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# Uncertainties in determining microbial biomass C using the chloroform fumigation–extraction method

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ABSTRACT

We tested the accuracy of the chloroform fumigation–extraction method, which is commonly used to determine soil biomass C concentrations. Accurate and precise determination of total microbial biomass is important in order to characterize soil properties and to develop predictive metal transport models for soils. Two natural soils, and individual soil components, including silica sand, montmorillonite, kaolinite, a humic acid, and *Bacillus subtilis* bacterial cells, were fumigated for 24 h. Following the fumigation, C from fumigated and unfumigated samples was extracted using a 0.5 M $K_2SO_4$ solution. The difference between the C content in the fumigated and unfumigated samples ideally represents C due to biomass because the fumigation procedure should lyse cells and release biomass C. We observed increased C release upon fumigation for bacteria-only samples, confirming the ability of fumigation to lyse cells. There was no difference in extracted C concentration between fumigated and unfumigated samples of silica sand and of humic acid, confirming that the fumigation process does not introduce additional organic C to samples of these soil components. However, the fumigated clay samples both showed increased C release relative to the unfumigated controls, indicating that significant concentrations of the fumigant, chloroform, adsorbed onto the clay minerals studied here. Additionally, we found significant chloroform remaining in the extracts from two fumigated natural soils. Attempts to remove the chloroform from the soils or soil components prior to extraction by increasing the evacuation time, or to remove chloroform in the extracts by sparging them vigorously with nitrogen gas, both failed. This research reveals that chloroform gas may adsorb significantly to clays and the clay fraction of natural soils. Thus, the fumigation–extraction method must be corrected to account for the added chloroform C and accurately assess the concentration of biomass C in soils that contain significant concentrations of clays.



## 1. Introduction

Soils are complex and variable mixtures of inorganic and organic components and free and adsorbed water. The inorganic fraction typically consists of minerals such as silica, feldspars, clays, and micas; the organic portion includes humic substances and microorganisms. These components represent a mixture of polar and nonpolar adsorbents that can strongly affect the distribution, speciation, and bioavailability of metals and organic pollutants in soils (e.g., Steffan and Akgerman, 1998). In order to develop quantitative adsorption models of contaminant distributions in soil systems, the amount of each component must be precisely determined.

Sequential extraction approaches (e.g., Tessier et al., 1979; Li et al., 1995) can be used to estimate the abundances of broad categories of minerals (carbonates, silicates, etc.) relative to organic components in soil. Furthermore, mineral identities and abundances in a soil sample can be reasonably estimated using x-ray diffractometry approaches (e.g., Brindley, 1984; Bish and Post, 1993). However, the approaches that have been developed to estimate biological cell mass or numbers in soils lack the precision that is necessary for the estimates to be useful in quantitative geochemical speciation and transport models. Commonly used methods of biomass determination include direct counting of bacteria using an optical microscope, and the use of adsorbent fluorescent dyes for spectrophotometric measurement of cell numbers. Bacterial counts based on optical microscopy or spectroscopy typically yield estimates of cell numbers with uncertainties of an order of magnitude or more, and fluorescent dyes can illuminate particles that are not bacteria leading to falsely high counts (Fægri et al., 1977; Poglazova et al., 1996). Lindahl and Bakken (1995) showed that physical dispersion methods to separate cells from soil, such as ultrasonication, blender, drill-gun, and shaking approaches, can damage cells. The percentage, viability, and purity of bacterial cells separated from soil also depend strongly on the dispersion technique and whether a surfactant is used in the separation procedure (Bakken, 1985).

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Jenkinson and Powlson (1976a,b) introduced a biocidal fumigation method to determine the cell biomass C in soils. Their work demonstrates that chloroform ($CHCl_3$) fumigation effectively lyses cells in 24 h. In this procedure, after the fumigation period, the fumigated soil sample and an unfumigated control soil are placed in an incubator. The C from organisms that were killed and lysed during the fumigation process is readily mineralized to $CO_2$, so that the difference in $CO_2$ gas evolution between fumigated and unfumigated samples is a measure of the biomass C (Smith et al., 1995). Vance et al. (1987) and Tate et al. (1988) introduced a similar method which involves a fumigation step, but in this procedure organic C is extracted from fumigated and unfumigated samples using a 0.5 mol $L^{-1}$ $K_2SO_4$ solution instead of an incubation approach. The $K_2SO_4$ solutions are then analyzed for total organic carbon (TOC), and biomass C is calculated as the difference in TOC in the extracted solutions from the fumigated and the unfumigated samples. The fumigation method is based on the assumption that the increased amount of organic C extracted from a sample relative to a control is due entirely from cell lysis caused by chloroform fumigation. Vance et al. (1987) found an empirical linear relationship between biomass C and organic C released by fumigation–extraction. Clearly, if chloroform adsorbs onto any of the soil components and is extracted using the $K_2SO_4$ solution, then the subsequent measurements of increased TOC would in part be caused by the presence of chloroform and not only by lysis of biological organisms.

