A PROTOCOL FOR RADIOCARBON DATING TROPICAL SUBFOSSIL CAVE GUANO

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ABSTRACT. We present accelerator mass spectrometry (AMS) radiocarbon dates on several organic fractions isolated from tropical guano deposits recovered from insular Southeast Asia. Differences were observed between ^{14}C measurements made on bulk guano as well as bulk lipids, the saturated hydrocarbon fraction, solvent-extracted guano, and insect cuticles extracted from the same bulk sample. We infer that ^{14}C dates from the bulk lipid fraction and saturated hydrocarbon fractions can be variably contaminated by exogenous carbon. In contrast, ^{14}C measurements on solvent-extracted guano and isolated insect cuticles appear to yield the most robust age determinations.

INTRODUCTION

An underutilized, yet potentially powerful paleoenvironmental archive can be found in guano deposits accumulated over millennia by caverniculous birds and bats (Bird et al. 2007; Wurster et al. 2008). These deposits often retain a high degree of stratigraphic integrity, but the guano, largely composed of eviscerated insect cuticles, is subject to post-depositional processes on the cave floor and after burial (Shahack-Gross et al. 2004). Interactions among drip water and guano, microbial and fungal communities result in sediments with complex mineralogy that are rich in phosphorus, sulfur, and potassium (Karkanas et al. 2002; Shahack-Gross et al. 2004). Because subfossil guano is rich in limiting nutrients, it has been extensively mined for use as a fertilizer (Constantine 1970).

Despite mining activity, substantial guano deposits can still be found in tropical (McFarlane et al. 2002; Bird et al. 2007), semi-arid (Des Marais et al. 1980; Mizutani et al. 1992; Wurster et al. 2008), and temperate locations (Maher 2006). Thus, cave guano sediments represent potential multiproxy environmental archives from regions such as continental tropical and semi-arid environments where environmental archives are often rare (Des Marais et al. 1980; Thomas 2008). Environmental proxy records from these regions are often biased toward lacustrine sites that may offer refugia for wet adapted species during colder and/or drier climate regimes and thus may not yield records of environmental change representative of broader regional environmental change (Bird et al. 2005). Previous research on cave guano has demonstrated that pollen can often be recovered (Maher 2006) and $\delta^{13}C$ and δD records derived from guano sediments have been used to interpret vegetation and climate change as far back as the Last Glacial Maximum (Bird et al. 2007; Wurster et al. 2008). Moreover, there is the potential to develop geochemical and microcharcoal records of environmental change from cave guano.

Interpretation of a proxy record of environmental change from cave guano requires the development of a robust chronology for the accumulation of a sequence of guano-derived sediments. Given that guano is composed of organic matter, radiocarbon dating should be applicable to guano-derived sedimentary sequences. However, it is likely that guano is susceptible to post-depositional diagenesis

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particularly by bacterial and fungal communities, and this process may alter ^{14}C activities. Despite the obvious potential for post-depositional diagenesis and contamination by exogenous carbon, several papers have used ^{14}C to date subfossil guano, assuming that traditional acid-base-acid (ABA) (or even total organic carbon only) ^{14}C measurements will provide an accurate chronology (e.g. McFarlane et al. 2002; Maher 2006). The assumption that simple pretreatment will yield reliable ^{14}C dates on guano has not been tested, and Wurster (2005) noted discrepancies in ^{14}C measurements on different fractions isolated from subfossil guano in a semi-arid region.

Here, we test several methodologies for pretreating guano for ^{14}C dating by isolating fractions from subfossil guano and dating each fraction separately, with the aim of identifying a pretreatment protocol that can reliably decontaminate subfossil guano samples prior to accelerator mass spectrometry (AMS) ^{14}C dating. We compared ^{14}C measurements on bulk guano with the following fractions isolated from subfossil guano: solvent-extracted guano (SEG), solvent extract from guano (SE), the saturated hydrocarbon fraction (SH), and insect cuticles (IC) isolated from the guano sediment.

