A Bat-Guano-Derived $\delta^{15}$N and $\delta^{13}$C Record of Paleoenvironmental Change: Ziditã Cave, Romania

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A Bat-Guano-Derived $\delta^{15}$N and $\delta^{13}$C Record of Paleoenvironmental Change:

Zidită Cave, Romania

by

Daniel Martin Cleary

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Geology
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DEDICATION

I would like to dedicate this thesis to my family and my fellow geologists Brandon Tabor, Amanda Houts, Dan Pawlak, Mike Barthelmes, Ellen Shank, James Ostrander, Matt Ash, Ni An, Luke Anderson, Wayne Hendrix, and Julian Peota.
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ABSTRACT

Because nitrogen isotopes are fractionated along the soil-plant-insect-bat-guano pathway, it may be possible to reconstruct environmental and climatic changes reflected in the nitrogen isotopic composition of guano. A 1.5-m core of bat guano from Zidită Cave (western Romania) provides a record of climatic and anthropogenic influence on the regional nitrogen cycle and paleoenvironmental controls on nitrogen transforming processes. Increasing and decreasing trends of nitrogen isotopic composition (δ¹⁵N values) correspond well with changes in the influence of farming practices, deforestation, and forest expansion. These influences likely had a significant effect on the openness of the nitrogen cycle, resulting in changes in δ¹⁵N values of soil, plants, and ultimately guano. While δ¹⁵N values have gradually decreased since AD 1700, decadal trends towards more positive values at AD 1840 and 1920 coincide with increases in tree pollen (forest recovery). Additionally, the accumulation of relatively ¹⁵N-depleted guano coincides with periods of increased fire frequency, deforestation, and crop/herbaceous pollen (agricultural practices).

δ¹³C record of bulk guano indicates that carbon isotopic variation results from changes in water availability. Comparison of δ¹³C to δ¹⁵N indicates that there is a significant correlation between nitrogen and carbon isotopic composition of guano. When water availability was high (low δ¹³C values), δ¹⁵N values of bulk guano trend towards higher values. Although this connection to climate is the opposite of the findings from previous work correlating δ¹⁵N values of plant foliage and precipitation amount, the relationship between nitrogen isotopic values and water availability still appears to have merit. Based on these findings, δ¹⁵N of guano appears to be a signal for changes in N cycling of the Mada region that occurs, primarily in response to the
precipitation received, further modified by intense changes in anthropogenic activity.
CHAPTER ONE:

INTRODUCTION

Guano deposits form large accumulations, sometimes of tens of meters thick, and the organic material can be precisely dated using radiocarbon (Bird et al., 2007; Wurster et al., 2007; Forray et al., 2015; Onac et al., 2015). Guano piles when located deep within caves are protected from surficial processes and are maintained at the constant temperature and humidity of the cave environment at short time scales (hundreds of years). Guano with stratigraphic integrity is preferred, and occurs when bioturbation and/or diagenetic processes are minimal. All these make guano (which occurs in many caves) a new potential archive from which paleoclimatic and paleoenvironmental proxies can be measured. Guano-derived proxies can be particularly useful when there is insufficient or lack of other potential proxies in a region.

Previous studies of guano have shown useful paleo-reconstructions derived from isotopic composition analysis of carbon (Mizutani et al., 1992b; Wurster et al., 2007; Onac et al., 2014; Forray et al., 2015), nitrogen (Mizutani et al., 1992a; Bird et al. 2007), hydrogen (in chitin; Wurster et al., 2010), chlorine (Johnston et al., 2010), pollen (Leroy and Simms, 2006; Maher, 2006; Geantă et al., 2012; Basumatary and Bera, 2014; Forray et al., 2015), and chemical composition (Bird et al., 2007; Wurster et al., 2015). In this study, stable isotope and elemental analysis of a 900-year old guano core from Zidită Cave (NW Romania) was examined to further assess the utility of the combined carbon and nitrogen isotopic composition of bat guano.
The aim of this project is to reconstruct past climate, environmental, and human impact, examining at high-resolution the nitrogen and carbon isotopic values of guano from Zidită Cave in NW Romania, along with elemental analysis (C and N) and pollen data. The metabolic processes that have previously been recognized to affect the nitrogen isotopic composition of soil, plants, and insects (stages in the nitrogen pathway to guano) were also considered. In order to accomplish this, isotopic analysis of present day samples of vegetation, insect remains, and recent guano were used to calibrate the isotopic signal and elucidate the fractionation of nitrogen as it moves from plant to insect to bat and finally guano. This calibration will be used to interpret the trophic position of bats in Zidită Cave, and to subsequently determine if there were any associated changes in the trophic position of the bats within the food web. In addition, nitrogen isotopic values of guano will be compared to vegetation dynamics reconstructed via guano-derived pollen assemblage and climate (based on carbon isotopic analysis) (Forray et al., 2015). This study represents the first attempt at characterizing the soil-plant-insect-bat nitrogen pathway and the first study of guano $\delta^{15}$N values in western Romania or any other European cave.

The hypothesis of this thesis is that nitrogen isotopic composition of bulk guano, alone or in combination with carbon isotope, pollen, and elemental analyses, serves a proxy of 1) trophic position and/or 2) climate/environmental information.

1.1 Thesis Structure

This thesis has six chapters. After a brief Introduction, Chapter Two describes in detail the study site (with respect to geology, climate, and vegetation assemblage) along with the zoology of present-day bats and those that have roosted within the cave. This chapter will also provide background information on applying $\%$C, $\%$N, and C:N values to infer the degree of guano decomposition and also the fractionating processes of carbon and nitrogen isotopes that occur
within soil, plants, and animals. Due to this work being relatively new, an exhaustive discussion of the methodology and procedures involved in guano core acquisition, sampling, sample preparation, and analysis is provided in Chapter Three. The results of carbon and nitrogen elemental and isotopic analyses are provided in Chapter Four. In Chapter Five I will attempt to interpret these results and determine the utility of carbon and nitrogen isotopic composition studies on guano, in hopes of providing a new method of elucidating climatic, environmental, and anthropic impact information. This thesis will end with a summary of all findings and conclusions in Chapter Six.
CHAPTER TWO:
BACKGROUND

While the author has visited the cave site, a majority of the necessary reference material is written in Romanian. As a result, published papers written in English will be relied upon for the details of this chapter.

2.1 Study Area and Site Description

2.1.1 Zidită Cave: Geomorphology, Geology, and Speleology

The Zidită Cave (hereafter ZC) is located on the left bank of the Geoagiuc River in the Metaliferi Mountains, part of the Apuseni Mountains in western Romania (Fig. 2.1A; Forray et al., 2015). The cave is located on the Pleșa Mare Hill in proximity to Mada Village. The Pleșa Mare Hill is adjacent to the Mada Gorge, which was developed by downcutting of the river through Late Jurassic limestones in which the cave is formed (Borcoș et al., 1981; Cocean, 1988; Forray et al., 2015) (Fig. 2.1B). The entrance of the cave is hidden behind a human built wall previously used to fortify the location in the late medieval time (Pinter et al., 2001; Fig. 2.1C). Vadose conditions currently exist within the 547 m long cave and guano deposits can be found throughout, although large deposits are only located in the Great Hall, Great Passage and Bat Room (Fig. 2.1C). The guano core that represents the basis of this project was recovered from a 1.5 m thick guano deposit accumulated in the Bat Room (Fig. 2.2). In addition to guano deposits, the cave floor is covered with fine detrital sediments and breakdown boulders frequently covered in guano from when bats inhabited these sections of the cave.
Figure 2.1: A) Location of Zidită Cave in the Apuseni Mountains, Romania; B) Geological map of the surrounding region (modified from Borcoș et al., 1981); C) Map of Zidită Cave with sample site marked by white star. Reprinted from Quaternary Science Reviews, Vol. 127, Forray et al., A Late Holocene environmental history of a bat guano deposit from Romania: an isotopic, pollen and microcharcoal study, pages 141-154, Copyright (2015), with permission from Elsevier.

Figure 2.2: The cone-shape guano deposit in the inner part of the Bat Room of Zidită Cave. The core was recovered from the apex of the guano pile (photo: F. Forray).
Due to the relative isolation of the Bat Room from the rest of the cave, the areas beyond the Great Hall feature constant temperature and relative humidity at ~13.5 °C and ~88.5 % year around, respectively (Forray et al., 2015). The onset of stable climate within the Bat Room likely coincides with the development of the narrow conduit separating the Bat Room from the Great Hall. The cave is well ventilated near the entrance and as far as the end of the Great Hall, with warm air entering along the cave ceiling during the summer, which then forces the cold air out of the cave near the floor. This process reverses during the winter when colder air enters along the floor of the cave (Forray et al., 2015).