Although the fumigation method is commonly used, and is often considered the preferred method for biomass determination in soil (e.g., Franzluebbers, 1999), control experiments for the approach have never been performed, and so the accuracy of the procedure remains untested. Specifically, in applying the fumigation methods, one implicitly assumes that the introduced chloroform can be completely evacuated from the soil after the 24 h fumigation period. Because chloroform itself contains organic C and could add to the extractable C pool, this assumption must be tested with control experiments involving each of the major components of soils. Haney et al. (1999, 2001) questioned the acceptability of the fumigation–extraction method to determine biomass C by showing that the amount of C extracted using a 0.5 mol $L^{-1}$ $K_2SO_4$ solution can vary significantly as a function of pH.

Chloroform is volatile under the experimental conditions, and volatile organic carbon (VOC) vapors can adsorb substantially to clays (e.g., Guo et al., 1998). Generally, montmorillonite, a 2:1 clay mineral, can adsorb 200–300 mg VOC per gram of clay, and the 1:1 kaolinite has an adsorption capacity of ¼ to ½ of that (Thibaud-Erkey et al., 1995). There is evidence that suggests that chloroform can adsorb to soils both from aqueous solution (Dural and Peng, 1995) and from the atmosphere (Farrell and Reinhard, 1994; Thibaud-Erkey et al., 1995; Yeo et al., 1997; Chen and Dural, 2002), and therefore may show up as biomass C upon extraction with $K_2SO_4$.

In this article, we test the validity of the fumigation–extraction method by performing fumigation control experiments with individual soil components, including humic acid, sand, clays, and bacteria, and with two natural soils. If the fumigation–extraction approach is valid, then we should observe no difference in TOC between the extracted solutions of individual components from the fumigated and unfumigated samples, except for the bacterial samples, where cell lysis should increase the extracted TOC in the fumigated samples. However, if chloroform adsorbs onto any of the surfaces to a significant extent, then we will observe increased TOC in extracted solutions relative to the unfumigated controls even though no biomass is present in those samples. Our control experiments will determine whether chloroform adsorbs onto common soil components or to the two natural soils included here. If chloroform adsorption does occur onto some soil components, then our experiments will provide insight into the types of soil for which the fumigation–extraction method may not yield accurate biomass C analyses.

## 2. Materials and methods

### 2.1. Soil component experiments

Two clays, a silica sand, a humic acid, and a pure strain of bacteria were used as control materials for testing the fumigation–extraction method. The average grain diameter, with 1σ uncertainties, of the silica sand (Accusand 40/50) was determined using scanning electron microscopy, and was found to be 469 ± 89 μm ($n = 84$). Schroth et al. (1996) analyzed a suite of Accusand grades and found the average diameter of the 40/50 grains to be 359 ± 10 μm ($n = 4$) using sieve analyses. The clays that were used in this study included a kaolinite (KGa-1b) and a Na-rich montmorillonite (SWy-2), both of which were obtained from the Source Clays Repository and have been characterized extensively. According to the Source Clays Repository, the clays were homogenized and subjected to a low-temperature steam fired tray drying following their excavation. Dogan et al. (2006) found the mean Brunauer-Emmett-Teller (BET) surface areas of KGa-1b and SWy-2 to be 13.1 $m^2\ g^{-1}$ and 22.7 $m^2\ g^{-1}$, respectively. Cerato and Lutenegger (2002) estimated specific surface areas of 15 $m^2\ g^{-1}$ for KGa-1b and 637 $m^2\ g^{-1}$ for SWy-2 using the ethylene glycol monoethyl ether (EGME) method. Also available are chemical analyses (Mermut and Cano, 2001), infrared analyses (Madejová and Komadel, 2001) and powder x-ray diffraction analyses (Chipera and Bish, 2001) of both clays. We conducted fumigation tests using dried forms of both the kaolinite and montmorillonite samples, and we also conducted a test using a wetted montmorillonite powder in order to test whether wetness of the clay affects the results. In this case, the SWy-2 montmorillonite sample was wetted by soaking the clay in excess water for 1 h in 250 mL centrifuge tubes. After soaking, the tubes were centrifuged for 10 min at 8500 g three times and the water decanted each time. The amount of water retained by the clay after centrifugation was calculated by determining the weight difference between wet and dry SWy-2 samples, measuring the mass of the wet and dry clay samples before and after drying each in an oven at 105 °C for 24 h. The dry SWy-2 sample contained 5.0 ± 0.2% water by weight and the wet SWy-2 sample contained 82.8 ± 1.3% water by weight. The humic acid experiments used a commercial humic acid, which has been characterized by Malcolm and MacCarthy (1986), obtained from the Aldrich Chemical Company (Milwaukee, WI). The silica sand, humic acid, and clays were used in experiments without washing or other modifications.

