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Mark Tibbett,[1] *Ph.D.; David O. Carter,*[2] *M.Sc.; Tamsin Haslam,*[3] *M.Sc.; Rebecca Major,*[3] *M.Sc; and Robert Haslam,*[3] *B.Sc.*

A Laboratory Incubation Method for Determining the Rate of Microbiological Degradation of Skeletal Muscle Tissue in Soil

ABSTRACT: A controlled laboratory experiment is described, in principle and practice, which can be used for the of determination the rate of tissue decomposition in soil. By way of example, an experiment was conducted to determine the effect of temperature (12°C, 22°C) on the aerobic decomposition of skeletal muscle tissue (Organic Texel × Suffolk lamb (*Ovis aries*)) in a sandy loam soil. Measurements of decomposition processes included muscle tissue mass loss, microbial CO_2 respiration, and muscle tissue carbon (C) and nitrogen (N). Muscle tissue mass loss at 22°C always was greater than at 12°C ($p < 0.001$). Microbial respiration was greater in samples incubated at 22°C for the initial 21 days of burial ($p < 0.01$). All buried muscle tissue samples demonstrated changes in C and N content at the end of the experiment. A significant correlation ($p < 0.001$) was demonstrated between the loss of muscle tissue-derived C (C_t) and microbially-respired C (C_m) demonstrating CO_2 respiration may be used to predict mass loss and hence biodegradation. In this experiment Q_{10} (12°C − 22°C) = 2.0. This method is recommended as a useful tool in determining the effect of environmental variables on the rate of decomposition of various tissues and associated materials.

KEYWORDS: forensic science, forensic taphonomy, muscle tissue decomposition, temperature, microbial activity, microbial carbon assimilation, CO_2 respiration, gravesoil, Ovis aries, Q_{10}

A multidisciplinary approach is necessary to understand the complex processes controlling human cadaver decomposition (1). In the past, workers have employed entomology (2–4), soil chemistry (5), soil microbiology (6,7), biochemistry (8), botany (9,10), and mycology (11) to gain an understanding of processes associated with cadaver decomposition in terrestrial ecosystem. Consequently, it is generally understood that edaphic variables such as temperature, soil type, and moisture content can effect cadaver decomposition in soil (12–14). To date, only factors influencing postmortem tissue desiccation rate (tissue water content, clothing, burial position) have been determined under controlled environmental conditions (15).

The current paper describes the principles and practice of a simple laboratory incubation method that can be used to quantify the effect of environmental and edaphic variables on muscle tissue decomposition in soil. The method represents an initial attempt at formulating a model that may be used to understand the complex processes associated with postburial decomposition. We believe that a preliminary understanding of more complex decomposition can be achieved by examining the breakdown of a relatively simple substrate (skeletal muscle tissue) in soil. This is not an attempt to replicate the decomposition of a cadaver with its enteric flora and numerous components.

[1] School of Earth and Geographical Sciences, University of Western Australia, Crawley, WA 6009, Australia.
[2] School of Pharmacy and Molecular Sciences, James Cook University, Townsville, QLD 4811, Australia.
[3] School of Conservation Sciences, Bournemouth University, Poole, Dorset BH12 5BB, United Kingdom.

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A demonstration experiment considers the effect of temperature on the rate of muscle tissue decomposition. We determine whether the soil microbiota can utilize muscle tissue as a source of nutrients and energy in the absence of the enteric microbial community and how temperature affects the decomposition of muscle tissue in soil. The measurement of decomposition processes included tissue mass loss, microbial carbon dioxide (CO_2) respiration, and changes in carbon (C) and nitrogen (N) content of the muscle tissue.

Background Principles

The mass loss of muscle tissue can give an estimate of its decomposition as a substrate. However, the remaining mass of any complex substrate in soil is not a definitive quantity. It may vary because of the mass of its decomposers and loss of its partially decomposed products from the solid phase (16). Determination of mass loss allows the effect of environmental parameters on muscle tissue decomposition in soil to be assessed.

