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Dear Editor,

How to preserve termite samples in the field for carbon and nitrogen stable isotope studies?

The measurement of stable isotopes of carbon (^{13}C) and nitrogen (^{15}N) is a powerful tool in ecological studies, since it can indicate food preferences of organisms.[1] This is possible because these isotopes are available in the environment and are acquired during feeding. Thus, the isotopic composition of animal tissues reflects the diet gained and assimilated throughout the animal's life.[2] Particularly for termites, whose diets vary subtly within a decomposition continuous from sound wood to highly humified material, isotope analyses can be rather revealing. In such cases, carbon isotope signals indicate the source (e.g. C3 or C4 plants),[3] whereas nitrogen isotopes generally reflect the decomposition degree of the diet[4,5] (although this is not straightforward for wood-feeding termites which are able to fix N_2 from the atmosphere).[6]

The analyses demand that specimens should be processed soon after collection to prevent deterioration and, therefore, isotopic changes. Termites, however, are highly prone to decompose due to their weakly chitinised and very fragile bodies. For this group, therefore, more attention to prevent deterioration and isotopic alteration is needed. Currently, for isotopic analysis, termites are immediately dried at 60°C for 24 h[1,7–9] or frozen after collection.[10] Very often, however, collections occur in remote regions, away from well-equipped labs. In such places, fast processing is unfeasible, as this demands the availability of techniques for specimen preservation to avoid sample decomposition.

The conventional technique for preserving termites is their immersion in ethanol 80% which maintains the morphological characteristics in the long term.[11] Being an organic compound with liposolvent capabilities, ethanol could affect the carbon content and, hence, be unsuitable for preserving samples for carbon isotopic analyses.[18] This theoretical unsuitability of ethanol, however, has not been always confirmed, for vertebrates and invertebrates.[12] Ethanol-preserved tissues of quails,[13] sheep,[13] turtles[14] and caddisflies, a *Trichoptera*,[12] have shown no alteration in carbon isotopic signature. Alternatives would include inorganic (carbon-free) preserving substances, of which sodium chloride (NaCl) seems the cheapest, the best known, and also available worldwide.

This work, therefore, aimed to establish a technique for preserving termites which would suit field work in remote regions while still allowing $\delta^{13}C$ and $\delta^{15}N$ isotopic analyses. To do so, the isotopic ratios and the C/N ratios of stored samples of *Cornitermes cumulans* termites were compared with values for samples analysed immediately after collection. The stored samples were kept in vials containing (i) NaCl solution and (ii) ethanol. Our rationale is that stored samples should vary in their carbon and nitrogen content, because (i) ethanol-preserved samples are subject to lipid loss, whereas (ii) NaCl-preserved samples should keep their original carbon and nitrogen content. Therefore, we hypothesise that freshly processed samples should not differ from NaCl-preserved samples and would differ in their carbon content from ethanol-preserved ones.

The experiment was performed using worker termites (third instar and beyond) from three field colonies of *Cornitermes cumulans* (Kollar) (Isoptera, Termitidae), in Viçosa, state of Minas Gerais, in southeastern Brazil. *Cornitermes* spp. are Neotropical termite species occurring in several habitats, including forests, 'cerrados' (Brazilian savannas) and man-modified habitats, such as pastures or even gardens within cities, where they feed on living and dead grass and herbs.[15]

As soon as mound fragments were taken to the laboratory, termite specimens were extracted and allocated to the appropriate treatments, as described below. Voucher specimens were preserved in 80% ethanol, labelled and identified by comparison with the collection of the Termite Section of the Entomological Museum (UFVB) of the Federal University of Viçosa, Viçosa, Brazil (UFV). This work was carried out from December/2009 to February/2010.

From each termite mound, 30 samples of five workers each were collected to compose ten replicates for each of the processing techniques below.

Freshly processed workers were placed in vials with distilled water, immediately frozen at –14°C for about 2 h and then freeze-dried. This is thought to be the optimal procedure for isotopic analyses, as it avoids sample degradation.

Preserved in ethanol workers were placed in vials with 80% ethanol, at room temperature, for 49 days until washed in distilled water and placed in vials with distilled water to be freeze-dried. The ethanol concentration was confirmed by an alcoholmeter, after adding distilled water to 92.8° GL commercial sugar cane ethanol (Miyako do Brazil Industria e Comercio Ltda, Guarulhos, Brazil). This is the traditional method for maintaining termite samples in collections, and it is expected to be only a partially suitable procedure, as it is bound to affect the $\delta^{13}C$ isotopic signal, because the alcohol could leach lipids and add carbon to the sample.