*Bacillus subtilis*, a gram-positive soil bacterial species, was grown from pure culture slants. Cells from the slant were transferred to 3 mL of trypticase soy broth (TSB) with 0.5% yeast extract and allowed to grow for 24 h at 32 °C. These cells were transferred to 1 L of the same media and allowed to grow for 24 h at the same temperature. Cells were then harvested in early stationary phase by centrifugation and washed three times in test tubes in a 0.1 mol $L^{-1}$ $NaClO_4$ solution.

The fumigation procedure that we used closely followed the methods of Jenkinson and Powlson (1976a,b), Vance et al. (1987), and Tate et al. (1988). Experiments were conducted as a function of the mass of a soil component, with six experiments at different masses performed for each component. We used 0–50 g kaolinite, 0–50 g dry montmorillonite, 0–80 g wet montmorillonite, 0–50 g silica sand, 0–200 mg humic acid, and 0–200 mg bacteria. For each soil component separately, a known mass of material was spread evenly into two glass Petri dishes: one dish was fumigated with chloroform gas for 24 h, and the other was an unfumigated control. The fumigation samples were placed in a glass dessicator lined with moist filter paper. A small beaker that contained approximately 25 mL of ethanol-free chloroform and a few boiling chips was placed in the center of the dessicator. Once sealed, the dessicator was evacuated for 2 min, causing the chloroform to boil, thereby exposing the samples to chloroform vapor. After 24 h in the dark, the beaker of chloroform was removed from the dessicator, and the dessicator was evacuated 8 times to remove

chloroform vapor from the samples. Evacuations were performed by connecting the dessicator to a vacuum for 3 min, then sealing the dessicator from the vacuum, and finally slowly opening it to the atmosphere to refill with air. No noticeable chloroform odor existed after this procedure. We performed kinetics experiments, measuring the amount of organic C remaining on samples of dry SWy-2 montmorillonite as a function of evacuation time. The samples exhibited extracted organic C concentrations that were independent of evacuation time, so all subsequent experiments were conducted with eight 3 min evacuations.

Organic C extractions of both fumigated and unfumigated samples were performed using identical procedures. That is, each sample was transferred into a 500 mL glass media bottle, and 200 mL of 0.5 mol $L^{-1}$ $K_2SO_4$ was added. The bottles were then placed on an oscillating shaker for 30 min to thoroughly mix the sample with the $K_2SO_4$ extractant solution. The supernatant was filtered using Whatman No. 42 filters, and collected for C analysis. We used an organic C analyzer (Shimadzu TOC-5000) to analyze for organic C in the extracts. Sets of six calibration standards for the TOC analyzer were made from a 1000 mg $L^{-1}$ organic C solution as potassium biphthalate (Ricca Chemical Company, Arlington, TX) in the same 0.5 mol $L^{-1}$ $K_2SO_4$ matrix as the samples. The ranges of organic C concentrations in each calibration set were selected to be appropriate for the expected C concentrations in the various samples. Samples and standards were acidified with 1.0 μL of 1.0 mol $L^{-1}$ trace grade HCl per milliliter of solution to remove dissolved inorganic C. The TOC instrument analyzed the C concentration in each sample 3 times, with a resulting error of less than 5% for all analyzed solutions. Control blanks containing only 0.5 mol $L^{-1}$ $K_2SO_4$ and the HCl acidification were included with every TOC set, and used to correct each dataset for any background C present in the reagents or from the instrument.

A set of two-component experiments was conducted using a constant 50 g of silica sand and between 0 and 200 mg of bacteria. The fumigation and extraction procedures for these experiments were identical to those conducted for the single-component experiments.