All respiration of aerobic soil microorganisms releases CO_2 into soil air, which diffuses into the atmosphere. This CO_2 can be measured to determine the rate of microbial activity and hence decomposition (17–20). Such estimates of decomposition ignore C immobilized into (taken up by) the soil microbial biomass (the mass of living microorganisms in the soil) and lost as partially degraded intermediates (e.g., fatty acids, amino acids) of the original substrate. As such, CO_2 respiration is used to gauge decomposition and is not a direct measure of degradation of the material of interest but of the microbial activity generated by its decomposition. The measurement of microbial CO_2 respiration also provides the basis of a method for the estimation of the soil microbial biomass (21,22). The soil microbial biomass is responsible for biodegradation and regulating nutrient transformation and storage (23).

TABLE 1—*Soil biophysiochemical parameters of sandy loam soil from Lindens farm, East Lulworth, Dorset, England.*

Determinant	Value
Loss on ignition (%)	5.9
Bulk Density (g cm^{-3})	1.12
Sand (%)	61
Silt (%)	18
Clay(%)	21
pH	6.4
Total P (mg kg^{-1})	308
Total C (%)	3.1
Total N (%)	0.15
Total Cd (mg kg^{-1})	0.140
Bioavailable Cd (mg kg^{-1})	0.078
Total Zn (mg kg^{-1})	30.9
Bioavailable Zn (mg kg^{-1})	2.14
Microbial biomass C (μg C g^{-1} soil)	347

The C to N ratio (C:N) provides the most common index of substrate quality in soil (24). A low C:N ratio equates to high substrate quality and encourages a rapid rate of decomposition. A high C:N ratio equates to low substrate quality and typically has a slower rate of decomposition depending on free N in the soil. Carbon and N can be measured by dry combustion chromatography, a commonly used technique for estimating total C and N simultaneously (25).

Material Preparation: Background

The soil used in this work was a sandy loam of the Fyfield series from Lindens farm, East Lulworth, Dorset, England. Biophysiochemical characteristics were determined to characterize the soil (Table 1). Preliminary work then was undertaken to standardize soil moisture and determine soil microbial decomposition characteristics. Organic lamb (Texel + Suffolk) (*Ovis aries*) skeletal muscle tissue was selected as a human muscle tissue analogue.

Standardizing Soil Moisture

Soil moisture is an important variable because it alters the environment in which microbial activity can occur (26,27). Thus, soil moisture affects the rate at which soft tissue decomposes (5,8,14). This is the result of reduced diffusion coefficients of gases (e.g., O_2, CO_2, CH_4, H_2S) in soil water compared with soil air (26) and may lead to a change in biochemical processes limited by gas diffusion. The determination of soil water content does not give a good comparative measure of the availability of soil water to microorganisms, which is dependent on the tension at which the water is held at, and between, particle surfaces (26). Soils of different porosity that contain the same mass of water (per unit mass of soil) will hold water at different tensions and consequently offer differing moisture regimes to organisms (26). Therefore, the soil water holding capacity (WHC), which is a crude approximation of soil moisture tension, should be used to balance soil moisture regimes as an otherwise confounding variable. As noted by Orchard and Cook (27), soil water potential (ψ) is the most accurate measure of the physical force with which water is held in soil. However, facilities to measure and manipulate water potential are not commonly available.

Because of the effect of WHC on soil microbial activity, care must be taken in selecting appropriate moisture levels for experimental work. A WHC of approximately 60% has been suggested as the optimal for maximum microbial activity (28) and typically is used in studies concerned with the measurement of microbial CO_2 respiration and biomass (29–31).

Soil Microbial Decomposition Characteristics

The period of peak microbial activity can be used to determine the relationship between tissue mass and microbial activity. If microbial activity in the microcosm becomes independent of tissue mass, then the maximum theoretical rate of decomposition has occurred. This phenomenon is similar to that of maximal reaction velocity (V_{max}) proposed by Michaelis and Menten (32) and used in the study of soil enzyme kinetics (33). If experimental material is introduced at a V_{max} mass, small errors in that mass or differences in initial (lag phase microbial colonisation and growth) tissue decomposition will have no bearing on CO_2 respiration.

