

A comparison of methods to determine the biodegradable dissolved organic carbon from different terrestrial sources

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Abstract

The importance of dissolved organic matter (DOM) in many soil processes is determined in large part by its availability to microbial uptake and decomposition, as this biodegradation can yield both energy and limiting nutrients. Despite its importance in soil ecology, there are no standard approaches to measuring the biodegradable fraction of DOC (BDOC) in soils. Here, we evaluate the comparability and reproducibility of methods employed in six laboratories including batch, kinetic, and bioreactor methods. Solutions from a variety of sources (throughfall, soil solution and soil extracts) were analysed using methods typically employed in each of the six participating laboratories. Our results show that the precision of various BDOC methods was similar (5–15%) across a broad range of BDOC (from 12% to 56% of total DOC). Differences in mean BDOC for the various test solutions were statistically significant when results were pooled across all the methods, and only a 90-day incubation resulted in consistently higher values for BDOC than the other methods. For 4 of 6 test solutions, measured BDOC increased by 6–13% with added nutrients. Current methods produce largely comparable results, providing the justification for comparisons among existing data sets collected with different methodologies. We recommend two standard methods for future studies: (1) a rapid determination of relatively labile DOC (measurement of DOC removal after 7 days of incubation with added nutrients) and (2) a 42-day incubation with repeated analysis of CO₂ production when determination of decomposition rate constants and a labile and relatively refractory component of DOC is desired.

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1. Introduction

Dissolved organic matter (DOM) plays a key role in a wide variety of chemical, physical, and biological processes occurring in soils (Kalbitz et al., 2000; Zsolnay, 1996, 2003). It also facilitates the transport of metals and hydrophobic organic contaminants through soils (Kalbitz et al., 1997; Temminghoff et al., 1997), and thus DOM degradation can alter the dynamics of pollutant fate and

transport in soils. DOM represents a potential supply of energy and organic nutrients (especially N and P) to soil microflora. Microbial degradation of DOM can regulate the production of greenhouse gases such as CH₄ and N₂O by both reducing the O₂ content of soils and by providing the electrons required for methanogenesis and denitrification (Yavitt, 1997; Zsolnay, 1996; Lu et al., 2000). Finally, degradation of DOM in soils can alter its delivery to aquatic ecosystems, where DOM plays an important role in food webs, contaminant transport, and absorption of harmful UV-B radiation (Engelhaupt et al., 2003; Findlay et al., 2003; Houser et al., 2003). Recognition of the

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importance of DOM in both terrestrial and aquatic ecosystems has grown dramatically over the past 15 years, and with this growth has come increased interest in understanding the biodegradation of DOM and its consequences for soil processes (Kalbitz et al., 2003a; Marschner and Kalbitz, 2003; McDowell, 2003; Zsolnay, 2003). Qualls and Haines (1992) and Kalbitz et al. (2003b) recognized that in long-term incubations DOM can be partitioned into two different fractions characterized by rapid and slow turnover. In addition to information on the pool size of rapidly and slowly degradable DOM, information is needed about the degradation rate constants of these two fractions to estimate the contribution of DOM to C storage in soils.

Research on DOM in soils has been hampered by the lack of a standard method for testing its biodegradability (Marschner and Kalbitz, 2003). Typically, researchers have focused on production of CO₂ or consumption of dissolved organic carbon (DOC) as measures of DOC degradability. As reviewed by Marschner and Kalbitz (2003), approaches used to date include (1) measurement of DOC disappearance in static batch experiments; (2) quantifying the kinetics of CO₂ production or O₂ consumption from batch experiments and (3) monitoring the disappearance of DOC as solution passes through a bioreactor with a heavy growth of microbes on sintered glass beads. The most widely used method appears to be batch incubations with measurement of DOC disappearance. At this time, the degree to which results obtained using different methods are comparable is unknown. To the best of our knowledge, no published papers compare different methods for determining the biodegradability of terrestrial DOM. Schnabel et al. (2002) recently examined the effects of filtering, microbial inoculum, and incubation time on the disappearance of DOC. Yano et al. (1998) showed that the source of inoculum does not affect measured biodegradable DOC (BDOC), whereas Trulleyova and Rulik (2004) found that biodegradation was greater when using an inoculum with attached rather than suspended microbes.

Here we compare different methods, with the following specific objectives: (1) determine the precision of different methods; (2) using a common set of test solutions, compare the results obtained with different methods; (3) examine the effects of nutrient addition and incubation time on

measured BDOC and (4) propose standard methods for measurement of BDOC.

2. Materials and methods

2.1. DOM sample collection and shipment

The samples employed in this study included throughfall, solution collected from lysimeters, and soil extracts. These solutions are typical of those analysed by the participating laboratories, and ranged in initial concentration from 15.2 to 144 mg l⁻¹ (Table 1). After initial collections described below, all samples were split into aliquots, frozen in polyethylene (HDPE) bottles, and shipped frozen to each of the participating laboratories. All the solutions and extracts arrived at the laboratories in a frozen state, and were kept frozen until analysis. The specific UV absorbance at 280 nm (UVIKON 930, BIO-TEK Instruments) was measured at a single laboratory to estimate the aromaticity of DOM (Kalbitz et al., 2003b). For the UV measurements the DOC concentration was adjusted to 10 mg C l⁻¹, the pH to 7.7, and the ionic strength to 1000 μS cm⁻¹ to ensure comparability of all DOM solutions (Kalbitz et al., 2003b). Total phenols (Folin–Ciocalteu reagent; Swain and Hillis, 1959) and proteins (Coomassie Brilliant Blue G-250 procedure of Bradford, 1976) were also measured at a single laboratory.