### 2.2. Natural soil experiments

We tested two natural soils with significant clay content to determine if chloroform adsorption impacts the biomass C concentrations given by the fumigation–extraction method. Samples included the Waterford Loam (WcnAl) and Auten Loam (AxvA). Both soils were collected from the B horizon at a depth of 20 to 30 cm during July 2009 in St. Joseph County, Indiana, USA. Important characteristics of these two soils are listed in Table 1. Immediately after collection, the fumigation–extraction method of Vance et al. (1987) was applied to determine biomass C in both soils.

To differentiate the contributions of soil biomass and chloroform to the total carbon pool in the fumigated soil extraction solutions, we employed the aqueous chloroform detection method of Pillai et al. (1999). Briefly, 1 mL of the soil extract, 1 mL of pyridine, and 2 mL of 5 mol $L^{-1}$ NaOH were vigorously mixed and placed in a 70 °C water bath for 3 min. The solutions were cooled in an ice-water bath and then 2 mL of glacial acetic acid was added to each solution. Finally, 1 mL of 10 mol $L^{-1}$ HCl and 2 mL 1% *P*-aminoacetophenone were added. After 10 min, the solution chloroform concentrations were measured using a spectrophotometer set to a wavelength of 520 nm. The unfumigated extraction solutions of each soil were treated in the same way and used as a blank and to make the chloroform calibration standards, so that the measured difference between the fumigated and unfumigated samples could be attributed solely to the presence of chloroform in the fumigated extraction solutions.

**Table 1**
Soil characteristics for the Auten and Waterford loams.

| | Auten Loam (AxvA)[a] | Waterford Loam (WcnAl)[b] |
|---|---|---|
| % Clay | 17.4 | 11.0 |
| % Silt | 38.9 | 11.7 |
| % Sand | 43.7 | 77.3 |
| % Organic C | N/A | 0.34 |
| % Total C | 0.36 | N/A |
| pH (1:1 water)[c] | 7.4 | 6.1 |
| pH (1:2 0.01 M $CaCl_2$)[c] | 6.6 | 5.4 |

[a] All data for the Bt1 horizon (Soil Survey Laboratory, 2010).
[b] All data for the Bw1 horizon (Soil Survey Laboratory, 2010).
[c] See Miller and Kissel (2010) for descriptions of pH 1:1 water and pH 1:2 0.01 M $CaCl_2$ methods.

## 3. Results and discussion

### 3.1. Soil components

The results of the bacteria experiment are depicted in Fig. 1, which relates the biomass initially placed in the Petri dish to the concentration of organic C extracted in the $K_2SO_4$ solution. Data for the fumigated samples and for the unfumigated controls are both plotted, and the calculated difference (the concentration of TOC in the extract from the fumigated samples minus the concentration of TOC in the extract from the unfumigated controls) is also shown. The unfumigated controls exhibit increasing extracted TOC with increasing initial biomass in the Petri dish, indicating that the extraction wash dissolves some of the organic C from untreated bacteria, and that the concentration of organic C in the wash is controlled by the amount of biomass exposed to the wash solution. The fumigated bacterial samples contain greater TOC in the extraction solution relative to that measured in the extraction solutions for the unfumigated controls for all initial biomasses studied, and the difference increases with increasing initial biomass. The increased TOC in the fumigated samples indicates that the chloroform treatment exposes the extraction wash solution to higher levels of organic C than in the unfumigated case, likely due to cell lysis from fumigation. For an ideal application of the fumigation–extraction approach, the unfumigated control in a bacteria-bearing system would exhibit little or no extracted TOC in the $K_2SO_4$ wash solution. However, the difference in TOC concentrations between the fumigated samples and the unfumigated controls also increases with increasing biomass, and this relationship strongly suggests that the fumigation procedure can be calibrated and used successfully in a bacteria-only system to relate extracted TOC to initial biomass in the sample.

For the non-bacterial soil components that we tested, the differences between the organic C concentrations that were extracted from the fumigated samples and the unfumigated controls should be zero for the fumigation–extraction procedure to be valid. That is,

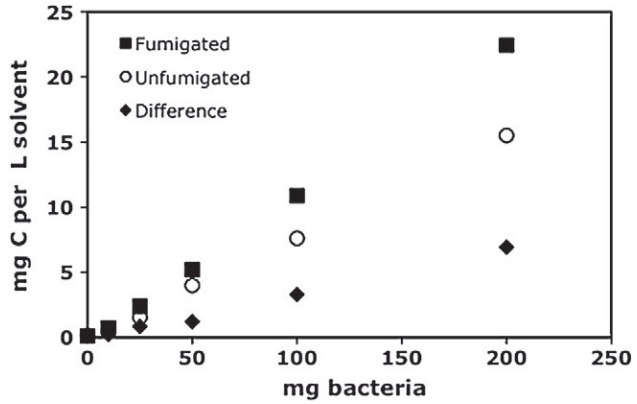


**Fig. 1.** Experimental results from the bacteria-only system, plotted in terms of the concentration of extracted TOC as a function of the initial mass of bacteria present in the sample.