2.1.2 Vegetation

At present, the vegetation in the ZC area consists of forests, meadows, orchards, and agricultural land. Along the Geoagiu River, the main species of trees are *Alnus glutinosa* (alder) and *Salix fragilis* (willow) (Forray et al., 2015). The primary trees and shrub taxa in forested locations are *Quercus pubescen* (pubescent oak), *Q. frainetto* (Hungarian oak), *Q. robur* (English oak), *Fagus sylvatica* (European beech), *Tilia tomentosa* (silver lime), *T. cordata* (small-leaved lime), *Acer campestre* (maple), *A. pseudoplatanus* (sycamore maple), *Crataegus monogyna* (common hawthorn), *Corylus avellana* (hazel). Species of shrubs (*Viburnum* and *Rhamnus*) are also present. Forests cover the Pleșa Mare and Dosu hills, two major geomorphic units of which the southern portion has been affected by deforestation (Forray et al., 2015). Agriculture is also prominent at lower elevations where crops, hay fields, and pastures cover sizable areas. Herbaceous taxa at these locations are *Apiaceae* (parsley), *Caryophyllaceae* (flowering plants), *Chenopodiaceae* (goosefoot), *Fabaceae* (legume), *Scrophulariaceae* (figwort), *Poaceae* (grasses), *Plantaginaceae* (flowering plants), and *Urticaceae* (nettles), while the main tree taxa are *Alnus glutinosa* and *Salix fragilis* (Pop and Hodîșan, 1957).
2.1.3 Climate

The current regional climate around the cave can be described as temperate continental, with a mean annual temperature and precipitation of ~10 °C and ~600 mm, respectively (data since 1960; NOAA). These data were recorded at the Deva Meteorological Station that is located 22 km southwest of ZC. This station is the closest in proximity to ZC, but it should be noted that Deva is located ~175 m lower than ZC and it is probable the reason for some differences between the climatic regimes of the two areas (Forray et al., 2015).

2.2 Regional Bat Zoology and Guano Composition

2.2.1 Bats of Zidită Cave

Systematic observations conducted between 1960 and 2014 indicated the dominant species of bat in Zidită Cave (hereafter ZC) was Rhinolophus euryale (Mediterranean Horseshoe Bat) (Decu et al., 2003). From 2012-2014 the Bat Room in ZC was home to 116 individuals during the spring/summer (maternity colony) and 128 individuals during the winter hibernacula (Forray et al., 2015). Over this time period there were also an insignificant amount of other species present in the cave during the winter (Myotis myotis and Rhinolophus ferrumequinum), but more species were observed in other sections of the cave during the spring/summer months (Rhinolophus hipposideros: 24 ind.; Rh. ferrumequinum: 30 ind.; and Miniopterus schreibersii: 60 ind.) (Forray et al., 2015). However, in June of 2015 a maternity of M. myotis was the overwhelmingly dominant species inhabiting the cave, with only a few R. euryale individuals and a single Myotis schreibersii observed (Fig. 2.3). The only other documented change in the predominant resident species of Zidită Cave was in 1952 when a large colony of M. schreibersii was present, with only a few individuals of Rh. hipposideros, Rh. ferrumequinum, Rh. blasii, and M. myotis (Dumitrescu et al., 1963).
When considering the dietary habits and preferences of *R. euryale* and *M. myotis* a few notable differences can be observed. *R. euryale* (Fig. 2.4A) feeds during the spring and summer months eating mostly Lepidoptera (moths), such as *Colotois pennaria* as well as Coleoptera (beetles), Clucidae (mosquitoes), and in smaller amounts Diptera (flies), at a maximum of 5 km from the roosting location (Fig. 2.5) (Russo et al., 2002; Miková et al., 2013; Forray et al., 2015). Those insects that are part of the bat’s diet, during their larvae stage feed primarily on grasses, with five exceptions that feed on tree leaves. *R. euryale* search for prey via aerial foraging practices near and within vegetation (Russo et al., 2002; Goiti et al., 2004).

Some *Myotis* species are known to eat larger prey such as fish (Aihartza et al., 2008; Aizpurua et al., 2013), but they likely have a primarily insect based diet (Arlettaz et al., 1997), similar to *R. euryale* (Miková et al., 2013). *M. myotis* (Fig. 2.4B) are opportunistic predators and the type of insects they ingest is dependent upon the abundance of a given species of insect, but also on the ratio between net energy gain and handling time (Arlettaz, 1996; Pereira et al., 2002).

Figure 2.3: *Myotis myotis* maternity colony (June 2015) in Ziditā Cave (photo: F. Forray).
Figure 2.4: The two observed bat species forming large colonies in Zidită Cave. 
A) *Rhinolophus euryale* (Observed colony from 2012-2014).
B) *Myotis myotis* (Colony in 2015).

Figure 2.5: A) Aerial view showing Zidită Cave in relation to the nearby village of Mada. B) Aerial view of the cave and the maximum foraging range (red circle) of *Rhinolophus euryale*. Images were produced on Google Earth.
Therefore, the diet of *M. myotis* has the potential to vary seasonally if there is a more profitable prey during different times of the year (Arlettaz, 1996; Pereira et al., 2002). Multiple studies have recognized predacious carabid beetles as the primary prey of *M. myotis* (Arlettaz, 1996; Pereira et al., 2002; Siemers et al., 2011), however, due to the optimal foraging nature of the species and a lack of observational data for the area, I cannot conclude this is the predominant prey of *M. myotis* in areas surrounding ZC. Although capable of catching prey in flight, in most cases *M. myotis* capture (via gleaning) their prey from the ground, listening for arthropod movement instead of echolocation (Arlettaz, 1996; Russo et al. 2007; Siemers et al., 2011).

### 2.2.2 Guano Composition

Guano deposits are primarily composed of unconsolidated organic material, such as commutated insect exoskeletons (McFarlane et al., 2002; Wurster et al., 2010; Onac et al., 2014) and may also include inorganic material (clays, silts, phosphate, and carbonate minerals). Insect remains are in the form of chitin, a biological polymer that is common in arthropods due to its strength and stability (Tellam et al., 2000). Wurster et al. (2010) and Mizutani et al. (1992) found that during the early stages of diagenesis, stable isotope values of chitin in guano have the potential to retain their initial values. Fresh guano is also likely to have a large fraction of urea, however, this is quickly lost via ammonia volatilization (McFarlane, 1995). This process has a more significant effect when moisture availability is high (Mizutani et al., 1992). Layers of silts and clays have been found within guano sequences at some locations as a result of past flooding events (Forbes and Bestland, 2010; Onac et al., 2014). Authigenic minerals are also common due to the reaction between phosphoric and sulfuric acid (produced by bacterial respiration and sulfur oxidizing bacteria) and limestone fragments or other sediments interbedded in guano piles (Shahack-Gross et al., 2004; Bird et al., 2007; Onac, 2012). Phosphate minerals (hydroxylapatite,
brushite, ardealite, taranaklite) are frequently found in guano, the type of which is dependent upon pH and moisture (Onac and Vereș, 2003; Pușcaș et al., 2013). Sulfate minerals such as gypsum are also common in guano and crystallize as the result of an interaction between host rock and H₂SO₄ in guano (Onac and Forti, 2011; Wurster et al., 2015). Presence of nitrate and sulfate minerals such as niter and gypsum is dependent upon relatively low moisture as both minerals are relatively soluble (Shahack-Gross et al., 2004).

2.3 Stable Isotope Notation

Carbon and nitrogen have multiple isotopes, meaning each element has multiple forms that differ by the amount of neutrons in their nucleus (Sharp, 2007). This project focuses on isotopes of carbon and nitrogen that do not radioactively decay (termed “stable isotopes”). Isotopic ratios for carbon (¹³C/¹²C) and nitrogen (¹⁵N/¹⁴N) are commonly expressed in delta notation: \( \delta = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000 \) where R is the ratio of the heavy isotope to the light isotope. This notation facilitates reporting small differences in isotopic ratios at natural abundance, the range of which is generally <0.1 or 100 per mil (‰). Standard used as scales for N and C are discussed in Table 3.1.