Sample 1 was a bulked throughfall sample collected beneath a northern red oak stand at the Harvard Forest Long-Term Ecological Research (LTER) site (Petersham, Massachusetts, USA; Nadelhoffer et al., 2004). The predominant tree species at this site are northern red oak (*Quercus borealis*), red maple (*Acer rubrum*) and paper birch (*Betula papyrifera*). Individual throughfall samples were collected during July–October 1997, filtered through pre-combusted 0.7 μm glass fibre filters (Whatman GF/F), and frozen following collection. For this study, the throughfall solutions collected in 1997 were thawed, bulked, and re-frozen.

Sample 2 was an aqueous litter extract obtained from a 150-year-old Norway spruce (*Picea abies* [L.] Karst.) stand located in the Fichtelgebirge of North-East Bavaria, Germany (Kalbitz et al., 2004). In May 2000, the litter layer was collected, the visible roots and organisms

Table 1
Origin, concentration, and composition of test solutions used in this study

Sample code	Origin	Collection method	DOC (mg l ⁻¹)	UV abs.(1mg C ⁻¹ cm ⁻¹)	Proteins (mg N l ⁻¹)	Phenolics (mg C l ⁻¹)
1	Hardwood throughfall	Throughfall collector	15.2	0.0181	0.301	3.12
2	Spruce litter	Batch extraction	20.6	0.0071	0.122	0.91
3	Spruce forest floor	Tension lysimeter	15.9	0.0266	0.410	1.84
4	Hardwood forest floor	Zero tension lysimeter	26.5	0.0231	0.345	6.19
5	Agricultural soil	Extraction by percolation	143.7	0.0217	0.830	3.27
6	Agricultural muck soil	Batch extraction	50.2	0.011	0.063	0.67

removed and the remaining litter sieved to pass 5 mm. The resulting sample was packed into polyethylene bags and stored at -20°C until extraction. Extraction of the litter was performed with ultra-pure water (litter:water ratio: 1:10 w/v), and the suspension was stirred manually (3 times within 24 h). The resulting slurry was then passed through a ceramic plate (pore size $1.0\ \mu\text{m}$) and filtered through $0.45\ \mu\text{m}$ membrane filters (cellulose acetate; OE 67, Schleicher and Schuell). The filtered solution was diluted to a concentration of about $20\ \text{mg C l}^{-1}$.

Sample 3 was a soil water sample collected beneath the Oa horizon of a Haplic Podzol in the Norway spruce stand described above (Sample 2). Tension plate lysimeters were held under near-continuous vacuum of 100 kPa (evacuated for 1 min every 5 min). Leachate was obtained from the lysimeters in autumn 2001, filtered ($0.45\ \mu\text{m}$, cellulose acetate; OE 67, Schleicher and Schuell) and diluted to a concentration of about $20\ \text{mg C l}^{-1}$.

Sample 4 was a soil water sample obtained from zero tension lysimeters set below the Oa horizon of a Dystric Cambisol at the Harvard Forest site described above (Sample 1). Soil solution from the lysimeters was collected within 24 h of rainfall events between May and November 1997. Solutions were transferred to the laboratory on ice and filtered through pre-combusted Whatman GF/F filters (nominal pore size $0.7\ \mu\text{m}$) within 4 h of collection. For this study, we used lysimeter solutions obtained from the control plots; further details of the site can be found in Nadelhoffer et al. (2004).

Sample 5 was obtained from a relatively organic-rich ($64\ \text{g C kg}^{-1}$) Ap-horizon (0–20 cm) of an arable field (Gleyic Fluvisol) located at Oderbruch, a former floodplain of the Oder River in north eastern Germany (Marschner and Bredow, 2002). After collection during autumn 1997, the soil was air-dried and sieved to pass 2 mm. The soil was moistened to 60% of its water holding capacity and incubated at 5°C for 24 h in the dark prior to placing it in leaching columns. Water-soluble material was collected by leaching the 80 g soil columns with 250 ml of 1 mM CaCl_2 for 6 h at a rate of $25\ \text{mm h}^{-1}$ under unsaturated conditions (Marschner and Bredow, 2002).

Sample 6 was collected from the upper 10 cm of an organic rich ($110\ \text{g C kg}^{-1}$) agricultural muck soil (Eutric Histosol) located in Imerhausen, north of Munich. The soil was air-dried after collection in September 2001 then extracted with 10 mM CaCl_2 at a ratio of 2:1 (v/w) in an overhead shaker for 10 min (Zsolnay, 1996). The DOC solution was obtained by filtering the extracts through polycarbonate filters (Nuclepore, $0.4\ \mu\text{m}$ pore size).

2.2. Biodegradation methods

We assessed DOC biodegradability using standard methods employed in our individual laboratories, including static, kinetic, and bioreactor methods. Each of these methods measures potential rather than in situ rates of degradation, as is typically the case in studies of the BDOC

content of soil solution. Most methods involved addition of a specific inoculum to the sample to be analysed, and 4 of the 7 methods added additional nutrients (Table 2). Two of the laboratories conducted long-term incubations to determine both the labile and relatively refractory components of the BDOC pool, but most methods employed were designed to measure only the relatively labile fraction of the DOC pool. For most methods, net transfer of DOC into microbial biomass was considered part of the BDOC pool, as the particulate matter produced during BDOC measurements was filtered prior to analysis of DOC at the end of the incubation.

Institutions involved in this inter-laboratory comparison were University of Wales, Bangor, UK (BG); University of Bochum, Germany (BO); University of Bayreuth, Germany (BT); GSF Institute for Soil Ecology, Munich, Germany (MN); University of New Hampshire, USA (NH); Agriculture & Agri-Food Canada, Ottawa, Canada (OT). Each laboratory carried out its standard DOC biodegradation protocol as summarized in Table 2 and detailed below.