Material Preparation: Practice

Standardizing Soil Moisture

To calculate WHC, a known volume of fresh soil was soaked in water for 24 h. Saturated soil then was placed in a covered Büchner funnel until water ceased to drip from the funnel (approximately 2 h). At this point, soil was saturated fully at field capacity (100% WHC). The WHC of soil then was calculated by measuring the mass of water lost on oven drying (m_w) at 105°C to a constant mass (m_s). Soil moisture (Θ_m) then was calculated in the normal way (Eq 1) (34).

$$\Theta_m = m_w/m_s \quad (1)$$

Soil Microbial Decomposition Characteristics

To determine the time of peak microbial activity after tissue amendment a time course of CO_2 evolution in experimental microcosms was undertaken. In this experiment, field fresh soil (100 g dry weight) was sieved (4.6 mm), adjusted to 60% WHC and placed in polyethylene incubation chambers (1285 mL) (Merck Ltd., United Kingdom, product no. 215044808). An approximation of O_2 requirement was based on the assumption that O_2 is consumed by soil microorganisms at a rate of 1.5 mL O_2 100 g^{-1} soil h^{-1}. Thus, the microbial biomass in 100 g of soil located in each incubation chamber would consume approximately 36 mL of O_2 every 24 h. The headspace in each incubation chamber is ~1000 mL, or ~200 mL O_2. Therefore, one would expect sufficient O_2 for 5 d of basal soil respiration before the air in the headspace would need to be changed. This allows a generous margin for greater respiration rates under muscle tissue amendment if air and alkali traps are changed every 24 h.

Soil microcosms were placed in the dark at 22°C for 48 h to equilibrate. Subsequently, cuboid tissue samples (1.0 g) were buried at the same depth (2.5 cm) in the soil. To determine the amount of CO_2 respired from the soil and decomposing tissue, 10 mL of sodium hydroxide (0.3 M NaOH) solution was placed in 20-mL vials (CO_2 traps) and suspended in the incubation chambers. The chambers then were sealed. The CO_2 traps and the air in the incubation chambers were replaced at 24-h intervals for 7 d. The NaOH solution from the CO_2 traps was back-titrated with HCl (0.1 M) into 10 mL $BaCl_2$ (1.0 M) and six drops phenolphthalein as color indicator (35,36). The experiment was replicated six times and controls (no tissue) were included. From this trial, it was determined that microbial CO_2 respiration peaks 48 h after tissue amendment (Fig. 1).

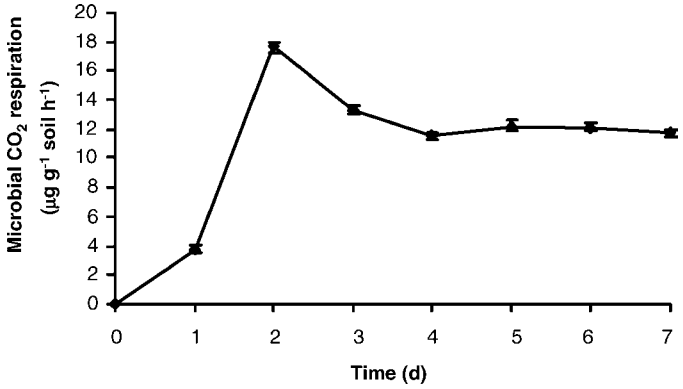

FIG. 1—*Time course of microbial CO_2 respiration ($\mu g\ g^{-1}$ soil h^{-1}) following the addition of 1.0 g of skeletal muscle tissue (Ovis aries) in 100 g dry weight of sandy loam soil of the Fyfield series, Lindens farm, East Lulworth, Dorset, England. Incubation was conducted at 22°C. Bars ± standard error, n=6.*

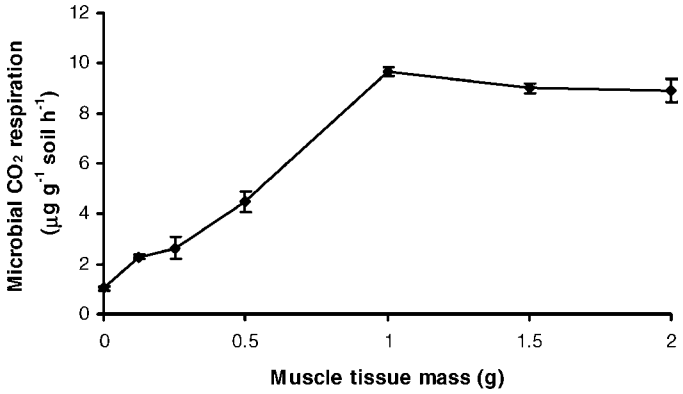

FIG. 2—*Microbial CO_2 respiration ($\mu g\ g^{-1}$ soil h^{-1}) 48 h after the addition of a range of skeletal muscle tissue (Ovis aries) mass (0.0 g, 0.125 g, 0.25 g, 0.5 g, 1.0 g, 1.5 g, 2.0 g) in 100 g dry weight sandy loam soil of the Fyfield series, Lindens farm, East Lulworth, Dorset, England. Incubation was conducted at 22°C. Bars ± standard error, n=6.*