because those single-component systems do not contain any bacterial cells, the fumigation should not lyse any cells and therefore should not introduce additional organic C to the $K_2SO_4$ wash solution. The humic acid (Fig. 2) and the silica sand (Fig. 3) systems show this relationship. In the case of humic acid, we expect the TOC concentration in fumigated and unfumigated extraction solutions to increase with the amount of humic acid because humic acid is largely composed of organic C. The amount of TOC in the fumigated and unfumigated extraction solutions from experiments with the same initial mass of humic acid is essentially the same, and is close to being equal to the entire mass of humic acid in each experiment, suggesting that almost all of the humic acid dissolves into the $K_2SO_4$ wash solution in each extraction. The difference between the two concentrations is close to zero for all humic concentrations tested. This result suggests that the presence of humic acid in a soil does not affect the amount of C attributed to biomass using the fumigation–extraction method.

The results from the silica sand-only systems (Fig. 3) demonstrate that virtually no TOC is extracted from each sample, and that this result is independent of initial sand mass and is independent of whether the sample was exposed to chloroform. As is the case for the humic acid system, the difference between extractable TOC concentrations in the solutions from fumigated and unfumigated samples is essentially zero and does not increase with increasing sand mass, indicating that the chloroform fumigation procedure does not add to the extractable C pool. The two-component experiment that involved a constant 50 g of the silica sand and varying amounts of bacteria demonstrates that the method may be difficult to apply to a sand-rich soil or aquifer material (Fig. 4). The results from the sand and bacteria system indicate that the presence of the silica sand reduces the efficiency of the fumigation–extraction procedure in extracting biomass C from the bacteria that are present in the sample. The silica sand does not adsorb chloroform and its presence does not totally block cell lysis due to fumigation. However, for a given mass of bacteria, the concentrations of extracted C from both the fumigated and unfumigated samples are lower when the silica sand is present than it is for the corresponding bacteria-only experiments. The dependence of the extraction efficiency on silica sand content of the sample suggests that the fumigation–extraction technique would need to be calibrated for a particular biomass:sand ratio, making it impractical for the determination of biomass in a sample where the ratio of biomass to mass of sand is unknown. However, Figs. 1 and 4 also show that the values of the difference between fumigated and unfumigated samples in the bacteria-only and in the bacteria and sand experiments are similar for a given biomass. This result may indicate that the method could be used successfully for determining biomass C in a sandy soil, but more tests would be required to determine if the difference values are independent of silica sand content.

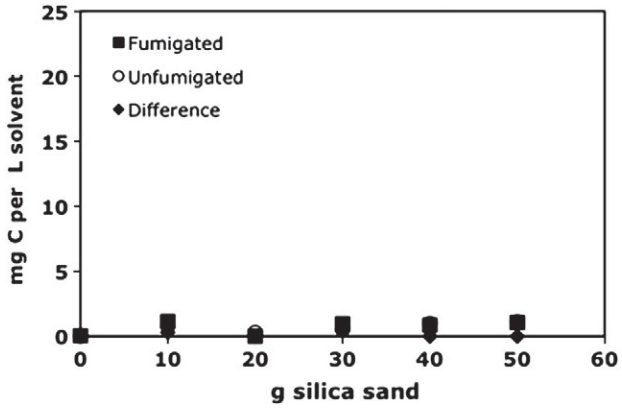


**Fig. 3.** Fumigation results for silica sand-only samples, plotted in terms of the concentration of extracted TOC as a function of the initial mass of sand present in the sample.