METHODS

Study Area and Materials

Two sites were sampled in the now tropical humid zone of insular Southeast Asia: Niah Cave (3°49′N, 113°46′E) and Gangub Cave in southern Palawan (8°40′N, 117°35′E). Pits of up to 2 m depth were excavated through the accumulated guano. Exposed profiles were sampled at 3–5 cm intervals, adjusted where necessary to ensure that sample intervals did not cross stratigraphic boundaries. Approximately 100 g of guano was sampled per increment and sealed in a plastic Ziploc® bag. Modern fresh guano from the surface was collected. Upon return to the laboratory, all samples were kept in a cold store at 4 °C until freeze-dried, and several samples selected for ^{14}C dating. Niah Cave contained up to 7 m of guano, and we took 2 samples from deep within this profile at ~670 cm (GU4-140) and ~700 cm (GU4-170) depth on the assumption that they were likely to be ^{14}C-dead.

We tested the IC protocol with 2 internationally available process standards to ensure no ^{14}C bias was introduced due to the technique. We used TIRI-A, a barleymash with a pMC consensus value of 116.35 ± 0.0085 pMC (Scott 2003), and IAEA-C5, subfossil wood with a consensus value of 20.05 ± 0.02 pMC (Rozanski et al. 1992). This protocol was used to extract insect cuticles from Gangub Cave from 7 bulk guano samples down ~1 m of the profile. A flow chart of the isolation of these analytes with brief description of the extraction protocol is given in Figure 1.

Measurement of Bulk Guano, Lipids Extracted, Lipid-Free Residue, and Insect Cuticles

Bulk guano was digested in 2M HCl (80 °C, 8 hr), washed free of acid with deionized (DI) water, then digested in 1M KOH (80 °C, 2 hr). The digestion was repeated using DI water until the solution appeared clear. The residue was rinsed free of alkali, digested in 1M HCl (80 °C, 2 hr), then rinsed free of acid, dried, and homogenized. Bulk guano was then combusted to CO_2 by heating with CuO in a sealed quartz tube. The gas was converted to graphite by Fe/Zn reduction for ^{14}C analysis by AMS.

Additionally, several fractions were extracted from the bulk guano using a sequential procedure. Initially, bulk lipids were extracted using a Dionex automated solvent extractor (ASE) using 2:1 (v/v) dichloromethane/methanol (DCM:MeOH) as the solvent with three 5-min extractions at 100 °C. The residue was purged for 90 s under high-pressure N_2 and collected as solvent-extracted guano (SEG). The extract was collected and the solvent evaporated under a stream of N_2 until dry to recover the solvent extract (SE).

Bulk Guano
(BG)

MeOH/DCM

Solvent-Extracted
Guano (SEG)

Solvent Extract
(SE)

Density Separation/
ABA

Further
treatment

Insect Cuticles
(IC)
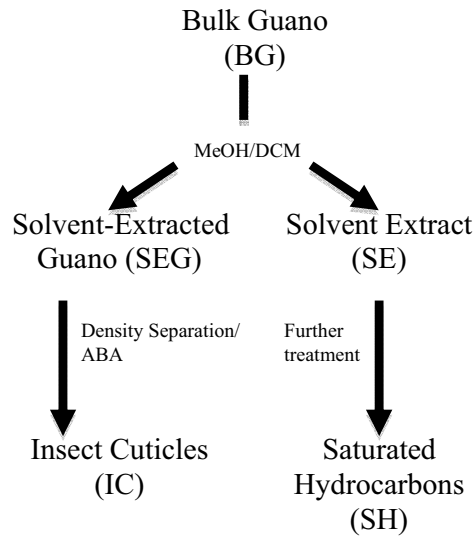
Saturated
Hydrocarbons
(SH)

Figure 1 Flow chart of isolation procedures to obtain various fractions used in AMS dating. See text for treatment details.