To determine the relationship between microbial activity and muscle tissue mass, seven different masses (0.125 g, 0.25 g, 0.5 g, 0.75 g, 1.0 g, 1.5 g, 2.0 g) of tissue were buried (2.5 cm) in soil. Microbial CO_2 respiration was measured after 48 h using the method described above. A tissue mass of 1.0 g—2.0 g in 100 g dry soil resulted in a rate of decomposition independent of tissue mass (Fig. 2).

Experimental: Temperature and Decomposition

An experiment was conducted to measure the effect of temperature on muscle tissue decomposition in soil. Field fresh soil (100 g dry weight) was sieved (4.6 mm), adjusted to 60% WHC and placed in polyethylene incubation chambers (1285 mL). Soil was placed in the dark at 12°C or 22°C for 48 h to equilibrate. Cuboid tissue samples (1.5 g) were buried at an equal depth (2.5 cm) in soil and incubated in the dark at 12°C or 22°C for 35 days.

Muscle tissue mass loss was measured at 7-d intervals using a sequential harvesting program. Tissue was removed from the soil, rinsed, dried, and weighed. Microbial CO_2 respiration measurements were conducted at 24- to 48-h intervals using the method described above. Basal microbial CO_2 respiration values were subtracted from tissue-amended CO_2 respiration values. The resulting levels of respiration represent microbial CO_2 respired as a direct response to tissue burial. A temperature coefficient value (Q_{10}) was used to assess the difference in microbial activity (CO_2 respiration) at 10°C intervals. Samples were replicated six times and controls (soil without tissue) were included.

TABLE 2—*Percent tissue C, N and C:N ratio (± SE) of fresh muscle tissue (Ovis aries) and muscle tissue buried for 35 days at 12°C or 22°C in sandy loam soil from Lindens farm, East Lulworth, Dorset, England.*

Sample	% C	% N	C:N ratio
Fresh	43.50 ± 5.14	7.97 ± 0.38	5.46 ± 0.40:1
35 day (12°C)	26.56 ± 1.66	4.60 ± 0.57	5.77 ± 0.23:1
35 day (22°C)	54.65 ± 2.05	4.46 ± 0.74	12.25 ± 1.75:1

Carbon and N measurements of fresh and buried tissue were carried out using dry combustion chromatography (Carlo Erba EMASyst 1106) after 35 d of burial. Benzylthiuronium chloride (Elemental Microanalysis, Devon, UK) was used as the standard. Samples were selected carefully in an attempt to minimize variation attributable to the intrinsic heterogeneity of skeletal muscle tissue (37).

Muscle tissue carbon (C_t) loss was predicted using accumulated CO_2-C respiration measurements and an estimate of microbially assimilated C. The CO_2-C measurements were summed over each 7-d incubation period to match mass loss measurement intervals. These were 7, 14, 21, 28, and 35 days. The CO_2-C can be calculated by determining the fraction of C for every unit of CO_2 respired. The molar mass of C is 12 g per mole (mol^{-1}). The molar mass of CO_2 is 44 g mol^{-1}. Therefore, C represents 12/44 (0.273) of every unit of CO_2 respired. However, the simple measurement of CO_2-C cannot account for all C decomposed in the burial environment. Microbial cells aerobically transform organic material into CO_2, H_2O, intermediates (e.g., amino acids), cellular materials (e.g., lipids, fatty acids) and energy (38). As a result, some C is assimilated into the cells of the microbial biomass. Assuming a microbial C assimilation efficiency of 0.33, (39) approximately 2 g C are respired as CO_2 per g C assimilated. Thus, microbially assimilated C (C_a) and microbially respired C (C_r) can be calculated as:

$$\begin{aligned} C_a + C_r &= C_m \\ C_m &= C_{resp} * 0.273 * 1.5 \end{aligned} \qquad (2)$$

Where C_m is microbially derived carbon (mg), C_{resp} equals respired CO_2(mg), 0.273 is the fraction of C present in CO_2, and 1.5 is the assimilation constant (value required to account for microbially assimilated C). The C_m then was compared with the percentage of C in the muscle tissue (C_t) (43.5%, Table 2).