The clays that we studied exhibit markedly different behavior than the other soil components. If chloroform does not adsorb onto mineral surfaces, then the model clays, like the silica sand, would show little or no total organic C upon extraction, and no difference between fumigated samples and unfumigated controls. Unlike the results for silica sand and humic acid, our experiments with wet and dry montmorillonite (Figs. 5 and 6) and kaolinite (Fig. 7) strongly suggest that chloroform vapor adsorbs substantially to the clays during the 24 h of fumigation. That is, the difference between the fumigated clay samples and the unfumigated controls is not zero, but increases with the mass of clay that is fumigated. Different surface chemistries and a lower surface area for the silica sand relative to the clay samples likely explain why chloroform adsorption onto silica sand was negligible in our experiments while we observed extensive chloroform adsorption onto both types of clay. The wet montmorillonite experiments demonstrate that natural soil samples that contain hydrated clays also adsorb chloroform during the fumigation–extraction method, adding to the organic C pool in the extraction solution. The wetted montmorillonite adsorbs more chloroform per unit dry weight than the dry montmorillonite samples, but there is a significant extent of chloroform adsorption in both cases. The dry and wetted montmorillonite samples contain 5 and 83% water by mass, respectively. Natural soil samples typically contain clays in this hydration range, so our results indicate that these clays adsorb significant concentrations of chloroform and that the fumigation–extraction approach cannot be

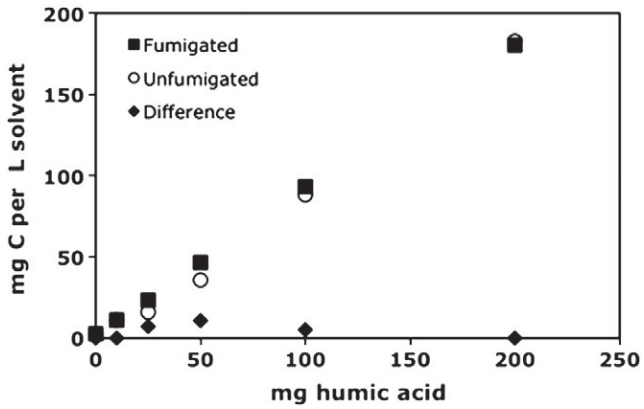


**Fig. 2.** Fumigation results for humic acid-only samples, plotted in terms of the concentration of extracted TOC as a function of the initial mass of humic acid present in the sample.

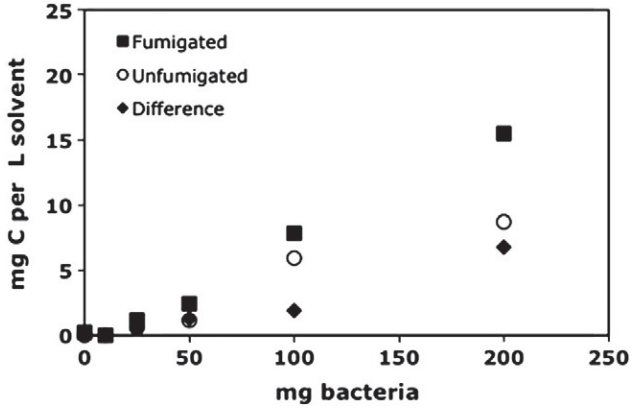


**Fig. 4.** Results for fumigation experiments with a constant 50 g of silica sand and varying amounts of bacteria, plotted in terms of the concentration of extracted TOC as a function of the initial mass of bacteria present in the sample.

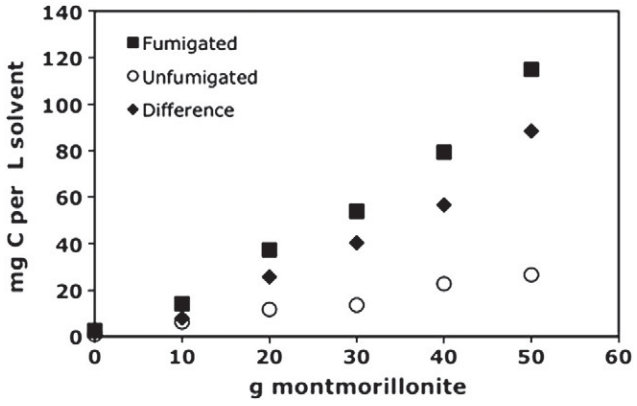


**Fig. 5.** Fumigation results for montmorillonite-only samples (SWy-2), plotted in terms of the concentration of extracted TOC as a function of the initial mass of montmorillonite present in the sample.