2.4 %C, %N, and C:N of Bulk Guano

The analysis of the weight percentage of carbon and nitrogen and the ratio of carbon to nitrogen has been used previously to determine if guano has experienced diagenesis via microbial decomposition (Bird et al., 2007; Wurster et al., 2007, 2010). Nitrification and ammonification are primary soil processes that can lead to fractionation of nitrogen isotopes between compounds within the bulk guano (Hogberg, 1997). However, N is not volatilized or otherwise lost from the bulk material in these reactions and therefore in the case that these processes occur, bulk guano would still
retain the $\delta^{15}$N signature of the original material. Alternatively, the fractionations associated with the production of ammonia (denitrification and ammonia volatilization) would lead to enrichment of $^{15}$N and $\%$N drops in bulk guano (Bird et al., 2007). McFarlane et al. (1995) found that ammonia volatilization occurs rapidly and soon after the guano is deposited, which results in a majority of the nitrogen from urea being volatilized. McFarlane et al. (1995) concluded that even in the presence of ammonia volatilization, the chitin in guano is unaltered and is therefore suitable for interpretation of isotopic analysis, while other researchers consider that guano in which low $\%$N, $\%$C, and, high C:N values suggest diagenesis occurs and therefore the $\delta^{15}$N values are unreliable (Bird et al., 2007; Wurster et al., 2007, 2010). Although, it remains possible that the $\delta^{15}$N of guano may retain its integrity as suggested by McFarlane (1995), low $\%$N values in this study will be treated with caution as there is no way to unambiguously conclude that the $\delta^{15}$N values have not been altered by digenetic processes (Bird et al., 2007). When values of $\%$C, $\%$N, and C:N for bulk guano remain consistent and therefore imply little diagenesis, the corresponding $\delta^{15}$N values will be considered appropriate for interpretation.

2.5 Carbon

2.5.1 C$_3$, C$_4$, and CAM Photosynthetic Pathways

The carbon isotopic composition of plants has been of particular interest for decades due to the values dependence on the type of photosynthetic pathway, the carbon source and environmental changes (Ehleringer et al., 1997). All plants discriminate against $^{13}$C but how efficiently they do is dependent upon the type of photosynthetic pathway utilized (Marshall, 2007). There are three photosynthetic pathways that plants can utilize to obtain CO$_2$ (C$_3$, C$_4$, and CAM) and each has a standard range of $\delta^{13}$C values (O’Leary, 1988; Cerling and Quade, 1993). The success of the C$_3$, C$_4$, or CAM pathways is dependent upon the climatic conditions and the
distributional abundance of plants with any given pathway can therefore indicate the characteristics of the climate at any given time (Ehleringer et al., 1997).

Figure 2.6: Range of $\delta^{13}$C values for C$_4$ plants (~ -10 to -16 ‰) and C$_3$ plants (~ -24 to -32 ‰). Modified from, O’Leary, Carbon Isotopes in Photosynthesis, BioScience, 1984, v. 38, no. 5, p. 328-336, by permission of Oxford University Press.

The Calvin Benson cycle (C$_3$ plants) results in $\delta^{13}$C values of biomass between -24 and -32 ‰ (Fig. 2.6). C$_3$ plants thrive in cooler environments with significant rainfall (Cerling and Quade, 1993). Deciduous trees and shrubs are common C$_3$ plants. Within C$_3$, C$_4$, and CAM plants, the fractionation associated with rate-limiting step of photosynthesis, reflects the overall fractionation that occurs in each respective pathway (O’Leary, 1988). The low $\delta^{13}$C values in C$_3$ plants are due to the high fractionation (~29 ‰) that carboxylation enzyme RuBisCO in combination with minor discrimination of $^{13}$C that occurs during CO$_2$ diffusion through the stomata (Marshall, 2007). However, in C$_3$ plants the diffusion and dissolution steps do not limit CO$_2$ uptake, as these processes occur faster than carboxylation (O’Leary, 1988).

The C$_4$ pathway was first described by Hatch and Slack (1966) and the values of these plants have $\delta^{13}$C values between -10 and -16 ‰ (Fig. 2.6) and the representative plants are better acclimated to climate regimes of high aridity and temperatures (Marshall, 2007). The ability to
survive under high water stress is important in their ability to thrive in this climate. These plants fare better in these conditions due to their ability to more efficiently enrich CO\textsubscript{2} metabolically than C\textsubscript{3} plants (Sharp, 2007). C\textsubscript{4} plants utilize phosphoenolpyruvate (PEP) carboxylase to catalyze photosynthesis, which produces malate or aspartate that is transferred to the bundle sheath cells (O’Leary, 1988; Marshall, 2007). Alternative to C\textsubscript{3} plants, the limiting step in C\textsubscript{4} photosynthesis is the stomatal diffusion of CO\textsubscript{2} (O’Leary, 1988). However, slow leaking of CO\textsubscript{2} from the bundle sheath also contributes to the low δ\textsuperscript{13}C values of C\textsubscript{4} plants (Hattersley, 1982; O’Leary, 1988). Typical plants that utilize the C\textsubscript{4} pathway are grasses, such as the southern prairie grasses of North America or savanna grasses of Africa (Cerling and Quade, 1993).

CAM plants have intermediate δ\textsuperscript{13}C values in comparison to C\textsubscript{3} and C\textsubscript{4} plants and represent succulents and cacti (O’Leary, 1988; Cerling and Quade, 1993). The same carboxylating enzymes used in C\textsubscript{4} plants are relied on by CAM plants, however, the activities of the enzymes are divided diurnally (Marshall, 2007).

### 2.5.2 Plant Water Use Efficiency

Water use efficiency (WUE) can be described as the ratio of net photosynthesis to transpiration (Marshall et al., 2007; Gagen et al., 2011; Manzoni et al., 2011). WUE increases during conditions of elevated CO\textsubscript{2} due to the closure and decrease in number of stomatal apertures of plants, which reduces evapotranspiration (Young et al., 2006; Gagen et al., 2011). In C\textsubscript{3} plants, variation in δ\textsuperscript{13}C is related to stomatal conductance that changes in response to variations in CO\textsubscript{2} concentrations (Loader et al., 2007; Bodin et al., 2013). In addition, plants will also reduce stomatal conductance to decrease transpirational loss of water in low soil moisture conditions (Stewart et al., 1995; Manzoni et al., 2011). This stomatal adjustment allows plants with a higher water use efficiency to better thrive under varying climatic conditions (Manzoni et
Therefore, differences in plant water use efficiency (WUE) can be associated with wet and dry periods. During more arid/dry climatic periods when water availability is low, plants with a high WUE will thrive, as they are able to minimize water loss (Stewart et al., 1995; Silva et al., 2010). This results in less discrimination of $^{13}$C and higher $\delta^{13}$C values. Therefore, in arid conditions, due to the reduced stomatal conductance, these high WUE plants have an advantage by minimizing the water loss (Forray et al., 2015). Alternatively, during more wet periods, no competitive advantage is afforded to plants with high WUE, leading to more discrimination against $^{13}$C and lower $\delta^{13}$C. Under these conditions the higher isotopic discrimination is related to a CO$_2$ partial pressure gradient between the atmosphere and foliage that changes the $\delta^{13}$C towards these lower per mil values. Under conditions when the canopy effect, temperature and salinity are not also influencing the C$_3$ assemblage, $\delta^{13}$C values can be used to interpret hydroregime changes in the associated region (Farquhar et al., 1982; Forray et al., 2015).

2.6 Nitrogen

In contrast to carbon isotopic studies of guano, relatively little work on guano has attempted to use nitrogen isotopic composition for more than elucidation of the trophic position of bats. For example, Bird et al. (2007) measured the nitrogen isotopic composition of guano and used the data to interpret bat diet in a guano pile in Makangit Cave, northern Palawan (Philippines). The roles of trophic position and diet in controlling $\delta^{15}$N values in hair, tissue, blood, and bone collagen of vertebrates is relatively well documented (DeNiro and Epstein 1981; Macko et al., 1982; Ambrose and DeNiro, 1986). Mizutani et al. (1992a) further demonstrated other controls on $\delta^{15}$N values of guano, for example, represented by latitudinal dependence of $\delta^{15}$N values in guano from caves in Jamaica and the southwestern United States. This section will review biogeochemical pathways of nitrogen in terrestrial ecosystems and the roles of
nitrogen isotope fractionation in the pathway from soil to plant to insect and bat, and ultimately guano.

Beyond guano studies, other ecological work has documented the role of various processes of global nitrogen cycling on $\delta^{15}$N values of various material (i.e. soil, foliage, roots, etc.), which can also be used to provide valuable environmental information (Robinson, 2001; Amundson et al., 2003). Recent work has also begun to elucidate some of the large to regional scale climatic controls on nitrogen cycling and $\delta^{15}$N values of soil and foliage (Austin and Vitousek, 1998; Amundson et al., 2003; Aranibar et al., 2003; Swap et al., 2004; Cheng et al., 2009). In addition, nitrogen isotope studies have begun to address the roles of anthropogenic activities, such as deforestation, fertilizer usage, and controlled fires in the global nitrogen cycle (Cheng et al., 2009). For example, disturbance of the environment by human activity (removal of cryptobiotic crusts) as well as a sudden increase in nitrogen content after ~1950 have been elucidated by $\delta^{15}$N record derived from peat (Esmeijer-Liu et al., 2012) and soil (Evans and Ehleringer et al., 1993). Thus, one may be able to use this knowledge bank to assess the degree to which these activities have impacted the environment surrounding a cave, as these processes may be reflected in the $\delta^{15}$N values of guano, which is ultimately derived from vegetation within the cave surroundings.