Method 1: DOC disappearance after 5 days of incubation with added nutrients (BO; Marschner and Kalbitz, 2003). The biodegradability of DOC was assessed with 15 ml aliquots of $0.45\ \mu\text{m}$ (cellulose nitrate) membrane-filtered solutions diluted to $10\text{--}30\ \text{mg DOC l}^{-1}$. The solutions were supplemented with 6 ml nutrient solution (0.1% NH_4NO_3 and 0.1% K_2HPO_4), inoculated with an unfiltered 90 μl soil extract which was prepared by shaking air-dried Hortico Anthrosol topsoil with 4 mM CaCl_2 (1:2) for 10 min, and then incubated at 20°C in Teflon vials in the dark. Initially and after 5 days, DOC was determined with a Shimadzu 5050 TOC Analyser after filtration ($0.45\ \mu\text{m}$), acidification and purging (NPOC). In each case 5 replicates were run. Samples with added glucose served as a control for the viability of the inoculum; under these conditions about 94% of the carbohydrate was degraded.

Method 2: DOC disappearance over 7 days with nutrients added (GSF; Zsolnay and Steindl, 1991; Zsolnay, 1996). The DOC solutions (8 ml) were added to small volume Teflon vials. In addition, 3 ml of a nutrient solution (0.1% NH_4NO_3 and 0.1% K_2HPO_4) and 50 μl of a microbial inoculum derived from an agricultural soil slurry (Eutric Histosol) were added. The vials were then incubated in the dark at room temperature for 7 days. The DOC content was quantified both before and at the end of the incubation after acidification and purging (NPOC) with a Shimadzu 5050 TOC Analyser. To ensure that the assay was functioning, control samples containing $20\ \text{mg l}^{-1}$ glucose were analysed and approximately 88% of this glucose was removed after 7 days. All samples were analysed in triplicate.

Methods 3 and 4: DOC disappearance during 7-day incubation without (Method 3) and with (Method 4) added nutrients (BG). Prior to measuring initial DOC concentrations, test solutions were centrifuged at 14,000g for 5 min to remove any particulate organic matter. Five millilitre of the

Table 2
Summary of the methods used to compare DOM biodegradation

Method	Laboratory	Nutrients added	Temperature (°C)	DOC separation (filter type)	MBC removal after incubation	Method
1	BO	Yes	20	Cellulose nitrate	Yes	DOC analysis before and after 5-day incubation in Teflon ^a
2	GSF	Yes	Room	Polycarbonate	No	DOC analysis before and after 7-day incubation in Teflon ^b
3	BG	Yes	20	Centrifugation	Yes	DOC analysis before and after 7-day incubation in polypropylene
4	BG	No	20	Centrifugation	Yes	DOC analysis before and after 7-day incubation in polypropylene
5	BT	No	20	Cellulose acetate	Yes	DOC analysis before and after 90-day incubation in glass ^c
6	NH	Yes	Room	Glass fibre	NA	Removal of DOC by passage through a biofilm for ca. 3 h ^d
7	OT	No	35	Cellulose nitrate	Yes	Kinetics of DOC removal during 42-day incubation in glass ^c
8	BT	No	20	Cellulose acetate	NA	Kinetics of CO ₂ release during 90-day incubation in glass ^c

A microbial inoculum was added to the test solutions prior to incubation in all methods except Method 6. MBC = microbial biomass carbon. NA = not applicable. Codes for the laboratories are as follows: BG = University of Wales, Bangor, UK; BO = University of Bochum, Germany; BT = University of Bayreuth, Germany; MN = GSF Institute for Soil Ecology, Munich, Germany; NH = University of New Hampshire, USA; OT = Agriculture & Agri-Food Canada, Ottawa, Canada.

^aMarschner and Kalbitz (2003).

^bZsolnay and Steindl (1991) and Zsolnay (1996).

^cKalbitz et al. (2003b).

^dYano et al. (1998).

^eGregorich et al. (2003).

test solution, 50 µl of a soil microbial inoculum, and 50 µl of a nutrient solution were added to polypropylene tubes and the tubes incubated at 20 ± 2 °C on a rotary shaker for 7 days. The DOC content of the samples was measured at day 0 and 7 with a Shimadzu TC/TN analyser after acidification and purging to remove inorganic carbon (NPOC). The day 7 samples were also centrifuged at 14,000g for 5 min to remove cellular material. Samples were run in triplicate. The nutrient solution consisted of 1.2 mM KCl, 0.5 mM CaCl₂, 0.5 mM KNO₃, 0.5 mM NH₄Cl, and 0.1 mM K₂HPO₄. The soil inoculum was prepared by taking 1 g of a brown earth soil (*Eutric Cambisol*) under grassland (*Lolium perenne*, *Trifolium repens*) and shaking with 10 g of distilled water. The DOC in the microbial inoculum was below detection limits (<0.01 mg l⁻¹). The effect of nutrient addition was investigated by doing a replicate incubation series where distilled water instead of the nutrient solution was added.

Method 5: DOC disappearance after 90 days of incubation (BT; Kalbitz et al., 2003b). Prior to performing the assays, the DOC solutions were filtered through 0.2 µm membrane filters (cellulose acetate; OE 66, Schleicher and Schuell) to remove any particles and microorganisms. Solutions with more than 20 mg C l⁻¹ were diluted to avoid an excessive growth of microorganisms. Sixty millilitre of each DOC solution were transferred to 120-ml incubation flasks (in triplicate) without nutrient addition. A glass-fibre

filter (Schleicher and Schuell GF 92, 47 mm, cut into quarters) was added to provide surface area for the establishment of biofilms. A standardized mixed inoculum extracted from four different soil types was added before the flasks were sealed and incubated in the dark at 20 °C for 90 days. More details about inoculation and incubation conditions are given in Kalbitz et al. (2003b). A glucose solution (20 mg C l⁻¹, with addition of N, P, and K) was used as a control to determine the activity of the inoculum. A second control with ultra-pure water and inoculum was also run to quantify the amount of DOC added with the inoculum and consumed during the 90-day incubation.