Preserved in NaCl workers were placed in vials containing a brine solution of table salt (300 g/L), at room temperature for 49 days, until freeze-dried. A brine solution was obtained by adding salt to distilled water, at room temperature, until the water could no longer dissolve any more salt. This is thought to be the best procedure to our aims, as it is cheap, straightforward, and prevents sample decomposition without masking the isotopic signals, as the compound contains no carbon or nitrogen.

To proceed with the isotopic analyses, all the samples were freeze-dried for 48 h, ground, sieved (mesh = 100) and placed in tin capsules.

The $\delta^{13}C$ and $\delta^{15}N$ isotopic ratios, and the C/N ratio in each sample (1.5 μg), were determined in an isotope ratio mass spectrometer (ANCA-GSL 20–20, SerCon Ltd., Crewe, UK), at the Laboratory of Stable Isotopes, Soils Department, Federal University of Viçosa.

The abundances of ^{13}C and ^{15}N in the samples were expressed in δ notation, defined as parts per thousand (‰) deviations from an International Standard, as given by the formula:

$$\delta X = R_{sample} - R_{standard} / R_{standard} * 1000 \quad (1)$$

where R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios, respectively, for the sample and the International Standard. X stands for the 'heavy' isotopes ^{13}C or ^{15}N. The standards are Pee Dee Belemnite (PDB) limestone for $\delta^{13}C$ and atmospheric nitrogen for $\delta^{15}N$. The δX values denote isotopic enrichment or depletion relative to the standard; positive values meaning that the sample contains more of the 'heavy' stable isotope. The estimated analytical precision of these measurements was 0.1‰ for carbon and 0.2‰ for nitrogen. One laboratory standard was analysed for every 12 unknown samples in each analytical sequence, allowing corrections to be made for instrument drift.

Generalized linear models were used to verify whether $\delta^{13}C$ and $\delta^{15}N$ isotopic signals of termite samples differed between processing techniques, i.e., termites freshly processed, preserved in ethanol, or preserved in NaCl. Analyses were performed independently for each y-variable ($\delta^{13}C$, $\delta^{15}N$ or C/N), and included mounds as a blocking factor. Contrast analysis inspected the statistical similarity between processing techniques. Modelling was performed using R,[16] with Normal errors confirmed by residual analysis.

Samples preserved in NaCl and ethanol did not show any sign of decomposition by the end of the experiment (49 days). The external morphology of the termite individuals was fully preserved by NaCl or ethanol.

The $\delta^{13}C$ signals for freshly processed termite samples did not differ from those for samples preserved in NaCl or in ethanol (Tables 1 and 3).

The $\delta^{15}N$ signals for freshly processed samples did not differ from those for all other processing techniques (Tables 1 and 3).

The C/N ratios for freshly processed termite samples differed from those for samples preserved in NaCl and did not differ from ratios for samples preserved in ethanol (Tables 2 and 3).

Our results are promising for termite studies demanding carbon and nitrogen isotopic analyses. We have shown that samples preserved in 80% ethanol or in NaCl for 49 days can present isotopic signals similar to those of freshly processed samples (Tables 1 and 3). This would make both methods potentially suitable for termite preservation, contradicting our expectations that ethanol would be an unsuitable preservative.

Table 1. Mean ± standard deviation (SD) for $\delta^{13}C$, $\delta^{15}N$ isotopic signals of termite samples subjected to different processing techniques

	Mean ± SD	
Technique	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
Freshly processed	−12.59 ± 0.15	17.79 ± 0.86
Preserved in NaCl	−12.21 ± 0.19	17.12 ± 1.23
Preserved in ethanol	−12.88 ± 0.15	16.40 ± 0.30

The scenario is not so straightforward, however. On one hand, salt-treated termite samples did not present alterations in $\delta^{13}C$ and $\delta^{15}N$ isotopic signatures, and that is consistent with the work of Ponsard and Amlou,[17] who tested the effects of salted water (33 g/L) on the isotopic signals of *Drosophila melanogaster*. On the other hand, the NaCl solution did increase the C/N ratio of preserved termite samples relative to those of fresh samples, and this was achieved by a larger depletion in the nitrogen than in the carbon content of such samples (Tables 2 and 3). The reasons for both (i) depletion of nitrogen and carbon and (ii) greater nitrogen than carbon depletion in salt-treated samples remain to be investigated. It is puzzling to realise, however, that despite such differences in carbon and nitrogen content, the isotopic signatures have been preserved in samples treated with NaCl. Caution prevents us from recommending such a technique to preserve termite samples before further research is carried out.