The limited amount of work dedicated to nitrogen isotopic composition of guano may in part be due to the more complicated nature of processes and isotopic fractionation in the nitrogen cycle (as compared to the carbon cycle) and/or due to potential uncertainty regarding secondary processes of nitrogen isotope fractionation during decomposition following guano accumulation (Bird et al., 2007; Wurster et al., 2007). Based on a study of ammonia volatilization in a guano heap (Cueva del Tigre, Mexico), McFarlane (1995) concluded that $\delta^{15}$N values of bat guano may
be suitable for analysis and interpretation of paleoenvironmental information. His conclusion was based on the consideration that the process occurs early via decomposition of bat urea, not insect chitin. All additional fractionating processes associated with the decomposition of guano (i.e., nitrification, etc.) do not result in volatile loss of nitrogen. As shown by Bird et al. (2007) and Wurster et al. (2007), the %N, %C, and C:N values can be indicative as to whether the isotopic composition of guano has been changed following its deposition. These authors have attributed the low %N values in older guano of their cores to the volatilization of ammonia. Decomposition and degree of fractionation increase with depth, therefore the youngest or shallowest guano in any given accumulation may be most useful to reconstruct the role of environmental change on δ¹⁵N values of guano. Young guano accumulations are also advantageous in nitrogen isotopic studies as they can be radiocarbon dated more precisely.

2.6.1 Sources of Soil Nitrogen

The primary source of nitrogen in terrestrial systems is ultimately the atmosphere, which is 78 % N₂ (Vitousek et al., 1998). A majority of nitrogen in the atmosphere is in the form of N₂ gas and the entire well-mixed reservoir of N₂ has a δ¹⁵N value of 0‰ (Michener and Lajtha, 2007). However, most plants are unable to use N₂ directly, but instead require reactive nitrogen in the forms of nitrate and ammonium (Galloway et al., 1995). Nitrogen reaches the substrate from the atmosphere either by direct deposition or by nitrogen fixation via either biological (bacteria) or industrial pathways (Evans and Ehleringer, 1993). Prior to the rise of the industrial age, biological fixation was the primary means of nitrogen input into the soil substrate (~110 Tg N/year; Fig. 2.7) (Shearer and Kohl, 1993; Galloway et al., 1995; Kendall et al., 2007). The nitrogen isotopic composition of biologically fixed nitrogen is in most cases similar to the atmospheric N-pool with a δ¹⁵N value of ~1 ‰ (Zhang et al., 2014). After biological fixation, lightning represents the other
natural source of nitrogen deposition. Lightning thermally oxidizes atmospheric nitrogen to nitrates, after which rainfall delivers the reactive nitrogen to the soil (Vitousek et al., 1998). Laboratory measurements have shown that reactive nitrogen produced by lightning also has an isotopic composition similar to the atmosphere with $\delta^{15}N$ values -0.5 to 1.4 ‰ (Hoering, 1957; Kendall et al., 2007). However by comparison, wet and dry deposition of reactive nitrogen fixed by lightning account for relatively minor nitrogen delivery to the soil (~5-10 Tg N/year; Fig. 2.7).

Figure 2.7: Illustration of the primary sources, sinks and processes that are associated with the production of reactive nitrogen for the terrestrial system nitrogen cycle. Values of Natural (blue) and anthropogenic (red) fluxes from the 1990’s are provided in TG N/year. Adapted by permission from Macmillan Publishers Ltd: [NATURE] (Gruber, N., and Galloway, J.N., 2008. An Earth-system perspective of the global nitrogen cycle. Nature, v. 45, p. 293-296), copyright (2008).

While these natural processes contribute a proportion of all reactive nitrogen to the substrate, human activities are increasingly dominating the nitrogen budget of the terrestrial
system (Galloway et al., 2004). One of the largest anthropogenic sources of nitrogen for the terrestrial nitrogen cycle is fertilizer (Fig. 2.7; Vitousek et al., 1998). Crop yield is most often nitrogen limited and thus the use of nitrogen fertilizers has fundamentally transformed agriculture during the “green revolution” (Bouwman et al., 2002). The two main types are natural nitrate fertilizers (“manure”) and synthetic fertilizers produced via the Haber-Bosch process (reduction of molecular nitrogen to ammonia) (Kendall et al., 2007). While synthetic fertilizers typically have $\delta^{15}N$ values closer to 0 ‰, natural fertilizer typically has a much wider range of higher values (~10–25 ‰) (USGS Resources on Isotopes). The type of fertilizer added can have a significant effect on nitrogen isotopic composition of the soil in the area of application (Cheng et al., 2009; Szpak, 2014). For example, Choi et al. (2003) found that soil five years after compost fertilizer ($\delta^{15}N = 17.4 \pm 2.0$ ‰) was applied to fields had $\delta^{15}N$ values of $8.8 \pm 2.0$ ‰. Alternatively the soils associated with the use of synthetic fertilizers ($\delta^{15}N = -1.6 \pm 1.5$ ‰) were less $^{15}N$-enriched ($\delta^{15}N = 5.9 \pm 0.7$ ‰) (Choi et al., 2003). Regardless of type, long-term fertilizer application can result in higher nitrification rates and increased gaseous loss of nitrogen (Zhang et al., 2012). Nitrification increases in response to the flux of a large amount of ammonium in fertilizers and the gaseous loss results in $^{15}N$-enrichment in the soil (Michener and Lajtha, 2007). Therefore, the use of a fertilizer must be considered when interpreting the nitrogen cycling of a region.

### 2.6.2 Stable Nitrogen Isotopic ($\delta^{15}N$) Processes in Soil and Associated Fractionations

The Earth’s $\delta^{15}N$ values of soil depend on the relative roles of a range of nitrogen biogeochemical processes (Hogberg, 1997). Processes within the soil that can transform nitrogen are ammonification, denitrification and nitrification. Microbially-mediated ammonification (i.e., transformation of organic nitrogen to ammonium) is accompanied by negligible fractionation of
The primary processes that fractionate $^{15}$N values of the soil reservoir are nitrification and denitrification (Hogberg, 1997; Tiunov, 2007). During nitrification, ammonium is oxidized to nitrate ($\text{NO}_3^-$), accompanied by a $\sim$15-35‰ enrichment (Robinson, 2001; Table 2.1) during an intermediate step of the reaction where ammonium is oxidized to nitrite. Finally, denitrification describes the reduction of nitrate and nitrite to nitrous oxide ($\text{N}_2\text{O}$) and eventually to nitrogen gas (Hogberg, 1997), and is accompanied by a $^{15}$N-enrichment of $\sim$28-32‰ (Robinson, 2001; Table 2.1). This process is mediated by anaerobic bacteria and some fungi and occurs more frequently in deep soil where oxygen concentration is low (Hogberg, 1997). Due to the large variation in $^{15}$N fractionations, soil $\delta^{15}$N values depend on the dominant processes within a given soil profile (Tilsner et al., 2003; Toyoda et al., 2005; Kendall et al., 2007). For example, denitrification is more prevalent in poorly drained reducing soils (Sharp, 2007), and this process results in a $^{15}$N-enriched nitrate pool.

Table 2.1: Soil nitrogen biogeochemical reactions and associated $^{15}$N-enrichments (Robinson, 2001).