Method 6: DOC removal after passage of test solutions through a heavily colonized microbial bioreactor (NH; Yano et al., 1998, 2000). The basic principle of the assay involves measuring the amount of DOC removed after passage of the solutions over an extensive biofilm growing on sintered glass beads contained within a 30 cm glass column. The difference between input and output concentrations is defined as the biodegradable portion of DOC. The biofilm was established by extracting a sample of fresh forest floor in cold, aerated ultra-pure water over a period of 2 days. The extract was then filtered using a Whatman GF/C filter (1.2 µm pore size) to remove large particles but retain most microbes and protozoa in the filtrate. This forest floor extract was used initially as a colonization solution and later as a maintenance solution. The solution

was continuously passed through the bioreactor column, and the colonization process was complete within 3–5 months (Yano et al., 1998). Inorganic nutrients (NH_4Cl , KH_2PO_4 , NaNO_3 , $\text{Ca}(\text{NO}_3)_2$, and Na_2SO_4) were added to the maintenance solution as well as the samples to achieve final concentrations of 1.82 mM N, 2.43 mM P, 0.070 mM S, and 2.42 mM K.

The sample solutions were thawed and analysed for DOC (after acidification and purging (NPOC) with a Shimadzu TOC 5000 Analyser) in triplicate before and after the biodegradability experiment. Assay solution was diluted to 10 mg DOC l^{-1} ($\pm 0.50 \text{ mg l}^{-1}$) and nutrient salts added. The DOC solutions were run through several bioreactor columns (either 3 or 2 depending upon the amount of solution available) at a rate of $0.33\text{--}0.37 \text{ ml min}^{-1}$ for a period of up to 8 h. Breakthrough, the point at which the test solution appeared in the output, occurred after approximately 2.5 h depending on flow rate. This breakthrough point was checked with a Br^- tracer. Output samples were removed every 30 min after the first 3 h of DOC test solution input, and each sample was treated as an individual result. The output samples were immediately filtered (Millipore sterile Durapore $0.22 \mu\text{m}$ filter) and analysed for DOC. Standard solutions of D-glucose, humic acid and ultra-pure water were run through the columns prior to and following the experimental test runs. The glucose solution was used to monitor microbial uptake of DOC, with about 85% typically removed during passage through the columns. Humic acid was used to monitor adsorption of DOC to the sintered beads with average removal values found to be 1.6% ($0.16 \pm 0.08 \text{ mg l}^{-1}$). The pure water addition was used to assess the microbial release of DOC, which was $0.18 \pm 0.08 \text{ mg l}^{-1}$.

Method 7: Kinetics of DOC disappearance during a 42-day incubation. In this assay (OT; Gregorich et al., 2003), 60 ml of each DOC solution were placed in a clean, acid-washed, 200-ml conical glass flask. Microbial inoculum ($100 \mu\text{l}$) was added to each flask and the flask was closed with a foam stopper. The inoculum solution was prepared by adding 45 ml of distilled/deionized water to 4 g of agricultural soil (Mollic Gleysol), shaking vigorously by hand, and incubating the suspension for approximately 24 h at 35°C . Prior to inoculation, the suspension was shaken and allowed to stand for about 30 min before transferring the top 30 ml to a clean plastic vial. The quantity of DOC in the inoculum was below the analytical detection limit ($<0.01 \text{ mg l}^{-1}$). The initial flask mass, mass of the solutions added, and total mass of the incubation flask were recorded. Flask masses were measured at least once a week and corrections were made for any water losses by evaporation. Inoculated flasks were incubated in the dark at 35°C on an orbital shaker for 42 days. Subsamples (5 ml) were removed from the incubation flasks at 1, 3, 7, 14, 28, and 42 days and centrifuged at 4500 rpm for 20 min. The supernatant solution was then passed through a $0.22 \mu\text{m}$ cellulose nitrate filter and analysed

immediately for non-purgeable organic C (NPOC) on a Shimadzu 5050 TOC Analyser. All tests were done in triplicate.

A double exponential equation for two distinct DOC pools with different mineralization rate constants was fitted to the measured data, using a least-squares optimization method (Quasi-Newton; Statistica '99).

Cumulative DOC uptake (in percent of initial DOC)

$$= a \times (1 - \exp^{-k(1)t}) + (100 - a) \times (1 - \exp^{-k(2)t}), \quad (1)$$

where t is time in days, a the portion of DOC that is readily removed in per cent of initial DOC (labile DOC), $(100 - a)$ the portion of DOC that is slowly removed in percent of initial DOC (refractory DOC), $k(1)$ the mineralization rate constant for the labile fraction, and $k(2)$ the mineralization rate constant of the refractory fraction (both in day^{-1}).

Method 8: Kinetics of carbon dioxide production measured over a 90-day incubation without added nutrients (BT; Kalbitz et al., 2003b). The fundamental conditions of incubation were the same as Method 5. Mineralization of DOC over time was quantified with CO_2 measurements (gas chromatograph HP 6890, Hewlett Packard, thermal conductivity detection) of the headspace of each incubation flask. At the beginning of the experiment, measurements were performed daily, but the measurement interval increased (up to 3 weeks) at the end of the incubation. The molar amount of CO_2 in the gas phase was calculated using the ideal gas law equation, and CO_2 in the liquid phase was calculated by using temperature, pH and tabulated solubility constants. Samples were gently shaken by hand every day to ensure equilibrium between the aqueous and the liquid phase. To calculate CO_2 in the liquid phase for each measurement, a linear change of the proton concentration during the incubation from the initial to the final pH of the solutions was assumed. The kinetics of DOC mineralization were analysed by a double exponential equation for two distinct DOC pools (labile and refractory) analogous to that used to analyse DOC removed as a per cent of initial DOC (Eq (1)).

2.3. Statistical analysis

Analysis of Variance (ANOVA) was used to analyse the effects of Test Solution and Method on BDOC. Post-hoc comparison of least-squares means was conducted with Tukey's Honestly Significant Difference (HSD). The ANOVA used was S–N–K (Student, Newman, and Keuls), and the significance threshold was $P < 0.05$. For the kinetic approach (Methods 7 and 8), in which multiple samples were taken over time, data collected at the end of the incubation were used to compare to the batch and biofilm approaches (Methods 1–6). All statistical analysis was conducted with SYSTAT version 11.