To recover insect cuticles, SEG was first immersed in sodium polytungstate (SPT) mixed with DI water to a specific gravity of 1.87 g/cm^3. This mixture was centrifuged for 20 min at 1800 rpm, resulting in organic material floating with heavier minerals sinking. The floatant was then poured into a separating funnel with additional SPT and allowed to settle overnight to ensure near complete separation between mineral and organics. After the organic material was collected, we followed a modified procedure to isolate natural chitin based on Schimmelman and DeNiro (1986). The organic floatant was collected, rinsed with DI water and immersed in 2M HCl for 3 hr to remove carbonates. The sample was then neutralized with DI-H_2O, and immersed in 1M NaOH for 30 min at 100 °C to de-proteinate the sample, neutralized again, and re-immersed in 1M HCl. After a final neutralization, the sample is lyophilized.

In order to retain as much organic material as possible, the sample was also filtered through a polycarbonate 0.4-μm filter (Whatman™) at each step to recover all particulates. The above procedure results in a %C increase from <3% in a bulk fossil guano matrix to >35% in a purified insect cuticle (IC) sample, which is within the range typical of fossil chitin (Miller et al. 1988). There were no further treatments for IC and SE fractions. However, the same pretreatment protocol as bulk guano was performed on submitted SEG fractions. These samples were combusted to CO_2 by heating with CuO in a sealed quartz tube. The gas was converted to graphite by Fe/Zn reduction for ¹⁴C analysis by AMS.

Measurement of Saturated Hydrocarbons

A modified version of the method from Huang et al. (1996) was used to extract saturated hydrocarbons from the sediment matrix. First, we solvent-extracted all lipids ultrasonically (3 × 15 min) using MeOH, followed by MeOH:DCM (1:1) and finally DCM. The supernatant was recovered after each extraction by centrifugation, combined, and concentrated under reduced pressure. DCM redissolved the extract, which was then fractionated into neutral and acid fractions by solid phase extraction (aminopropyl solid phase; Bond-Elut, Varian). Further silica gel flash column chromatography using hexane as eluant was performed to isolate a hydrocarbon fraction. A few drops of RuO_4 solution were added to the isolated aliphatic hydrocarbons. The RuO_4 oxidation was repeated 2 to 3× until the bright yellow color (of RuO_4) persisted, then the sample was dried under a stream of N_2.

The sample was then passed through a small silica gel column using hexane as eluant to yield the saturated aliphatic hydrocarbon fraction, which was again dried under a stream of N_2.

The saturated hydrocarbons were frozen, dissolved with DCM, transferred to a quartz tube, and dried in a stream of oxygen-free nitrogen. The total carbon in a known weight of the pretreated sample was recovered as CO_2 by heating to 900 °C with CuO in a valve-sealed quartz tube, following evacuation on a vacuum line with a turbomolecular pump. The gas was converted to graphite by Fe/Zn reduction.

Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) is widely used in chemical characterization of natural polymers, including chitin and its major derivative, chitosan (Duarte et al. 2002). In this study, FTIR was used to characterize the IC fraction from 2 samples (GAN 1 and GAN 2), in order to confirm that this fraction represented material derived from natural chitin. For comparison, the results were compared with results obtained from the IC fraction isolated from modern, fresh bat guano, obtained from commercial Mexican desert bat guano fertilizer. Samples were diluted by grinding with solid KBr, and pressed into pellets for analysis with a Nicolet FTIR instrument. Absorbance values were determined over the range 4000–400 cm[1]. Spectral bands were identified by comparison with published assignments for natural chitin and *N*-acetyl-D-glucosamine, the monomeric unit of the chitin polymer (Focher et al. 1992; Seoudi et al. 2005).