<table>
<thead>
<tr>
<th>Process</th>
<th>Reaction</th>
<th>Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonification</td>
<td>Organic N $\rightarrow$ NH$_4^+$</td>
<td>$\sim$0–5</td>
</tr>
<tr>
<td>Nitrification</td>
<td>NH$_4^+$ $\rightarrow$ NO$_2^-$ $\rightarrow$ NO$_3^-$</td>
<td>15–35</td>
</tr>
<tr>
<td>Denitrification</td>
<td>NO$_3^-$ $\rightarrow$ NO$_2^-$ $\rightarrow$ N$_2$O $\rightarrow$ N$_2$</td>
<td>28–32</td>
</tr>
<tr>
<td>Ammonia Volatilization</td>
<td>Urea $\rightarrow$ NH$_3$ (gas)</td>
<td>40–60</td>
</tr>
</tbody>
</table>

2.6.3 Stable Nitrogen Isotopic ($\delta^{15}$N) Processes in Plants and Associated Fractionations

The nitrogen isotopic composition of plants is dependent upon the $\delta^{15}$N of their nitrogen source in the soil, but metabolic processes can also result in intra-plant fractionations (Evans, 2001). Plants use their roots to obtain inorganic nitrogen in the form of nitrate and ammonium. Frequently plants do so through a symbiotic relationship with mycorrhizal fungi on their roots.
that help in competition with soil microbes and in accessing nitrogen that is strongly adsorbed to soil particles (Dawson, 2001) (Fig. 7.1). Once a plant has obtained nitrogen either through fixation from the atmosphere (through symbiotic bacteria) or uptake through the root system, the metabolic processes further fractionate nitrogen isotopes. There is some debate as to the degree of fractionation during assimilation via the root system of plants (Cheng et al., 2009). However, it has been demonstrated that $^{15}$N-enrichment of the fungus does typically occur during the transfer of isotopically depleted nitrogen from mycorrhizal and ectomycorrhizae fungi to plants (Hogberg, 1997; Hobbie et al., 2000). Ammonium is assimilated directly after uptake at the root system and therefore fractionation does not occur following this event (Bloom et al., 1988, Evans, 2001). Nitrate can alternatively be translocated to other parts of the plant and be assimilated during various metabolic processes that all discriminate against $^{15}$N (Evans, 2001). The result is an intra-plant nitrogen source that becomes progressively more $^{15}$N-enriched as multiple assimilation events occur. Therefore $\delta^{15}$N values of the foliage are generally higher than those of roots (Evans et al., 1996, 2001). Based on these processes, Hobbie et al. (2000) hypothesized that greater reliance on mycorrhizal fungi for nitrogen uptake leads to lower $\delta^{15}$N values in foliage. Other studies have found similar results with respect to the reliance on ectomycorrhizal fungi (Spriggs et al., 2003). However regardless of fungal association, a general relationship between the $\delta^{15}$N value of soil and foliage has been recognized. Fertilizer studies have shown that when the nitrogen isotopic composition of the soil increases (decreases), foliage $\delta^{15}$N values are typically higher (lower) (Zhong et al., 2007; Zhang et al., 2012).
2.6.4 Stable Nitrogen Isotopic ($\delta^{15}$N) Processes in Insects and Bats and Associated Fractionations

Nitrogen isotopes can be further fractionated within insects and bats after ingestion as a result of metabolic processes. Following ingestion, nitrogen is assimilated into an organism’s tissues where a majority of it is converted to ammonia and excreted as urea (Adams and Sterner, 2000). $^{14}$N is preferentially utilized during ammonia to urea conversion, resulting in the organism becoming relatively $^{15}$N-enriched (~3 ‰) compared to its dietary source (i.e., insect is more $^{15}$N-enriched than the plant) (Kelly, 2000). Although there is also fractionation associated with assimilation of nitrogen to various animal tissues, urea production is the dominant effect on whole body $\delta^{15}$N values (Michener and Schell, 1994). $^{15}$N-enrichment occurs within insect larvae and again within the bats as urea is produced and lost by each organism (Fig. 2.8).

Figure 2.8: Diagram showing the pathway of nitrogen from soil to insect to bat, and finally guano.
While increasing \( \delta^{15}N \) values with increasing trophic level has been globally recognized (Kelly, 2000), from the description above it becomes clear that the \( \delta^{15}N \) at any trophic level is dependent upon the initial \( \delta^{15}N \) of the plant, which is closely related to the soil N pool.
CHAPTER THREE:

METHODOLOGY

3.1 Coring and Sampling Procedure

A 1.5 m core was recovered in 2012 from a guano pile in the inner part of the ZC (Bat Room) using a Russian corer (Forray et al., 2015). This type of corer is beneficial in extracting loose sediment as it protects the guano from compression that is a common effect when using piston samplers (Maher, 2003). The corer consists of a 1 m long steel chamber (diameter 7.5 cm) and of 1.5 m long rods. These are linked one to each other using small screws. The corer chamber has a rotating blade made of steel with a sharp end. When turning the blade 180º, the guano is shuffled into the chamber and remains enclosed in relatively undisturbed condition. The corer was pressed into the soft guano sediment from the top of the guano pile with the blade closed so that no guano will enter in the core before it reaches the desired depth (Fig. 3.1; 3.2).

Figure 3.1: Coring the guano deposit in Zidită Cave (left) and the upper 1 m of the core (right) (photos: A. Giurgiu).
When the corer chamber entered completely into the guano (1 m), the blade is slowly rotated 180° with the help of a handle attached to the top of the corer. Doing so, the guano is transferred into the chamber. Once the corer is closed, it is pulled up using handles. To avoid contamination with material from the upper 1 m of the borehole, a second core was then retrieved from a parallel location (within 10 cm from the first one). This time the corer chamber stopped at -1.5 m, thus recovering another 0.5 m of new guano material.

![Schematic of Russian corer](image)

**Figure 3.2:** Schematic of Russian corer used in acquiring guano.

While still in the cave, the stratigraphy of the entire core was thoroughly documented, photographed, and then sampled for radiocarbon, pollen, and isotope analyses (Fig. 3.3). This
reduces the potential of contamination during transport. Samples were separated into aliquots at 2 cm ± 2 mm resolution and stored in a cooling box at 4 °C until analyzed.

Figure 3.3: Sampling the guano core while still in the cave (photo: A. Giurgiu).

Preparation of guano samples for isotopic analysis follows the procedure implemented by Onac et al. (2014) whom modifying a previous method of Wurster et al. (2007). Sample preparation was completed at Babeș-Bolyai University (Cluj, Romania). Upon starting the procedure, each entire un-weighted sample was first placed into a small beaker and dried at 40°C for 3 hours. The dried samples were then homogenized in an agate mortar that was cleaned with hydrogen peroxide to remove organics in between each sample grinding. To determine carbonate fraction, half of each aliquot was mixed with 1 ml (or more if necessary) HCl (10 %) until no visible reaction is observed. The remaining portion was not treated with HCl. The samples were then placed in a drying oven (at 40 °C) overnight to ensure all inorganic carbon was removed from the samples. After drying, samples were subsequently re-homogenized in an agate mortar a second time. Each homogenized sample was then stored in glass vials to avoid contamination.

3.2 Core Description

Since the author was not present during initial core acquisition and sampling, this core description represents the work originally completed by Forray et al. (2015). The guano sequence from ZC appears as alternating brown and black layers, although the composition is the
same throughout (Fig. 3.4). Large insect remains (2-3 mm), hairs and fungi filaments are represented in the loose guano from 0 – 70 cm. In this section, at 57 cm, there was also an accumulation of bat bones. After 70 cm the guano transitions from loose material at the top of the core to more compacted guano. At this transition, hydroxylapatite, quartz, muscovite, and leucophosphite minerals reside in a 1 cm clayey lens.

Figure 3.4: The stratigraphy of the ZC guano core and associated descriptions for each visible layer. White rectangles represent the location of radiocarbon samples, with the calibrated ages (AD) located adjacent. Reprinted from Quaternary Science Reviews, Vol. 127, Forray et al., A Late Holocene environmental history of a bat guano deposit from Romania: an isotopic, pollen and microcharcoal study, pages 141-154, Copyright (2015), with permission from Elsevier.

The composition from 70 - 125 cm is the same as the guano from 0 - 70 cm, with a high concentration of fungi filaments and insect remains. The insect remains in this section are much smaller (<1 mm) in comparison. The base of the core (125-150 cm) is drier and more compact
than the previous section. There are also two significant accumulations of brushite and hydroxylapatite nodules found at 96-103 cm and 125-147 cm.

3.3 Present Day Vegetation, Insects, and Guano

Vegetation samples and *Myotis myotis* guano were collected by F. Forray in March and June of 2015 from the area surrounding the cave (vegetation) that the bats are expected to feed and within the Bat Room (guano samples) near the core site. The *Rhinolophus euryale* guano sample was recovered similarly during a visit to ZC in May, 2013. Two insect remains (*Timarcha goettingensis* and *Triphosa goettingensis*) were also collected within the entrance part of the cave in March 2015. *Timarcha goettingensis* is a type of herbivorous beetle that was found on a plant near the cave entrance. Similar remains and appendages of *Timarcha goettingensis* were found in the most recent guano and therefore can be considered as part of the bat diet (Forray et al., 2015). Wings of *Triphosa goettingensis* (a type of moth) were chosen to be analyzed due to their scattering along the cave floor of Zidită Cave between the entrance and Great Hall (Forray et al., 2015). Each homogenized vegetation sample was weighed out at 5-6 mg each to account for the low %N, whereas guano and insect remains were done so at ~2 mg.