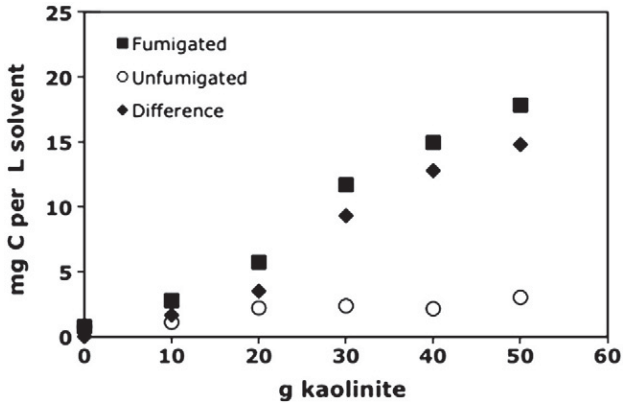


**Fig. 7.** Fumigation results for kaolinite-only samples (KGa-1b), plotted in terms of the concentration of extracted TOC as a function of the initial mass of kaolinite present in the sample.

accurately used to determine biomass for these types of samples. The unfumigated control experiments demonstrate that there is a measurable amount of carbon associated with the natural clays. Cannan Cabar (1999) demonstrated that increased amounts of humic substances associated with clays can decrease the adsorption of carbon tetrachloride and trichloromethane; thus, the amount of humic substances associated with the clays likely has a strong impact on the degree of chloroform adsorption to the clay surfaces.

The amount of chloroform adsorbed in our experiments adds an average of 1540 μg C per g montmorillonite and 350 μg C per g kaolinite into the extraction solution. The fumigation–extraction method, applied to ten different naturally occurring soils by Vance et al. (1987), yielded between 60 and 1220 μg biomass C per gram of soil. The concentration of adsorbed chloroform in our clay experiments is comparable to this range, and our results indicate that adsorbed chloroform could contribute substantially to the C pools of these soil samples. In soil samples that contain significant quantities of clay mineral surfaces, exposure of the sample to chloroform leads to chloroform adsorption onto the clays. The subsequent $K_2SO_4$ wash desorbs the chloroform into the wash solution, and because chloroform contains organic C, its presence on the clays and in the wash solution contributes to the organic C concentration that is determined for the sample. Thus, soil samples with significant concentrations of clay minerals could yield artificially high results for biomass C during the fumigation–extraction process due to adsorption artifacts.

### 3.2. Attempts to remove chloroform from extraction solutions

Having determined that chloroform adsorbs to clays and subsequently appears in the 0.5 mol $L^{-1}$ $K_2SO_4$ extraction solutions, we conducted additional evacuations to determine if the adsorbed chloroform could be removed prior to TOC analysis. After a sample is fumigated for 24 h, the standard method calls for 8 evacuations of the fumigation chamber by vacuum for 3 min each. Using 50 g samples of the fumigated montmorillonite, we varied the duration of each of these 8 evacuations from 0 min (no evacuation, sample extracted directly) to 4 min. Essentially no difference is observed in the measured total organic C in these samples (Fig. 8).

We also conducted tests to determine if the chloroform that is present in the extraction solution could be removed by further gas sparging of the solution. In these tests, we used extracts of fumigated kaolinite samples. Immediately following extraction, 5 mL of each sample solution was vigorously sparged with $N_2$ gas for 30 min. The samples were subsequently acidified with HCl as reported in the Materials and methods section, and analyzed for total organic C. During organic C analysis, the DOC analysis instrument sparges each sample with $N_2$ for an additional 4 min. While the sparging did remove some of the chloroform from these solutions, the effect is minimal (Fig. 9). The maximum effect of the sparging that we observed was a decrease in DOC of only 10.5% and a strong linear correlation still exists between the mass of kaolinite in each sample and the concentration of organic carbon present in the extraction

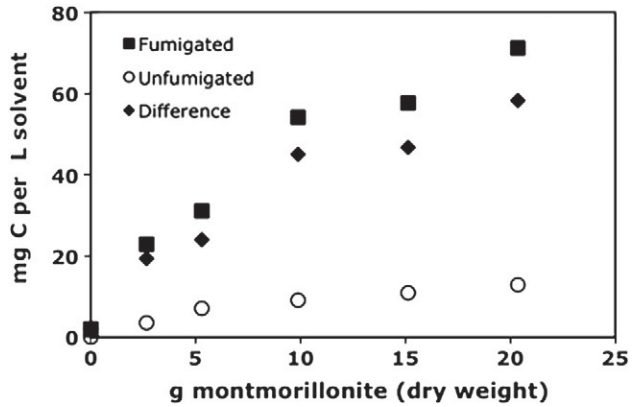


**Fig. 6.** Fumigation results for wet montmorillonite samples (SWy-2), plotted in terms of the concentration of extracted TOC as a function of the initial dry mass of montmorillonite present in the sample.