RESULTS

In order to obtain an appropriate process background using material with a matrix similar to that of the sample extracts, we used extracts from presumed ^{14}C-dead samples GU 140 and GU 170. When comparing BG, SEG, and SE extracts from these samples, both BG (ANUA-22304) and SEG (SUERC-6524) pMC (% modern carbon) are indistinguishable from the background pMC. However, the SE (SUERC-6516) and SH (SUERC-13877) fractions both yield measurable ^{14}C activities with 8.08 ± 0.28 and 16.08 ± 0.13 pMC, respectively (Table 1). Moreover, saturated hydrocarbons (SH) extracted from subfossil guano sediments yield conflicting results. A surface sample taken at Gangub Cave yielded a ^{14}C age of 2626 ± 46 BP (SUERC-13878), while an age of $14{,}683 \pm 65$ BP (SUERC-13877) was obtained on a sample expected to be similar to background (Table 1).

Table 1 Comparison of different fractions of surface and assumed ^{14}C-dead guano samples.

Sample identifier[a]	Lab code	[14]C enrichment (pMC ±1 σ)	Conventional ^{14}C age (yr BP ±1 σ)	δ^{13}C (‰ VPDB)	Analyte
Surface samples					
GAN1 0-7 (IC)	SUERC-17475	103.03 ± 0.47	modern	−25.6	Insect cuticles
GAN mod 4 (SH)	SUERC-13878	72.11 ± 0.41	2626 ± 46	−30.8	Saturated hydrocarbons
Expected ^{14}C-dead samples					
GU4 140 (BG)	ANUA-22304	0.25 ± 0.01	indistinguishable from background	−29.0	Bulk guano
GU4 140 (SEG)	SUERC-6524	0.20 ± 0.03	indistinguishable from background	−27.4	Solvent-extracted guano
GU4 140 (SE)	SUERC-6516	8.08 ± 0.28	20,212 ± 277	−30.1	Solvent extract
GU4 170 (SH)	SUERC-13877	16.08 ± 0.13	14,683 ± 65	−28.8	Saturated hydrocarbons

[a]See text for method.

As guano lipids, and by inference bulk guano-containing lipids, contained exogenous carbon, we endeavored to recover originally deposited insect cuticles for AMS dating. Firstly, we ran the IC protocol on 2 standard materials (TIRI barleymash and IAEA-C5) to see if any ^{14}C bias was introduced during the processing. We also immersed each reference material with the polycarbonate filter in DI-H_2O to test if there might have been any contamination introduced by filtration. Although an initial TIRI barleymash (SUERC-17480) did result in 1.5 ± 0.5 lower pMC (114.82 vs. 116.35 pMC) than the consensus, subsequent measurements on both TIRI barelymash (SUERC-17480) and IAEA-C5 (SUERC-20988) indicated that results were within 1 standard deviation of the consensus value (Table 2).

Table 2 ^{14}C measurement results of process standard materials.

Sample identifier	Lab code	^{14}C enrichment (pMC ±1 σ)	Conventional ^{14}C age (yr BP ±1 σ)	$\delta^{13}C$ (‰ VPDB)
TIRI barleymash (consensus value = 116.35 ± 0.0084 pMC)				
TIRI barleymash filter[a]	SUERC-20991	116.47 ± 0.51	modern	−25.9
TIRI barleymash FP[b]	SUERC-20989	116.55 ± 0.50	modern	−25.2
TIRI barleymash FP[b]	SUERC-17480	114.82 ± 0.50	modern	−25.2
IAEA (consensus value = 23.05 ± 0.02 pMC)				
IAEA-C5 filter[a]	SUERC-20990	23.08 ± 0.14	11,779 ± 48	−24.8
IAEA-C5 FP[b]	SUERC-20988	23.01 ± 0.14	11,803 ± 49	−24.8

[a]Reference material was immersed with polycarbonate filter overnight in DI-H_2O.
[b]Reference material underwent exact same preparation used to extract insect cuticles from guano sediment (IC, see text for details).