3.4 Isotopic and Elemental Analysis

Aliquots of 1-2 mg were weighted and placed in tin cups and then measured for $\delta^{15}$N, $\delta^{13}$C, %C, %N, and C:N using a Costech ECS4010 Elemental Analyzer (Costech Analytical Technologies ECS) coupled to a Delta V Advantage Isotope Ratio Mass Spectrometer (ThermoFisher Scientific) hosted in the School of Geosciences Stable Isotope Laboratory (USF). Samples within tin capsules are dropped into the combustion reactor (incinerated at 1700-1800 °C) of the EA, whereupon they react with oxygen. Upon combustion, the bulk guano is broken down into its elemental components (i.e. N$_2$, CO$_2$, etc.) and the gaseous forms
of each are then separated as they pass through the gas chromatographic separation column (Fig. 3.5; Sulzman, 2007). After separation the gases are transferred to the IRMS where the element of interest is isolated and continues while gases of other elements are removed as waste. The remaining gas is then impacted with accelerated electrons from an ion source to create positively charged particles (Sharp, 2007). This ion beam is repelled by an electrical field in the direction of the flight tube where the beam is further accelerated and focused to a thin beam (Sulzman, 2007). Now within the mass analyzer, the ion beam is bent via a magnetic field orientated perpendicular to the path of the beam (Fig. 3.5).

The amount of bend with respect to the charged ions is dependent on their mass (Sharp, 2007). Therefore, heavy isotopes (i.e., $^{15}$N and $^{13}$C) bend less than lighter isotopes (i.e. $^{14}$N and $^{12}$C). The beams are then collected via Faraday cups (positioned to capture specific masses) and passed through a resistor that creates an output voltage (Sulzman, 2007). Lastly, a computer system is used to convert this voltage signal to a $\delta$-value relative to a standard. A protein standard B2155 (Elemental Microanalysis Ltd.) and glutamic acid
(internal laboratory standard) were used during bulk guano analysis. The resulting average values for δ¹⁵N, %C, %N, and C:N for each standard are provided in Table 3.1.

Table 3.1: Average values of standards following isotopic analysis and precision of each parameter.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Protein Standard (B2155)</th>
<th>Internal Glutamic Acid Standard</th>
<th>Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ¹⁵N</td>
<td>5.94 ± 0.08‰</td>
<td>-6.28 ± 0.08‰</td>
<td>0.08‰</td>
</tr>
<tr>
<td>%N</td>
<td>13.32 ± 0.33%</td>
<td>9.54 ± 0.42%</td>
<td>0.37%</td>
</tr>
<tr>
<td>%C</td>
<td>46.5 ± 1.18‰</td>
<td>41.37 ± 1.78%</td>
<td>1.48%</td>
</tr>
<tr>
<td>C:N</td>
<td>3.49 ± 0.02</td>
<td>4.34 ± 0.05</td>
<td>0.04</td>
</tr>
</tbody>
</table>

3.5 Radiocarbon Dating and Age Depth Model

Cosmic rays interact with atmospheric nitrogen to produce ¹⁴C, a radiogenic isotope, which is then oxidized twice to ¹⁴CO and again further to ¹⁴CO₂ (Trumbore, 2000). This is a process that naturally produces radiocarbon via bombardment and is constantly occurring in the atmosphere (Taylor, 1997). Radiocarbon dating can be used on material less than 55,000 years old and is based on the radioactive decay (β-decay) of ¹⁴C with a half-life of 5,730±40 years to ¹⁴N (Libby, 1955; Godwin, 1962). Bat guano can be radiocarbon dated because ~1 % of all ¹⁴C produced is incorporated into plants via photosynthesis (Taylor, 1997) and is then transferred through the food web to bats. It is assumed that the ¹⁴C content of the organisms in this food web are at equilibrium with atmospheric ¹⁴C concentrations as long as they are alive, however, once guano is deposited the radiocarbon clock begins (Taylor, 1997). Since bats congregates in large colonies on the ceiling of caves, the oldest accumulation will always be near to the cave floor, with guano getting progressively younger towards the top of the pile.

An issue with radiocarbon dating is that the amount of cosmic rays reacting with atmospheric nitrogen, and as a result ¹⁴C in the atmosphere, is not constant through geologic time.
Therefore it is necessary to calibrate radiocarbon ages, with respect to this variation. Calibration requires the use of data sets that include records of atmospheric $^{14}$C specific activity (Stuiver et al., 1993). These data sets are compiled via $^{14}$C measurements of tree rings that have a known age and U-series dated carbonates. In addition, due to atmospheric nuclear weapons testing, radiocarbon in the atmosphere has doubled since 1950 (Reimer et al., 2004). To account for this, samples with a high $^{14}$C activity are calibrated using CALIBomb (Reimer and Reimer, 2009).

In this study twelve accelerator mass spectrometer ages were obtained on bulk guano samples at Poznań Radiocarbon Laboratory in Poland (Forray et al., 2015). All calibrated ages are reported in years AD (Appendix 2). Using the R software environment (R Development Core Team, 2013), the Clam 2.2 code (Blaauw, 2010) was run to produce the age depth model (Forray et al., 2015). Samples PZ1 to PZ8r had resulting $^{14}$C ages with high radiocarbon activity and were therefore calibrated using CaliBomb (Reimer and Reimer, 2009) with the calibration dataset IntCal13 (Reimer et al., 2013) and NHZ1 bomb curve extension (Hua, 2013; Forray et al., 2015). IntCal13 is a revision of the IntCal09 calibration curve through updated data of $^{14}$C measurements on speleothems, corals, foraminifera, and tree rings (Reimer et al., 2013). Alternatively, Clam code via IntCal13 calibration curve (Blaauw, 2010; Reimer et al., 2013) was utilized to calibrate the oldest samples (PZ10, PZ11r, PZ12, and PZ13r).

The resulting calibrated ages indicate that the upper 100 cm of the core represents from AD 1964 – AD 2011. The lower 50 cm (100-150 cm depth) is much older with an age range of AD 1000 – AD 1964 (Fig. 3.6). The reason for the longer interval being represented in the less thick accumulation of guano at the base of the core is likely due to compaction from the overlying guano as deposition progressed. More dates would be required to determine if there was a hiatus when bats were not present in the cave.
3.6 Suess Correction of $\delta^{13}C$ Values

Since the rise of the industrial revolution, the atmospheric $\text{CO}_2$ concentration has risen by $\sim 40\%$ (Francey et al., 1999; Verburg, 2007). Due to fossil burning and deforestation incorporating primarily $^{13}C$ to the atmospheric carbon reservoir, $\delta^{13}C$ values of the atmosphere have been progressively decreasing over the past 200 years (Verburg, 2007). This effect is known as the “Suess Effect” and indicates any change in the carbon isotope abundance as related to anthropogenic activities (Keeling, 1979). The “Suess Corrected” time-series reported in this work, essentially subtracts the Suess effect over the investigated time period, using the global record of atmospheric $^{13}\text{CO}_2$. 

Figure 3.6: The age-depth model based on 12 radiocarbon ages on bulk guano from Zidită Cave (see text for more information). Reprinted from Quaternary Science Reviews, Vol. 127, Forray et al., A Late Holocene environmental history of a bat guano deposit from Romania: an isotopic, pollen and microcharcoal study, pages 141-154, Copyright (2015), with permission from Elsevier.
CHAPTER FOUR:

RESULTS

4.1 %C, %N, and C:N of Bulk Guano

There are prominent peaks in %N, %C, and C:N representing significantly less nitrogen and carbon at 64 (5.1 %) and 66 (3.2 %) cm depth. Since at these locations values are much lower (~7 %) than those in the upper 100 cm of the core, these will be excluded as they signify significant decomposition of guano (explained in section 5.1).

Although there are minor fluctuations in %N [9.9-12.1 %] and %C [35.8-44.9 %] from 0 to 100 cm in depth, the overall percentage of each element can be considered constant across this range (%N: 11.3 ± 0.45; %C: 40.8 ± 2.31; Fig. 4.1). The average value of %N between 0 and 100 cm depth (11.3 ± 0.45) is also almost similar to the youngest guano value (10.7 %N) measured at the top of the core (0 cm). Similarly, carbon maintains a high concentration to 115 cm in depth (average of 40.6 %C). The C:N appears to have a decreasing trend in the upper 100 cm. Instead, between 100 and 115 cm, %N decreases, whereas the %C values remain constant, resulting in a large peak in C:N within this interval (Fig. 4.1).

Following this peak, the C:N approaches values similar to those in the upper 30 cm of the core. The higher C:N values indicate that nitrogen is decreasing more than carbon, implying that ammonia volatilization triggered by microbial processes removed nitrogen from the guano deposit more significantly than carbon. Both %N and %C decrease significantly in the lower 50 cm reaching the lowest values in the guano core.
Figure 4.1: Variation of %N, %C, and C:N in bulk guano with depth and age.