3. Results

3.1. BDOC of test solutions

When pooled across all methods, there were clear differences in measured BDOC among our 6 test solutions, and our samples included those of both low and high BDOC content (Fig. 1). Using single factor ANOVA, we found that Test Solution significantly affected % BDOC ($df = 5$, F -ratio = 46.366, $P < 0.000$). Post-hoc pairwise comparison of means showed that most differences among sample pairs in measured BDOC were statistically significant (Fig. 1). Samples 2 and 6, which were both batch extracts of organic horizons (litter, muck), had the highest fraction of biodegradable DOC (Fig. 1). The lowest values were obtained for the two lysimeter samples collected in the field beneath the Oa horizon (Samples 3 and 4) and for the percolate from the agricultural soil (Sample 5). The best predictor of the variation in %BDOC among our test

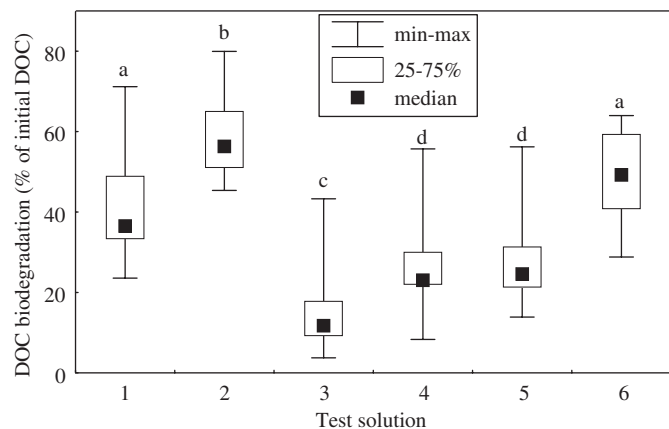


Fig. 1. Box plot (median, 25% and 75% confidence intervals) of % biodegradable DOC (BDOC) determined for each test solution by different methods. Different letters indicate statistically different values of BDOC as determined by post-hoc comparison of least-squares means using Tukey's HSD. Refer to Table 1 for identity of the 6 test solutions.

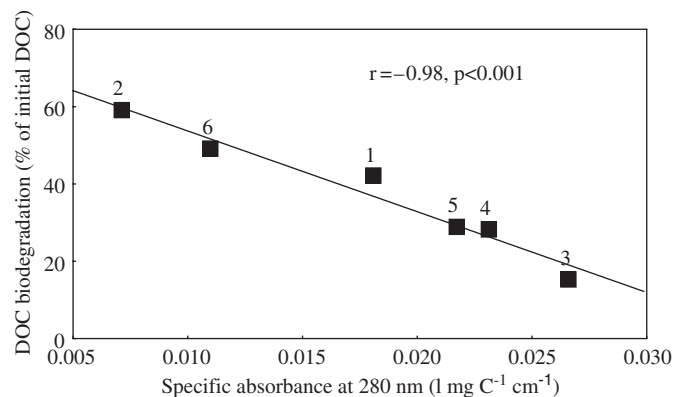


Fig. 2. DOC biodegradation (mean of all methods) as a function of specific absorbance at 280 nm (numbers in the figure indicate the test solutions as described in Table 1).

solutions was specific UV absorbance of the original solutions, with high UV absorbance associated with low BDOC (Fig. 2). BDOC was not correlated with total DOC, phenolic content, or protein content of the test solutions.

3.2. Precision and comparability of methods and laboratories

Among the different methods, the coefficient of variation for replicate analyses of the same sample ranged from 5% to 15% (Fig. 3). The methods with shorter incubation times (Methods 1–4) tended to show higher variability among replicate analyses than those obtained with the bioreactor (Method 6) or the longer-term incubations used in kinetic analyses of BDOC (Methods 5, 7 and 8).

There was a statistically significant interaction between Test Solution and Method, meaning that differences in % BDOC observed among methods varied according to the samples being analysed (ANOVA, $df = 35$, F -ratio = 26.578, $P < 0.000$). Overall, however, most methods deviated less than 20% from the mean values obtained with all the methods (Fig. 4). Results obtained with method 5 (90-day incubation measuring DOC loss) produced consistently higher BDOC values than those obtained with the other methods, and showed the greatest deviation from mean values (Fig. 4). In pairwise comparisons among methods, Method 5 was the only method that consistently provided statistically significant differences from the others (post-hoc comparison of pairwise mean differences).

3.3. Effects of operating conditions

Addition of nutrients caused a pronounced increase in measured BDOC for 4 of our 6 test samples during 7-day incubations (Fig. 5). Without nutrients added, the average % BDOC across all 6 test samples was 26%; with added nutrients, the average % BDOC obtained was 32%. The overall increase in measured BDOC with added nutrients was statistically significant (ANOVA, $df = 1$, F -

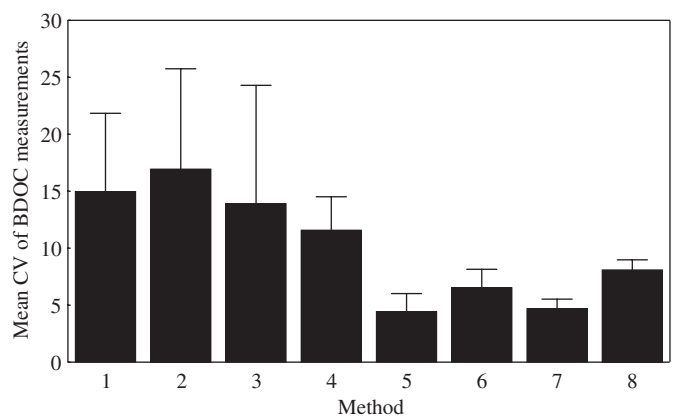


Fig. 3. Mean coefficient of variation (%; standard error of the mean shown with error bars) for determination of % BDOC by each method. Methods 1–4 are short-term incubations; 6 is bioreactor; 5, 7 and 8 are long-term incubations.