We wished to see if we recovered originally deposited material via the IC protocol. The material recovered was clearly remnants of insect cuticles (Figure 2A), and FTIR analysis from 2 samples from Gangub confirmed that we recovered the remains of original insect chitin that underwent significant degradation (Figure 2B). Both samples of fossil IC (GAN 1 and GAN 2) subjected to FTIR analysis contain a range of signals characteristic of the chitin macromolecule. Secondary amides are represented at 3270 cm^{-1} for symmetric NH stretching, and between 2880 and 2960 cm^{-1} for CH stretching vibrations (Seoudi et al. 2005). Although visible, these signals are much weaker in the spectra for the fossil samples when compared to fresh guano. In all spectra, the majority of signal is located between 900–1700 cm^{-1}. Bands at 1653 and 1558 cm^{-1} for C-O stretching in amide I and N–H deformation of amine II (Van de Velde and Kiekens 2004) are merged in GAN 1 and GAN 2, indicating a relatively high degree of deacetylation, as previously observed for degraded chitin (Stankiewicz et al. 1998; Liu et al. 2008). The formation of a broad band centered around 1616 cm^{-1} may reflect either degradation products or enhanced signal from chitin components with higher resistance to degradation, such as phenolic moieties (e.g. Stankiewicz et al. 1998). In addition, although signals at 1030 to 1150 cm^{-1} for C-O-C vibrations in the chitin monomer (Duarte et al. 2002) remain, the individual peaks are coalesced into a broad signal with maxima at 1030 and 1176 cm^{-1}, indicating breakage of the β-glycosidic linkages and degradation of the polysaccharide structure in the fossil samples. AMS ^{14}C analysis of 7 insect cuticles extracts from the guano deposit at Gangub Cave indicated 1 minor reversal out of 7 total ^{14}C measurements (Table 3, Figure 3).
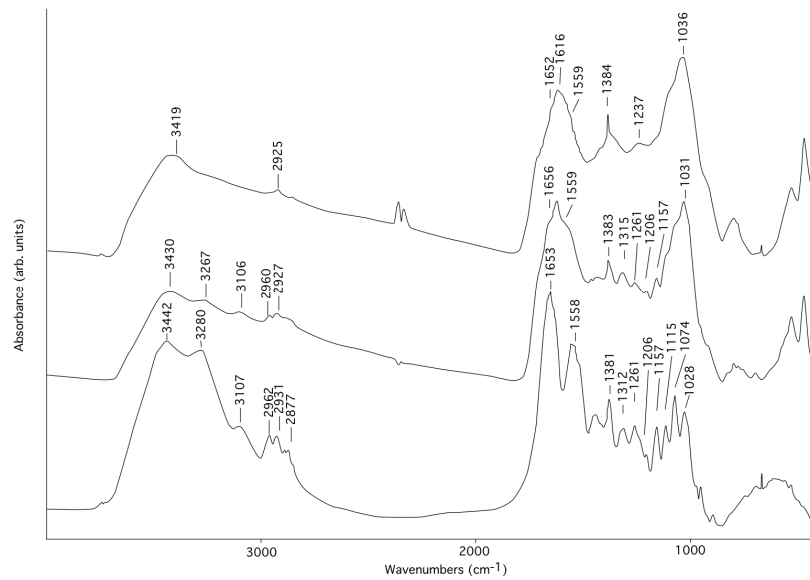
A.

B.

Figure 2 A. Binocular and scanning electron micrograph (inset) images of insect fragments isolated from the fossil guano matrix sample GAN2 35-40. B. FTIR of 2 fossil samples (GAN2 35-40-top spectra; GAN1 35-40-middle spectra) compared with fresh bat guano (bottom spectra), with major chitin diagenetic peaks labeled. These traces indicate that the subfossil guano is composed of degraded chitin polymers.

Table 3 ^{14}C results of insect cuticles recovered from a guano profile at Gangub Cave, Palawan.