Statistics

Descriptive and inferential statistics were carried out using Microsoft Excel 2000 and SPSS version 11.0.1. Normality was assessed with Kolmogorov-Smirnov test and equality of variance with Levene's test. Nonparametric data were analyzed using Mann-Whitney U tests. Confidence levels were set at 95%. Pearson's correlation coefficient and linear regression analysis was used to test for a relationship between C_t and C_m data.

Results

Tissue mass loss was greater in samples incubated at 22°C than at 12°C ($p < 0.001$) (Fig. 3). Tissue buried at 22°C lost approximately 50% of its mass after 7 d of burial. Tissue buried at 12°C required 14 d to lose a similar amount of mass (Fig. 3). The rate of

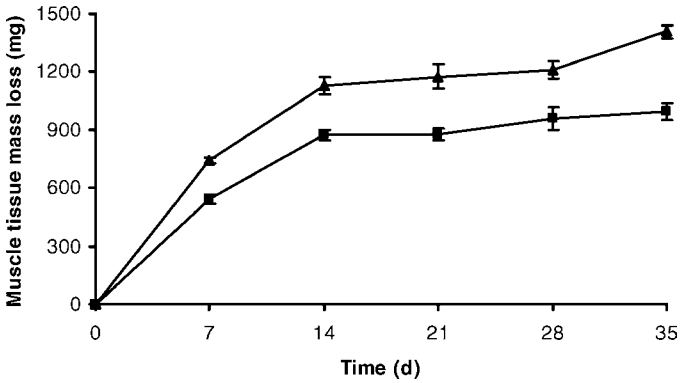

FIG. 3—*Mass loss (mg) of skeletal muscle tissue (Ovis aries: 1.5 g) buried at 12°C (■) and 22°C (▲) in a sandy loam soil of the Fyfield series, Lindens farm, East Lulworth, Dorset, England. Bars ± standard error, n=6.*

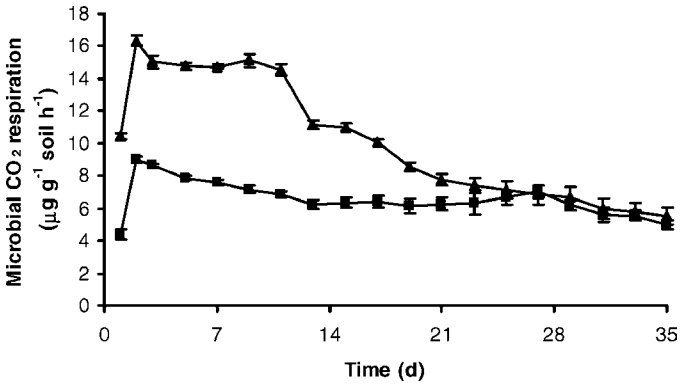

FIG. 4—*Microbial CO_2 respiration over a period of 35 d after the addition of 1.5 g skeletal muscle tissue (Ovis aries) in 100 g dry weight sandy loam soil of the Fyfield series, Lindens farm, East Lulworth, Dorset, England. Samples were incubated at 12°C (■) and 22°C (▲). Bars ± standard error, n=6.*