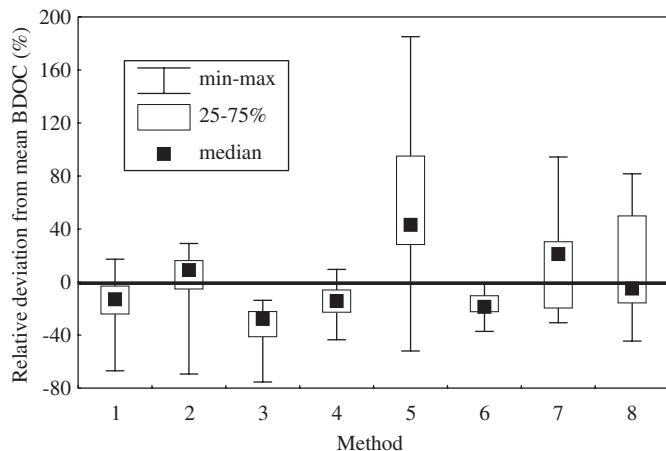


Fig. 4. Relative deviation of individual methods from mean BDOC obtained by all methods. Refer to Table 3 for a description of each method. For purposes of comparison, data from the last day of the kinetic analyses (Methods 7 and 8) are shown here.

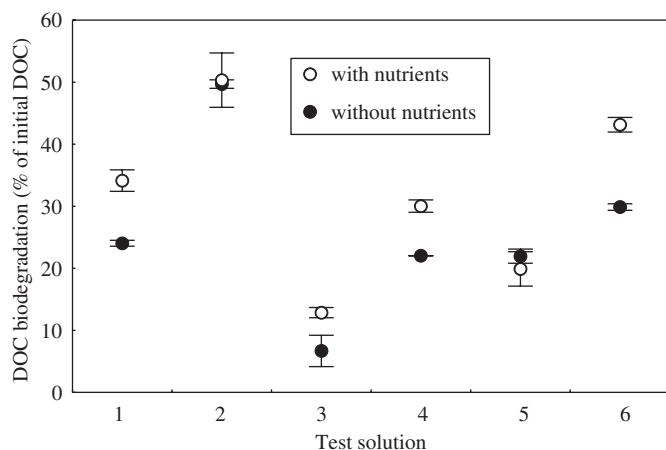


Fig. 5. Biodegradable DOC (%) measured during incubations with (open circles) and without (filled circles) added nutrients. Vertical line is standard error of the mean.

ratio = 31.34, $P < 0.000$), and the interaction between Nutrient Addition and Test Solution was also statistically significant (ANOVA, $df = 5$, F -ratio = 4.80, $P < 0.003$).

Longer incubations tended to consume more DOC and thus gave higher values of % BDOC (Fig. 4). The time course of DOC mineralization over 90-day incubations also showed that longer incubations consumed more DOC (Fig. 6). After 1 week of incubation, clear differences in % DOC mineralized emerged among the individual samples and a large part of the DOC degradation that occurred long-term had already occurred by 1 week.

Measurement of BDOC by DOC consumption (Method 5) resulted in higher values than measurement by CO_2 evolution (Method 8; Fig. 4). Methods 5 and 8 were identical except for the method used to quantify biodegradation.

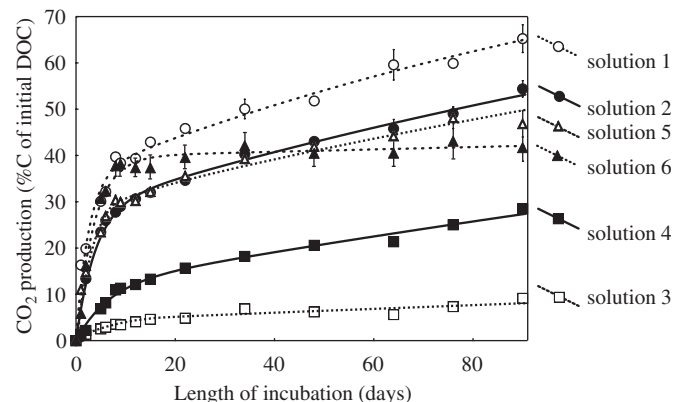


Fig. 6. Time course of DOC mineralization to CO_2 during 90-day incubations.

3.4. Kinetic analyses

Separation of BDOC into relatively labile and refractory fractions was successful with both kinetic methods, although the agreement between the two methods (7 and 8) was not particularly good (Table 3). In all cases the data fit the 2-component exponential decay model well. The lowest coefficient of determination for any sample with either method was 94%. Of the two kinetic methods employed here, the DOC removal method provided larger estimates of BDOC than mineralization.

4. Discussion

4.1. Biodegradability of DOC from different terrestrial sources

Our test solutions provided a robust test of different BDOC methods, as they spanned the full range of biodegradability typically reported in the literature (Marschner and Kalbitz, 2003). The portion of DOC that was biodegradable was highest (~50–60%) in batch extractions of organic horizons (litter, muck) and lowest (15–20%) in samples collected beneath the Oa horizon with lysimeters. It is not known whether this pattern can be generalized across a broader range of samples. Specific UV absorbance seems to be a good surrogate of DOM properties that control its biodegradability. The strong negative correlation between measured BDOC and UV absorbance indicates that aromatic compounds might be the most stable and recalcitrant (Kalbitz et al., 2003b).