4.2 $\delta^{13}$C of Bulk Guano

The values for $\delta^{13}$C of bulk guano and the Suess-corrected values are represented in Figure 4.2. The $\delta^{13}$C values decrease gradually from AD 1000 until ~AD 1375, where after a decreasing period to -26.2‰ occurs (Fig. 4.2). The constant values occur during the transition from the Medieval Warm Period to the Little Ice Age. There is a brief rise in $\delta^{13}$C values back to values similar to those between AD 1000 and ~AD 1375 before subsequently decreasing again to -26 ‰ (Fig. 4.2). Between AD 1500 and AD 1800 there is little change in $\delta^{13}$C values, however there is a rapid rise and fall in values at AD 1820, a time near the end of the Little Ice Age. The low interval at ~AD 1856 represents the lowest $\delta^{13}$C value in the series. After this low point, the values gradually rise until AD 1950. It is at this point when the Seuss correct values deviate from the uncorrected guano $\delta^{13}$C (Fig. 4.2). There is an approximate 0.5-2 ‰ difference between the Seuss correction and
uncorrected values that increases during the Industrial Age. Between AD 1968 and AD 1988 there are frequent (0.5-1.5 ‰) fluctuations in δ¹³C. There is a subsequent 1 ‰ decrease and rise between AD 1988 and AD 1999. From this point there is a general lowering trend in δ¹³C until AD 2008 when there is a slight increase to -26 ‰.

Figure 4.2: δ¹³C values of bulk guano (solid lines) and the Suess corrected values (dotted line).

4.3 δ¹⁵N Composition: Present Day Vegetation, Insect, and Guano

Excluding the young branch sample, vegetation had a mean δ¹⁵N value of -1.6 ± 1.4‰ (Table 4.1). The two insect samples (3.5 ± 0.02 ‰) were 3 ‰ higher than the mean of all
vegetation analyzed. There is a ~ 4-5 ‰ difference between the $\delta^{15}$N of guano for *M. myotis* (7.9 ± 0.05 ‰) and *Rhinolophus euryale* (12.4 ± 0.05 ‰).

Table 4.1: Isotopic and elemental results from analysis of vegetation and guano.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Type</th>
<th>Species</th>
<th>$\delta^{15}$N</th>
<th>$\delta^{13}$C</th>
<th>%N</th>
<th>%C</th>
<th>C:N</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV1</td>
<td>Leaf</td>
<td><em>Fagus sylvatica</em></td>
<td>-1.5</td>
<td>-30.5</td>
<td>1.1</td>
<td>44.6</td>
<td>41.9</td>
<td>$\delta^{15}$N 0.07</td>
</tr>
<tr>
<td>MV2</td>
<td>Leaf</td>
<td><em>Quercus sp.</em></td>
<td>-0.8</td>
<td>-29.6</td>
<td>1.1</td>
<td>45.8</td>
<td>42.0</td>
<td>$\delta^{13}$C 0.05</td>
</tr>
<tr>
<td>MV3</td>
<td>Hay (various leafs)</td>
<td><em>Unidentified grasses</em></td>
<td>-1.0</td>
<td>-29.1</td>
<td>2.1</td>
<td>39.7</td>
<td>18.5</td>
<td>%N 0.14</td>
</tr>
<tr>
<td>MV4</td>
<td>Leaf</td>
<td><em>Unidentified grass</em></td>
<td>-4.3</td>
<td>-27.6</td>
<td>1.1</td>
<td>35.5</td>
<td>32.2</td>
<td>%C 0.45</td>
</tr>
<tr>
<td>MV6</td>
<td>Catkins</td>
<td><em>Alnus serrulata</em></td>
<td>-1.7</td>
<td>-28.7</td>
<td>1.1</td>
<td>45.2</td>
<td>41.5</td>
<td></td>
</tr>
<tr>
<td>MV8</td>
<td>Kernel and Pod</td>
<td><em>Robinia pseudoacacia</em></td>
<td>-0.4</td>
<td>-27.6</td>
<td>4.1</td>
<td>44.6</td>
<td>11.0</td>
<td></td>
</tr>
<tr>
<td>MV10</td>
<td>Young Branch</td>
<td><em>Salix sp.</em></td>
<td>5.0</td>
<td>-27.5</td>
<td>2.0</td>
<td>49.3</td>
<td>24.5</td>
<td></td>
</tr>
<tr>
<td>MI1</td>
<td>Insect</td>
<td><em>Timarcha goettingensis</em></td>
<td>3.4</td>
<td>-29.1</td>
<td>10.7</td>
<td>45.8</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>MI2</td>
<td>Insect</td>
<td><em>Triphosa dubitata</em></td>
<td>3.5</td>
<td>-27.4</td>
<td>11.5</td>
<td>43.9</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>MMM</td>
<td>Guano</td>
<td><em>Myotis myotis</em></td>
<td>7.9</td>
<td>-25.5</td>
<td>9.9</td>
<td>44.9</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>FGRF</td>
<td>Guano</td>
<td><em>Rhinolophus euryale</em></td>
<td>12.4</td>
<td>-26.4</td>
<td>10.4</td>
<td>39.1</td>
<td>3.7</td>
<td></td>
</tr>
</tbody>
</table>

4.4 $\delta^{15}$N Values of Bulk Guano

As plotted in Fig. 4.3, a majority of the $\delta^{15}$N values are concentrated between ~AD 1963 and 2012 (0-100 cm of core), compared to the previous ~850 years of the record. Overall, $\delta^{15}$N values show a decreasing trend from AD 1000 to 1300 (1.1 ‰ decrease), followed by a 2.5 ‰ rise from AD 1378 to 1507, after which values remain relatively high from AD 1507 to 1880 (12.5 - 14 ‰; Fig. 4.3A). This interval (AD 1200 – 1900) coincides with the Little Ice Age. Within this interval, there is a relatively minor decline in $\delta^{15}$N values from ~AD 1500 (14.0 ‰) until AD 1720 (12.5 ‰). This is followed by a rapid decrease of 3.6 ‰ at AD 1880 and subsequent increase (2.9 ‰) to AD 1950. For the next 50 years the $\delta^{15}$N values gradually decrease until AD 2011 (Fig. 4.3B). At 1966 there is a rapid decrease of 4.3 ‰ (Fig. 4.3B). This sample was re-analyzed and the value remained unchanged between runs.
Figure 4.3: A) Time series of $\delta^{15}$N values of bulk guano for the Zidită guano core from AD 1000 to 2012. B) Enlargement of the time series from AD 1960 to 2012.
CHAPTER FIVE:

DISCUSSION: PALEO-RECONSTRUCTIONS USING δ^{13}C AND δ^{15}N OF BULK GUANO

5.1 Guano Decomposition as Indicated by %C, %N, and C:N

Guano from ZC is useful for nitrogen paleoenvironmental and paleoclimate studies in that it is a relatively modern accumulation (900 years). Unlike the cores previously analyzed by Bird et al. (2007) and Wurster et al. (2007), the %N and %C of ZC remains very high throughout most of the core (0 to 100 and 115 cm, respectively; Fig. 4.1). While C:N values appear similar at 120-150 cm depth to values near the youngest parts of the core (Fig. 4.1), the decrease in %C is responsible for this ratio. Although C:N is generally stable, both %C and %N are noticeably lower at the base of the core (Fig. 4.1). The down-core stability of %N, %C, and C:N indicates that little diagenesis has occurred since AD 1700 (Fig. 5.1). In the aforementioned studies, ^{15}N-enrichment typically occurred at points within the core where %N and %C decreased markedly. Stable %N and %C implies that the relatively high δ^{15}N values of guano at lower depths in the core are not associated with the microbial decomposition of organic matter within the guano, but instead could be related to primary changes in bat guano accumulated, which can be related to factors such as the trophic level, hydro-climate conditions, etc. The apparent ^{15}N-enrichment in the lower 0.5 m of the core (AD 1000 – 1700), is coupled with a significant decrease in %N (~9 %). This is likely the result of diagenesis and thus the δ^{15}N values at these depths are not considered to reflect the primary δ^{15}N values of the guano. These results suggest that
interpretation of nitrogen isotopic data from bulk guano is appropriate between AD 1700 and 2012, but not prior to this period (excluding two points at 64 and 66 cm; see Results).

5.2 Climatic History Based on $\delta^{13}$C values in Bulk Guano

5.2.1 Comparison to Previous Work

A Pearson correlation test demonstrates that the $\delta^{13}$C results of this study are significantly similar to those of Forray et al. (2015) ($p < 0.0005$). One minor difference between the two time series occurs between AD 1550 (Fig. 5.1) where there is an ~0.5‰ difference between peaks.