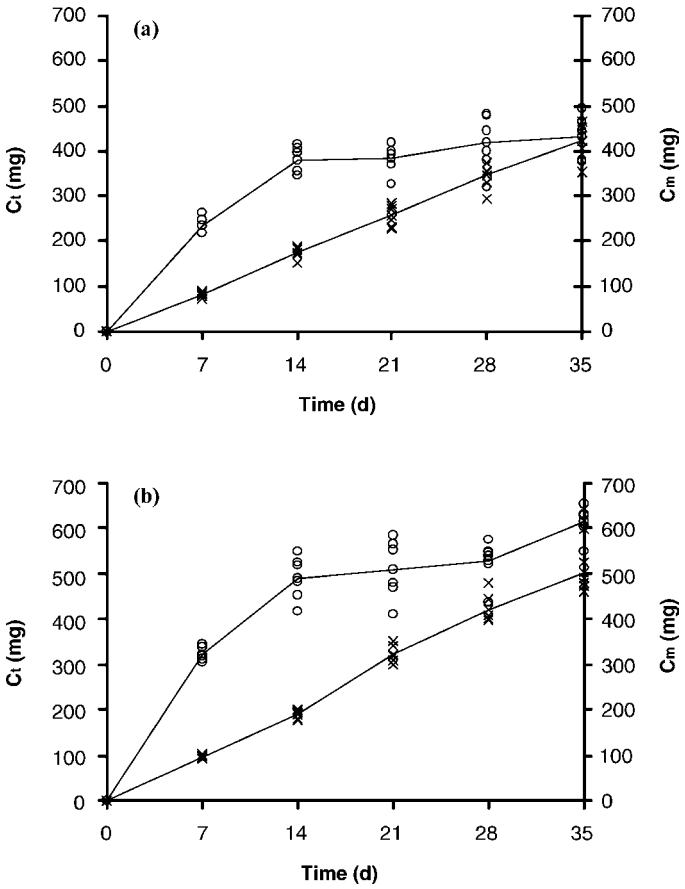

FIG. 5—*Comparison of muscle tissue-derived (Ovis aries) C loss (C_t: symbol o) and respiration-derived C loss (C_m: symbol x) incubated at (a) 12°C and (b) 22°C in a sandy loam soil of the Fyfield series, Lindens farm, East Lulworth, Dorset, England. Curves represent the mean of six replicates.*

tissue decomposition declined after approximately 14 d of burial. Microbial CO_2 evolution illustrated that the majority of microbial activity occurred during the first 14 d of burial (Fig. 4). These respiration rates were similar after 21 d of burial and remained so for the duration of the experiment ($p > 0.05$). In this experiment Q_{10} (22°C – 12°C) = 2.01.

The C:N ratio of tissue incubated at 22°C was higher than that of fresh tissue and tissue incubated in soil at 12°C ($p < 0.05$) (Table 2). The C:N ratios of fresh tissue and tissue incubated at 12°C were not significantly different ($p > 0.05$) (Table 2). Muscle tissue samples buried at 12°C demonstrated a decrease in %C and %N (Table 2). Estimates of percentage of C lost from muscle tissue (C_t) and from microbial CO_2 respiration (C_m) at both 12°C and 22°C are compared in Fig. 5. Throughout the incubation $C_t > C_m$ but at the end of the experiment these data converged. Muscle tissue samples buried at 22°C demonstrated an increase in %C and a decrease in %N (Table 2). A significant correlation was demonstrated between C_t and C_m measurements at both 12°C (Pearson's R = 0.723; $p < 0.001$) and 22°C (Pearson's R = 0.821; $p < 0.001$). Regression equations are shown in Fig. 6.

Discussion

Results show that muscle tissue was decomposed more rapidly at warmer temperatures. This finding is in accordance with the existing literature (3,12,14,40). The rate of mass loss decreased through time. This may be because of the loss of readily available substrate and the formation of stable humic substances recalcitrant to decomposition (41). Microbial CO_2 evolution data indicated that microbial activity associated with buried tissue decreased as a result of diminishing muscle tissue (substrate) supply. A Q_{10} value of 2.0 was anticipated as an approximate doubling of microbial activity occurs with each 10°C rise in temperature between 5°C and 30°C to 35°C (24). The application of this principle is important because it may prove a useful indication of the effect of soil temperature on the decomposition of muscle tissue in other settings.

The use of CO_2-C evolution proved to be a reasonable determinant of tissue mass loss by the end of the experiment. Although correlations show a strong relationship between C_t and C_m throughout the experiment, the curves comparing C_t and C_m (both at 12°C and 22°C) diverge for the first 14 d of incubation before converging for the remainder of the experiment. Therefore, we suggest that preliminary CO_2 respiration is not a good predictor of mass loss and longer incubations should be used. Measurement of CO_2respiration may be particularly useful when applied to the decomposition of amorphous substrates that do not lend themselves to mass loss estimation (e.g., hair, semen, excrement, blood). These could be dealt with in the same way described in this paper. However, it should be noted that assimilation efficiencies of microbial communities may increase with lower nutrient availability (39).