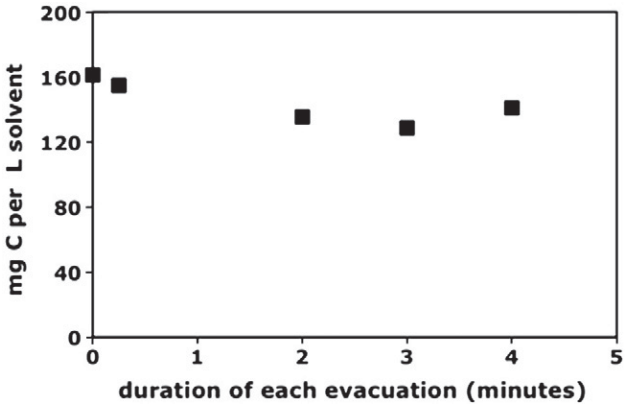


**Fig. 8.** Effect of evacuation time on the TOC content of the montmorillonite extraction solution. The TOC concentration is plotted as a function of the duration of each of the 8 evacuation steps in the procedure.

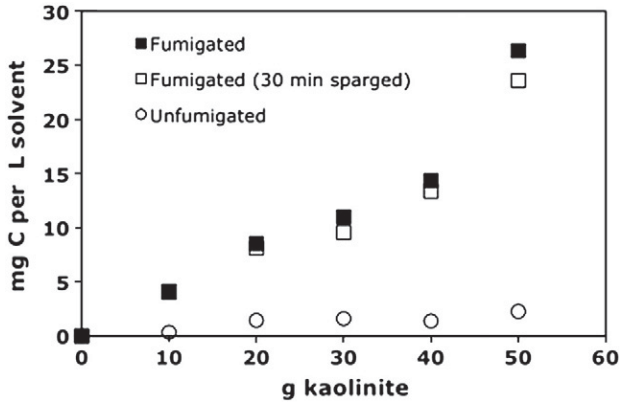


**Fig. 9.** The effect of an additional 30 min $N_2$ gas sparge of the extraction solution on the TOC content of the solution. Open squares, closed squares, and open circles represent the samples without the extra sparging, the samples with the extra sparging, and the unfumigated controls, respectively.

solution. These results demonstrate that sparging time does not strongly affect chloroform removal from the extraction solutions.

### 3.3. Natural soils

We conducted the fumigation-extraction procedure on 25 g of two natural loams (Table 1) in order to determine whether the chloroform adsorption that we observed for clays occurs onto clay-rich soils as well, using a spectrophotometric approach to measure chloroform concentration directly in the extraction solutions. Prior to fumigation, the soils were sieved using a #8 (2.38 mm mesh) sieve. We measured 83.0 μg biomass C per gram of soil in the Waterford Loam (WcnAl); of this 32.5 μg per gram was measured to be chloroform C using the spectrophotometric method. Thus 39% of the organic C pool interpreted to be due to biomass in the $K_2SO_4$ extracts of this soil was due to the presence of chloroform, and the remaining 61% was due to biomass. We measured 58.7 μg biomass C per gram of soil in the Auten Loam (AxvA), of which 8.3 μg per gram was due to chloroform C. For the AxvA sample, 14% of the carbon pool that would normally be interpreted as biomass C was in fact chloroform C. The soils studied here were collected from the B horizon, which contains less biomass C than a typical A horizon soil. An A horizon soil may typically contain between 200 and 2000 μg biomass C per gram of soil. Our results show that 32.5 and 8.3 μg chloroform per gram of soil were adsorbed to the two soils studied. Combining the range of typical A horizon biomass C and the chloroform adsorption observed in our B horizon soils, we can calculate that between 0.4 and 16% of the carbon extracted from an A horizon soil may be due to chloroform adsorption during the fumigation step. Clearly, the accuracy of the measurement in a range of natural soils is adversely affected by the adsorption of chloroform onto the soils. In natural soils that contain a significant clay fraction, such as the soils tested here, chloroform adsorption may artificially inflate the extracted carbon pool that is interpreted to be biomass C.

## 4. Conclusions

Our results show that the chloroform fumigation–extraction approach may need to be corrected in soils containing substantial amounts of clay minerals. It may be possible to use the fumigation–extraction method to estimate biomass C concentrations for samples that are poor in clay, such as a silica sand groundwater aquifer that also contains humic acid and/or bacteria. However, many soils contain substantial clay mineral fractions. We therefore suggest that the fumigation–extraction method may require a correction factor to accurately determine biomass C in soils containing a substantial amount of clay. The determination of a correction factor is likely to be a difficult task, because factors including pH, and clay amount, surface area, and type may influence the degree of chloroform adsorption during the soil fumigation step.

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