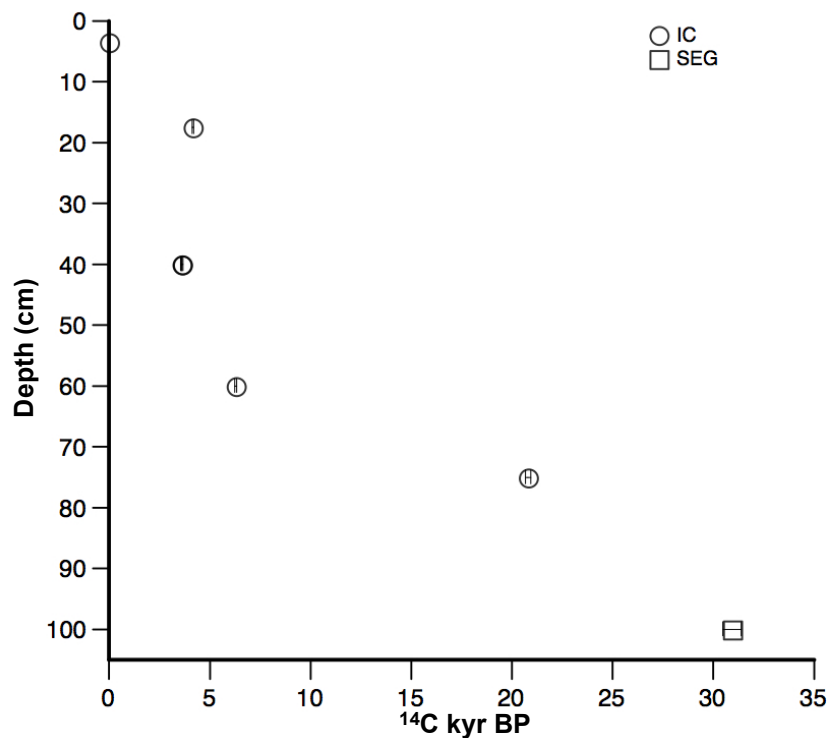| Sample identifier[a]	Lab code	[14]C enrichment (pMC ±1 σ)	Conventional ^{14}C age (yr BP ±1 σ)	δ^{13}C (‰ VPDB)
GAN1 0-7 FP	SUERC-17475	103.03 ± 0.47	modern	−25.6
GAN1 15-20 FP	SUERC-20542	59.66 ± 0.28	4150 ± 37	−22.2
GAN1 40-45b FP	SUERC-17463	63.99 ± 0.28	3586 ± 35	−26.6
GAN1 40-45a FP	SUERC-17462	63.67 ± 0.30	3627 ± 37	−26.7
GAN2 15-20 FP	SUERC-20543	45.77 ± 0.26	6279 ± 45	−21.0
GAN2 30-35 FP	SUERC-17464	7.51 ± 0.12	20,797 ± 128	−18.1
GAN2 55-60 (SEG)	SUERC-20993	2.13 ± 0.12	30,926 ± 448	−24.8

[a]See text for method.

Figure 3 Uncalibrated conventional ^{14}C results from a tropical guano profile at Palawan, Philippines. Different fractions were measured as described in text: (□) solvent-extracted guano; (○) insect cuticles. Error bars are ±1 σ.

DISCUSSION

Overall, these ^{14}C measurements indicate that the traditional method of ABA pretreatment and total organic carbon (TOC) dating is inadequate for the removal of exogenous carbon in these samples. This conclusion is broadly applicable to both bat and bird guano from vastly different environments. Wurster (2005) had previously demonstrated that TOC dates from a semi-arid bat guano deposit contained several reversals that were virtually eliminated by using a simple solvent extraction protocol to remove lipids. Our data demonstrate that lipids are considerably younger when compared with BG or SEG. A ^{14}C measurement on bulk lipids from a presumed ^{14}C-dead sample yielded 8.08 pMC, while a measurement from the residue of the same sample was within ±1-σ error of

blank. Interestingly, the bulk sample was also measured and found to be within ±1 σ of blank, suggesting that in this case the lipids were a minor component of the total sample. However, as bulk guano may contain variable amounts of solvent extractable carbon (unpublished data), dates on bulk guano should be considered unreliable.