A better picture arises from ethanol-preserved samples, whose carbon and nitrogen isotopic signals did not differ from those of fresh samples (Tables 1 and 3), a result which is in agreement with those from other studies on insects such as caddisflies,[12] despite being in disagreement with results on *Drosophila* flies[17] and ants.[18] It is, however, reassuring to note that the C/N ratios of alcohol-treated samples did not differ from that of fresh ones (Tables 2 and 3), implying that in the time frame here considered (49 days), termite samples had their natural carbon and nitrogen preserved. Ethanol preservation would seem alluring to termitologists, because termites are traditionally collected and preserved in such a chemical. A single collection, hence, could provide specimens for isotopic analyses and voucher samples. In addition, ethanol can also be used to preserve termite samples for DNA analyses.[19]

Different mechanisms have been proposed to explain how preservation techniques may affect $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios in samples. Among them it has been hypothesised that an enrichment can occur through the loss of molecules carrying the 'lighter' isotope (e.g., lipid molecules or nitrogenous excreta), or by the assimilation of the 'heavier' one possibly present in the preserving agent.[12,14]

This would easily explain the absence of isotopic alterations in NaCl-preserved samples compared with freshly processed ones, but not their increased C/N ratio. NaCl is supposed to only promote dissection of tissues, without loss of constituent materials.

Another result remains to be explained: the absence of alterations in carbon isotopic signals of ethanol-preserved samples. Samples subjected to organic solvents such as ethanol may suffer changes in carbon isotopic signals by both loss of dissolved lipids or gain of the solvent's constituent carbon. By removing lipids, which are depleted of ^{13}C and rich in ^{12}C, ethanol would enlarge the $^{13}C/^{12}C$ value for the sample,[20] thus amplifying its ^{13}C signal. Furthermore, carbon from the ethanol might be incorporated and thus alter the isotopic signals of the samples.[21] Contrary to our expectations, the termite samples analysed here kept their natural carbon isotopic signals, despite being preserved in

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Table 2. Carbon and nitrogen content (mean ± standard deviation) of termite samples subjected to different processing techniques

	Mean ± SD		
Technique	C (%)	N (%)	ratio (C/N)
Freshly processed	38.23 ± 0.36	6.50 ± 0.07	5.89 ± 0.05
Preserved in NaCl	7.80 ± 0.50	1.21 ± 0.05	6.32 ± 0.20
Preserved in ethanol	35.90 ± 0.38	6.39 ± 0.09	5.64 ± 0.07

Table 3. Models and contrasts used to inspect the effects of processing techniques (x variable) on the $\delta^{13}C$, $\delta^{15}N$ isotopic signals and C/N ratio of termite samples (y variables). Treatment levels are: Fresh = freshly processed, NaCl = preserved in NaCl solution and ethanol = preserved in 80% ethanol. Contrasts were performed between the full model (m1) and a model in which the concerned treatment levels were amalgamated into a single level. Amalgamation is indicated by the symbol &. The termite mounds were included as a blocking factor. Models with Normal error distribution. NS = $p > 0.05$ and = $p < 0.05$

Model										
m1: y ~ mound + treatments (Fresh, NaCl, ethanol) m2: y ~ mound + treatments (Fresh&NaCl, ethanol) m3: y ~ mound + treatments (Fresh&ethanol, NaCl)										
Source		$\delta^{13}C$			$\delta^{15}N$			C/N		
	df	F	p		F	p		F	p	
Contrasts										
m1 × m2	1	3.05	0.0844	NS	0.37	0.5456	NS	6.72	0.0112	*
m1 × m3	1	1.69	0.1972	NS	1.60	0.2087	NS	2.17	0.1447	NS
Error	86									

ethanol (Tables 1 and 3). A similar result was found for tissues of quails,[13] sheep,[13] turtles[14] and caddisflies,[12] preserved for up to 60 days and compared with samples dried immediately after collection (freeze-dried or at 60°C). Reasons for such a result would include fast exposure to solvent preventing changes in the chemical composition of samples or carbon losses from dissolved lipids being compensated for by gains from the ethanol molecule. Support for this last hypothesis could be found from the fact that we used ethanol from sugar cane (a C4 plant) and the termite studied (*C. cumulans*) may feed on C4 grasses. Whether or not these occurred to our samples is beyond the scope of our work. It seems, however, more conservative to suspect that no gains or losses have occurred. In fact, the alternative hypothesis would call for carbon losses from termite tissues to be counteracted – equally and simultaneously – by carbon gains from the preserving alcohol, which would seem a more convoluted explanation.

In conclusion, 80% ethanol can be safely recommended as a preservative for termite samples for up to 49 days prior to carbon and nitrogen isotopic analyses. NaCl preservation is a promising technique, still demanding more conclusive research. Additional studies are needed to evaluate the suitability of such methods for longer preservation periods.

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