate anaerobes are “specialists” in that they utilize oxalate as their sole growth-supportive substrate, converting it to CO₂ and formate as end products [1]. From initial Geisberg soil suspensions, stable (serially maintained) oxalate-consuming broth cultures, some of which formed detectable levels of formate, were established. However, our attempts to isolate the organisms responsible for the observed oxalate-consuming activity in these cultures were not successful.

3.2. Anaerobic oxalate consumption by different forest soils

Regional forest soils (Table 1) and their resident microbiota displayed differential activities relative to the anaerobic consumption of oxalate (Fig. 5). At the native pH of the soil, oxalate was consumed under anaerobic conditions by near pH-neutral soils (Geisberg [Ah horizon], Wendelingrund, and Oschenberg) whereas acidic soils (Hohe Warte, Waldstein, and Eremitage) were not active in the anaerobic consumption of oxalate during the time period examined (up to 90 days). Many soil bacteria and their associated processes are negatively impacted by low soil pH [22]. Indeed, compared to near pH-neutral soils, acid soils (<pH 3.5) possess reduced anaerobic capacities relative to the formation of acetate [16] and fixation of N₂[17].

4. Summary

Unlike microbial processes which occur in classical anaerobic habitats (e.g., aquatic sediments and gastrointestinal tracts), our understanding of the nature of anaerobic activities that occur in soil microsites and how these microbial activities impact the overall consumption of carbonaceous substrates in soils is far from complete. In the present study, the collective results demonstrated that forest soils were competent in the consumption of oxalate under anaerobic conditions. However, soil suspensions were designed to reveal the anaerobic capacities of soil microsites and, as such, may not reflect in situ capabilities. Thus, whether oxalate consumption actually occurs in situ under anaerobic conditions cannot be stated with certainty based on the results of the present study. Nonetheless, what is clear and noteworthy from this study is that oxic terrestrial soils harbor microorganisms

capable of consuming oxalate under anaerobic conditions and that these organisms, once adapted, can engage in the immediate and rapid consumption of significant amounts of insoluble oxalates. Since little is known about the microbial consumption of oxalate in soils, these findings provide insight into the types of environmental factors that can potentially regulate oxalate concentrations in soils and ultimately influence the nutritional-toxicological status of terrestrial ecosystems. Additional studies will be needed to determine (i) the identities of anaerobic oxalate-consuming microorganisms present in terrestrial soils and (ii) the impact that these soil microorganisms have on nutrient availability and oxalate-metal interactions. Studies that include soils that are frequently exposed to extended periods of water saturation (e.g., wetland soils) are particularly warranted.

Acknowledgments

Support for this study was provided by the Bundesministerium für Bildung, Wissenschaft, Forschung, und Technologie (0339476A0).

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