We found that the saturated hydrocarbons yielded conflicting data. A presumed ^{14}C-dead sample (SUERC-13877) contains significant ^{14}C activity, while a surface sample of extracted saturated hydrocarbons did not yield modern ^{14}C activities under an active roost (SUERC-13878). Although it is possible that the protocol introduced bias, bulk alkanes may still be a source for young carbon contamination, so this method is not recommended for cave guano deposits. Both bulk lipids and saturated hydrocarbons contained young carbon, an unexpected result as these fractions are hydrophobic, and therefore not as susceptible to remobilization as other components in guano.

We infer from these data that insect cuticles isolated from subfossil cave guano deposits yield the most reliable ^{14}C measurements upon which to develop a chronology for interpreting proxy environmental change records in the guano. Firstly, we demonstrated that there is no bias introduced using the extraction protocol. Results from standard materials were within error, and indicated that the IC protocol could yield reliable ^{14}C results. Secondly, we demonstrated that insect cuticles dominate the IC fraction both by visual inspection and via FTIR analysis. Earlier work has shown that robust ^{14}C chronologies can be constructed by using insect cuticles recovered from archaeological sites (Hodgins et al. 2001; Tripp et al. 2004). Tripp et al. (2004) found that simple washing with organic solvents and treating the samples with acid to yield accurate AMS dates in line with archaeological expectation. Although Hodgins et al. (2001) were more skeptical, finding inconsistency in mixed soil deposits between insect cuticles and *Carex* seeds, further work showed a simple solvent wash yielded an AMS ^{14}C date consistent with a redated peat sample from a homogenous deposit. A bat guano deposit from the Grand Canyon, USA, also yielded inconsistent ^{14}C dates on total organic carbon (TOC) material that were largely resolved using a simple solvent wash (Wurster 2005; Wurster et al. 2008). Finally, we show 8 ^{14}C measurements on the IC and SEG fractions from a guano deposit in southern Palawan (Gangub Cave) that appear to yield robust and internally consistent AMS ^{14}C dates (Table 3, Figure 2). [14]C measurements on IC samples from the surface under active roosts indicated the presence of modern carbon as expected, and remaining ^{14}C analyses from different depths in the guano suggest that a reliable chronology can be developed from IC and SEG fractions, although 1 minor reversal was apparent out of 7 total ^{14}C measurements. This could be due to bioturbation in the upper section of the profile. Moreover, when δ^{13}C values from the Gangub profile are compared against these ^{14}C measurements, we find savannah present during the Last Glacial Maximum and rainforest present during the Holocene, agreeing with a proposed model from this region (Bird et al. 2005).

CONCLUSION

Subfossil cave guano represents an underutilized source of proxy paleoenvironmental information, but interpreting such information requires the development of reliable chronologies. This study has demonstrated that the bulk guano (ABA), and solvent-extractable (SE) components of guano are not suitable for dating at these sites, being variably subject to contamination from exogenous carbon.

While further work may be required in order to fully validate the accuracy of ^{14}C dating the solvent-extracted guano (SEG) and insect cuticle (IC) fractions recovered from other guano deposits, this study at least confirms that the pretreatment procedure does not introduce bias for these fractions and reliable ^{14}C analyses can be obtained. Guano deposits may still suffer from bioturbation, particularly in the tropics where abundant and diverse coprophagous communities can exist (e.g. Ferreira et al.

2007). Thus, they may never provide high-resolution paleoenvironmental information, and as such, ^{14}C dates on the IC and SEG guano fractions appear suitable for reliably dating subfossil guano.

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