Although there were no differences in C:N ratios between fresh tissue and tissue buried for 35 d at 12°C, the percentage of C and N

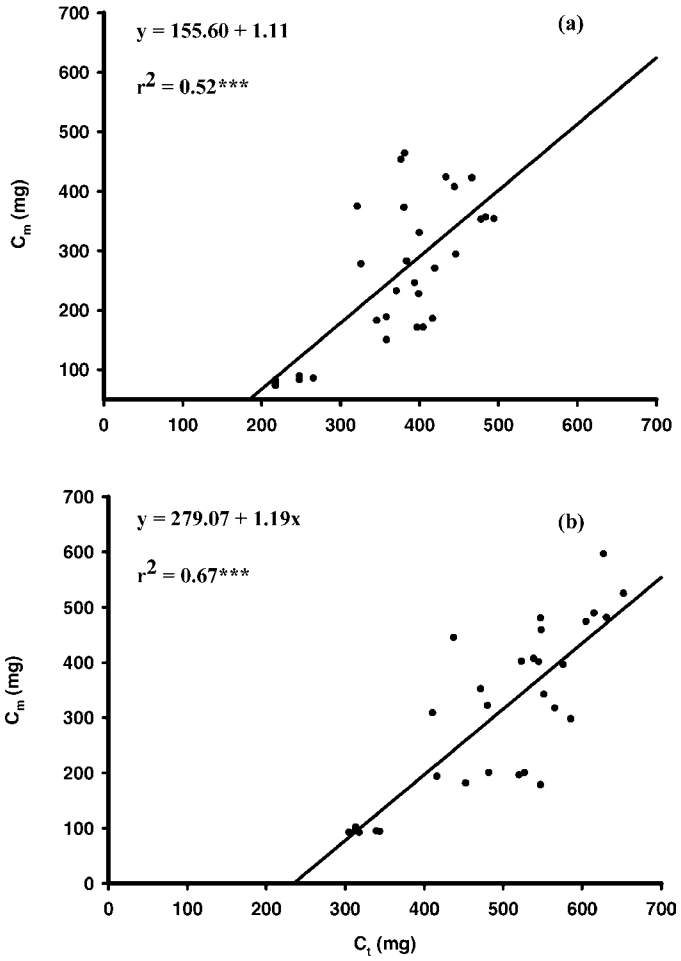

FIG. 6—*Linear regression relationship between muscle tissue-derived (Ovis aries) C (C_t) and microbial respiration-derived C (C_m) in samples incubated at (a) 12°C and (b) 22°C. n = 30 (*** $p < 0.001$).*

had both fallen. At 22°C, the C:N ratio had widened, which represents a reduction in the quality of the muscle tissue as a substrate. In this case, a greater percentage carbon (55%) in the remaining tissue suggests only the more recalcitrant and perhaps partly humified fractions remain.

Efremov (42) proposed that it is possible to gain an understanding of the processes of decomposition through the investigation of burial conditions. The findings from the current work have serious implications for forensic science and forensic taphonomy in particular. We have shown that soil microorganisms can play a rapid and substantial role in the early phase decomposition of discrete muscle tissue. This challenges the suggestion that the soil microbiota play their most significant role during the later stages of decomposition (e.g., 43).

The use of other human tissue analogues, as well as complete cadavers, could be tested for their rate of decomposition in the incubation system we describe. Given the scale of the current experimental work, the method might apply chiefly to smaller cadavers and their components. The measurement of aerobic and anaerobic gases (i.e., hydrogen sulfide, methane, ammonia) produced on decomposition may provide an insight into how anaerobic processes affect the rate at which buried remains decompose. These methods are in development and would use the same principles of replication and sequential or repeated measurements. The current demonstration of a relationship between temperature, muscle tissue decomposition and soil microbial respiration suggests that further quantifiable processes (related to the decomposition of muscle tissue in soil) may be developed. Future work should examine the effect of soil type, moisture, and pH on decomposition processes in soil. Forensic taphonomy also might benefit from the use of enzymatic, lipid, nucleic and substrate utilization analyses of burial environment soils. These analyses can provide information concerning structural (lipid and nucleic acid analysis) and functional (enzymatic and substrate utilization analysis) processes of the microbial community. Understanding successional changes in the microbial communities might prove a novel approach in determining postburial interval (see 11), and changes in microbial communities may be enduring indicators of former burials.

Acknowledgments

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Additional information and reprint requests:
Mark Tibbett, Ph.D.
School of Earth & Geographical Sciences
University of Western Australia
35 Stirling Highway
Crawley, WA 6009
Australia
E-mail: Mark.Tibbett@uwa.edu.au