4.2. Comparisons among methods

We conclude that all methods used in this study are equally suitable for comparisons among sites, seasons, or other variables by a single laboratory or investigator. Each method employed in our study had adequate precision, and gave similar results in terms of the relative biodegradability of DOC from the test solutions (Figs. 1 and 3). The one

Table 3
Results of kinetic analysis of DOC loss during long-term incubations in which DOC loss is measured directly (Method 7) or as CO₂ loss (Method 8)

Sample	Labile fraction (% of total DOC)		$k(1)$ (day ⁻¹)		$k(2)$ (day ⁻¹)	
	DOC loss	CO ₂ prod.	DOC loss	CO ₂ prod.	DOC loss	CO ₂ prod.
1	18.9	35.6	2.145	0.392	0.0040	0.0068
2	56.0	28.6	2.189	0.258	0.0120	0.0047
3	4.5	4.1	0.180	0.171	0.0036	0.0005
4	11.5	11.9	0.415	0.153	0.0033	0.0021
5	44.4	29.3	2.534	0.339	0.0056	0.0037
6	42.0	39.8	1.909	0.273	0.0122	0.0004

The percentage of total DOC that is labile and the decomposition rate constants for the labile [$k(1)$] and refractory [$k(2)$] fractions of total DOC have been calculated using a two-component exponential decay model (Eq. (1)). Conditions of incubation for the two methods are given in Table 2.

method that produced a consistent, statistically significant difference from the others was the 90-day static incubation (Method 5). Our data suggest that comparisons among previously published studies using different methods can be made with some confidence, especially if the incubation times are similar. Schnabel et al. (2002) also reported that incubation time was a critical parameter in determining DOC consumption.

The kinetic methods offer considerable additional information on the biodegradability of DOC. By providing estimates of the labile and refractory portions of DOC, they provide valuable information for modelling the fate of DOC in soils. Our comparison of the kinetics of DOC loss by the Ottawa lab (OT; Method 7) and CO₂ production by the Bitoek lab (BT; Method 8) showed excellent agreement (within 2% of total DOC) in 3 of our 6 test samples, but poor agreement in the others. Differences in the $k(1)$ and $k(2)$ values obtained by the different approaches were also very large, often showing 10-fold variation between the two methods (Table 3). Differences in temperature and the method of quantification (DOC loss vs. CO₂ production) both could have contributed to the differences between the rate constants obtained with these two kinetic methods. More inter-laboratory comparisons that directly compare the kinetics of BDOC consumption using both methods appear to be warranted.

4.3. Effects of operating conditions on measured BDOC

Despite the fact that each method gave broadly comparable results, we have identified several variables that do affect the measured BDOC in a solution. First, as expected, methods using longer incubation times tend to result in greater net removal of DOC from solution and thus higher values for BDOC (Fig. 4). This is particularly evident when comparing the results of batch 90-day incubations (Method 5) to shorter batch incubations (Methods 1–4). It is also evident from the kinetics of DOC removal during 42-day incubations, in which DOC concentrations declined throughout the incubation period (Table 3). The responsiveness of BDOC analysis to nutrient additions is also clearly indicated by our results. Some

samples showed clear signs of nutrient limitation to DOC consumption, while others did not (Fig. 5), in agreement with past studies (as reviewed by Marschner and Kalbitz, 2003).

Our data suggest that matching a DOC source with an inoculum that is derived from that particular site is not necessary. In our analysis, for example, samples from Harvard Forest (Solutions 1 and 4) were analysed with a bioreactor developed with microbes taken from soil at Harvard Forest (Method 6), as well as with microbes from a wide variety of other sources (all other methods). Despite the mismatch between inoculum source and DOC solution, we obtained similar results across the methods. This suggests that matching inoculum to DOC source is not critical in BDOC analysis. Previous work supports this conclusion, although there are few studies that directly test the effect of microbial inoculum on biodegradability of DOC (Yano et al., 1998). It seems that the type of inoculum may be more important than its source. Trulleyova and Rulik (2004) showed that biodegradation during 42-day incubations is more complete when microbes in the inoculum are attached to surfaces (biofilm) rather than suspended in the test solution, and Schnabel et al. (2002) found that filtration that allowed passage of particles of 2 µm or larger provided an adequate microbial inoculum.

4.4. Establishing standard methods for biodegradable DOC

Choice of a standard method is dictated by a number of factors. They include reproducibility, accuracy, ease of use, and requirements for specialized instrumentation or facilities. The methods we have tested and compared span a wide variety of experimental conditions, including short and long-term incubations, addition of nutrients, use of a biofilm or added inoculum, and measurement of DOC loss or CO₂ production.

We suggest that two standard methods should be considered for use in studies of DOC dynamics in soils. First, for analysis of the relatively labile fraction of DOC, we recommend analysis of DOC loss during a 7-day incubation at room temperature (20 °C) with added

nutrients and a small microbial inoculum. Controls should be run with nutrients and inoculum added to deionized high-purity water. We have chosen the 7-day incubation period as it captures a significant fraction of long-term degradation (Fig. 6), is long enough to clearly differentiate the BDOC content of various samples (Fig. 6), and is practical in the laboratory. Because BDOC analyses in the laboratory are all potential rather than in situ measurements, we recommend that nutrient addition be used routinely in the measurement of BDOC. Without nutrient additions, nutrient deficiency could restrict the degradation of DOC and not measure its true potential (Fig. 5). Addition of nutrients will facilitate inter-site comparisons of DOC degradability, and should also tend to decrease the effects of incubation time on measured BDOC. We propose routine use of an added inoculum to speed the decomposition process, and suggest addition of the inoculum on glass fiber filters as a means of increasing the surface area for microbial attachment and growth during the incubation (Kalbitz et al. 2003b; Method 5).

Second, in addition to short-term batch incubations for measurement of BDOC, we suggest that 42-day incubations with measurement of headspace CO₂ concentrations should be employed routinely in studies where measurement of the labile and refractory components of DOC is important. As with the short-term batch experiments, we suggest that these kinetic studies should be conducted at room temperature with an added inoculum and added nutrients. The headspace analysis allows repeated sampling over time with a minimal number of incubation vessels, and can be accomplished with very small volumes of gas removed. Both of these suggested standard methods are designed to determine the potential BDOC by eliminating possible nutrient limitations and by adding a microbial inoculum. In this way, DOC from different sampling sites or dates is characterized strictly in terms of its inherent degradability, independent of site specific or temporal variations of other factors controlling microbial activity. If these other controlling factors are of interest, the recommended standard methods can be supplemented by additional assays, without addition of nutrient and/or inoculum.

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References

- Bradford, M.A., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* 72, 248–254.
- Engelhaupt, E., Bianchi, T.S., Wetzel, R.G., Tarr, M.A., 2003. Photochemical transformations and bacterial utilization of high-molecular-weight dissolved organic carbon in a southern Louisiana tidal stream (Bayou Trepagnier). *Biogeochemistry* 62, 39–58.
- Findlay, S.E.G., Sinsabaugh, R.L., Sobczak, W.V., Hoostal, M., 2003. Metabolic and structural response of hyporheic microbial communities to variations in supply of dissolved organic matter. *Limnology and Oceanography* 48, 1608–1617.
- Gregorich, E.G., Beare, M.H., Stoklas, U., St-Georges, P., 2003. Biodegradability of soluble organic matter in maize-cropped soils. *Geoderma* 113, 237–252.
- Houser, J.N., Bade, D.L., Cole, J.J., Pace, M.L., 2003. The dual influences of dissolved organic carbon on hypolimnetic metabolism: organic substrate and photosynthetic reduction. *Biogeochemistry* 64, 247–269.
- Kalbitz, K., Glaser, B., Bol, R., 2004. Clear-cutting of a Norway spruce stand—implications for controls on the dynamics of dissolved organic matter in the forest floor. *European Journal of Soil Science* 55, 401–413.
- Kalbitz, K., Schwesig, D., Schmerwitz, J., Kaiser, K., Haumaier, L., Glaser, B., Ellerbrock, R., Leinweber, P., 2003a. Changes in properties of soil-derived dissolved organic matter induced by biodegradation. *Soil Biology & Biochemistry* 35, 1129–1142.
- Kalbitz, K., Schmerwitz, J., Schwesig, D., Matzner, E., 2003b. Biodegradation of soil-derived dissolved organic matter as related to its properties. *Geoderma* 113, 273–291.
- Kalbitz, K., Solinger, S., Park, J.H., Michalzik, B., Matzner, E., 2000. Controls on the dynamics of dissolved organic matter in soils: a review. *Soil Science* 164, 277–304.
- Kalbitz, K., Popp, P., Geyer, W., Hanschmann, G., 1997. β -HCH mobilization in polluted wetland soils as influenced by dissolved organic matter. *Science of the Total Environment* 204, 37–48.
- Lu, Y.H., Wassmann, R., Neue, H.U., Huang, C.Y., 2000. Dynamics of dissolved organic carbon and methane emissions in a flooded rice soil. *Soil Science Society of America Journal* 64, 2011–2017.
- Marschner, B., Bredow, A., 2002. Temperature effects on release and ecological properties of dissolved organic matter (DOM) in sterilized and biologically active soil samples. *Soil Biology & Biochemistry* 34, 459–466.
- Marschner, B., Kalbitz, K., 2003. Controls of bioavailability and biodegradability of dissolved organic matter in soils. *Geoderma* 113, 211–235.
- McDowell, W.H., 2003. Dissolved organic matter in soils—future directions and unanswered questions. *Geoderma* 113, 179–186.
- Nadelhoffer, K., Boone, R., Bowden, R., Canary, R., Kaye, J., Micks, P., Ricca, A., McDowell, W., Aitkenhead, J., 2004. The DIRT experiment: litter and root influences on forest soil organic matter stocks and function. In: Foster, D., Aber, J. (Eds.), *Forests in Time: The Environmental Consequences of 1,000 Years of Change in New England*. Yale University Press, New Haven, CT, pp. 300–315.
- Qualls, R.G., Haines, B.L., 1992. Biodegradability of dissolved organic matter in forest throughfall, soil solution and streamwater. *Soil Science Society of America Journal* 56, 578–586.
- Schnabel, R.R., Dell, C.J., Shaffer, J.A., 2002. Filter, inoculum and time effects on measurements of biodegradable water soluble organic carbon in soil. *Soil Biology & Biochemistry* 34, 737–739.
- Swain, T., Hillis, W.E., 1959. The phenolic constituents of *Prunus domestica*—I—the quantitative analysis of phenolic constituents. *Journal of the Science of Food and Agriculture* 10, 63–68.
- Temminghoff, E.J.M., van der Zee, S.E.A.T.M., de Haan, F.A.M., 1997. Copper mobility in a copper-contaminated sandy soil as affected by pH and solid and dissolved organic matter. *Environmental Science and Technology* 31, 1109–1115.

- Trulleyova, S., Rulik, M., 2004. Determination of biodegradable dissolved organic carbon in waters: comparison of batch methods. *Science of the Total Environment* 332, 253–260.
- Yano, Y., McDowell, W.H., Kinner, N.E., 1998. Quantification of biodegradable dissolved organic carbon in soil solution with flow-through bioreactors. *Soil Science Society of America Journal* 62, 1556–1564.
- Yano, Y., McDowell, W.H., Aber, J.D., 2000. Biodegradable dissolved organic carbon in forest soil solution and effects of chronic nitrogen deposition. *Soil Biology & Biochemistry* 32, 1743–1751.
- Yavitt, J.B., 1997. Methane and carbon dioxide dynamics in *Typha latifolia* (L.) wetlands in central New York State. *Wetlands* 17, 394–406.
- Zsolnay, A., Steindl, H., 1991. Geovariability and biodegradability of the water-extractable organic material in an agricultural soil. *Soil Biology & Biochemistry* 23, 1077–1082.
- Zsolnay, A., 1996. Dissolved humus in soil waters. In: Piccolo, A. (Ed.), *Humic Substances in Terrestrial Ecosystems*. Elsevier, Amsterdam, pp. 171–223.
- Zsolnay, A., 2003. Dissolved organic matter: artefacts, definitions, and functions. *Geoderma* 113, 187–209.