![Graph showing $\delta^{13}$C values over time](image)

Figure 5.1: A. Comparison of results between Forray et al. (2015; red lines) and the results produced at the University of South Florida (black lines); B. Close up view of the section marked by a rectangle in panel A.

Forray et al. (2015) interpreted this as a short warming period between ~AD 1500 and ~1475, followed by a major cooling event at AD 1500. The $\delta^{13}$C data for this project generally
agrees with this interpretation. There is the potential for within-sample heterogeneity, but of the samples that have been reanalyzed this does not appear to be the case. There is also a peak representing a brief warm period at ~AD 1845 that is not seen in the results reported by Forray et al. (2015). Between AD 1960 and AD 2011 there appears to be a little more variation between the records, but the overall trend and a majority of the peaks visually appear to be similar. These minor differences may be due to analysis being performed in two different labs and instruments. However, since the two records are based on the same samples, they also serve as inter-laboratory comparison.

5.2.2 Climate History since AD 1000

The results indicate an assemblage of predominantly C₃ vegetation since AD 1000 (δ¹³C values from ~ -27 to -23 ‰). It has been shown that within the C₃ pathway, one of the most significant factors controlling variation in δ¹³C values of large-scale biomass is plant water-use efficiency (Lambers et al., 2008; Bodin et al., 2013). The region has an abundance of shrubs and only patches of dense forest, indicating a lack of a canopy effect (Forray et al., 2015). Using the conclusion of Forray et al. (2015) that WUE was the dominant control of δ¹³C values in plants and therefore guano, these values are interpreted here as a proxy for soil water availability or ecosystem-level hydrologic regime.

Suess-corrected δ¹³C values of bulk guano display a gradual decrease between AD 1000 and AD 1350, indicating climate was becoming progressively cooler and wetter at this time (Fig. 5.2A). This suggests a steady transition into the Little Ice Age. There is a brief increase towards drier/warmer climate at AD ~1450 before subsequently shifting at ~AD 1475 to a much wetter and colder regime. Following this variation, the climatic conditions remained relatively constant. The Little Ice Age ends with a brief return to warm temperatures before the wettest and coldest period
in the record occurs at ~AD 1865. After this cold spike, δ¹³C values of guano rapidly trend towards the highest values of the core. From ~AD 1950 to AD ~1990, δ¹³C values decreased by 2.5‰,

reaching the driest and warmest period between AD 1000 and AD 2011 (Fig. 5.2B). δ¹³C values over these 40 years fluctuate frequently (0.5-1.5 ‰), which is interpreted to indicate rapid variation between warm/dry and cold/wet intervals. At ~AD 1993 there is a gradual change to wetter conditions, which is within the error (AD 1993 ± 2) of the cold event recorded by Popa and Kern (2009) in AD 1991 in their study on the width of tree rings. There is a subsequent
return to drier conditions at ~AD 1997, which is followed by a trend towards lower $\delta^{13}$C values representing cooler temperatures towards the end of the interval.

### 5.3 Controls on Bulk Guano $\delta^{15}$N values

#### 5.3.1 Challenges in Interpreting $\delta^{15}$N values

One of the most significant challenges to interpreting $\delta^{15}$N value of plants is to constrain the $\delta^{15}$N value of the soil source N pool (Robinson, 2001). Changes in nitrogen cycling and variation in biogeochemical nitrogen transformations can result in temporal changes to the $\delta^{15}$N value of the source N pool and the $\delta^{15}$N of plants that access it. While it is not feasible to identify the $\delta^{15}$N value of the soil nitrogen source at any given time, it is possible to interpret changes in $\delta^{15}$N values as related to human activity, bat dietary changes, or climatic forcing. Fractionation processes associated with deposition of guano and metabolic processes within bats and insects follow conservative pathways, which are unlikely to have changed since AD 1700. We can presume this to be the case as the insects, bats, plants and microbes involved and their nitrogen isotopic pathways are the product of natural selection and remain fixed by the metabolic reactions involved at a basic level. Therefore, in the absence of post-depositional diagenesis, bulk guano $\delta^{15}$N values likely reflect changes in the $\delta^{15}$N of the source area. Although nitrogen isotopes have gone through fractionating processes along the soil-plant-insect-bat-guano pathway, reconstructing the decadal trends in $\delta^{15}$N related to anthropogenic, climatic and metabolic influences should be possible when integrating palynological, isotopic, and observational (bats in ZC) records.

#### 5.3.2 Characterization of Nitrogen Isotopic Pathway

Multiple studies have confirmed an approximate 3-4 ‰ enrichment in whole body $\delta^{15}$N compared to diet through comparison of $\delta^{15}$N values between organisms at different trophic positions.
(DeNiro and Epstein, 1981; Peterson and Fry, 1987; Koch et al., 1994; see Chapter 2). With limited isotopic analyses of present day vegetation, insect remains, and guano we also attempt to characterize the isotopic pathway of nitrogen from plant-insect-bat-guano for the environment of ZC.

The difference between the average $\delta^{15}N$ value for 5 vegetation samples (-1.6 ± 1.38 ‰; n = 5; Fig. 5.3A) and mean of the two insect samples (3.5 ± 0.02 ‰; Fig. 5.3B) appears to be slightly higher than the expected enrichment from plant to primary consumer (~4-5 ‰). While the “young branch” sample is representative of regional vegetation, it is likely not part of the diet for insects we would expect in the diet of bats and is thus excluded from the calculated vegetation mean.

![Figure 5.3](image.png)

**Figure 5.3:** Plot of present day $\delta^{15}N$ and $\delta^{13}C$ values for vegetation (A), insect (B), and guano (C).

Since vegetation values have the potential to vary temporally (Goodale et al., 2015), only the *M. myotis* guano will be considered here as it was collected in the same year as the vegetation.
The higher than anticipated enrichment in $^{15}$N between bat guano and insect (~4.4 ‰) could be explained by the fact that bats are ingesting invertebrates instead of vertebrates. Carnivores that have a high proportion of invertebrates in their diet have been shown to have an enrichment up to ~5.5 ‰ in comparison to carnivores ingesting vertebrate organisms (enrichment closer to ~3 ‰) (Kelly, 2000). Kelly (2000) recognized this difference when compiling literature data but did not offer any conclusions as to why this occurs for terrestrial carnivores. Since the $\delta^{15}$N of feces has been demonstrated to be very similar to whole body $\delta^{15}$N values (Sutoh et al., 1987), this value (4.4 ‰) appears to be good approximation for the fractionation of nitrogen isotopes that occurs between ingestion of herbivorous insects by bats and eventual guano. Due to a small sample size, these results should not be taken as conclusive but as a beginning estimation. In the future more intensive sampling of vegetation, insect remains and also bat fur will be required to better constrain the total enrichment. However, these preliminary results indicate that there is on the order of a 10 ‰ increase in $\delta^{15}$N values from the initial plant foliage to those of fresh guano.

5.3.3 Trophic Position of Bats

*Rhinolophus euryale* has been shown to be a secondary consumer with the primary insect of their diet (Lepidoptera) being herbivorous (Mikova et al., 2013; Goiti, et al., 2004). *Myotis myotis* similarly has a typical diet that includes a high proportion of herbivorous insects, but can also potentially ingest carnivorous beetles. Approximately 99 % of all Lepidoptera species feed on plants (Common, 1990; Pierce, 1995), which indicates that any change in the $\delta^{15}$N value of guano, if plant $\delta^{15}$N values are not drastically fluctuating year to year, should be associated with a change in the diet of the bats or bat species. Siemers et al. (2011) conducted a study on bats (hair and muscle tissue) with diets featuring tertiary consumers (*M. myotis*) and secondary consumers (*M. blythii oxygnathus*) to determine if their trophic position could be recognized by $\delta^{15}$N. The
relationship did exist as the *M. blythii oxygnathus* had $\delta^{15}N$ values of $6.2 \pm 0.6 \%$, whereas *M. myotis*, which ate more carnivorous insects, had an average value of $8.0 \pm 0.5 \%$. Note that the aforementioned study is based on isotopic analysis of hair and muscle tissue and it is likely that additional fractionation of nitrogen isotopes occurs during digestion and guano deposition.

Although we did not measure the $\delta^{15}N$ of the bats in this study, any minor fractionation that occurs between bat tissues and guano would presumably be the negligible for both species (Sutoh et al., 1987). Our $\delta^{15}N$ value for fresh *M. myotis* guano ($7.9 \pm 0.05 \%$) is $\sim 1 \%$ higher than the isotopic signature of the ZC guano sample at the top of the core (0 cm; $7.0 \% \pm 0.08 \%$) when *Rhinolophus euryale* was the dominant species in the bat room (AD 2012). However, guano of *Rhinolophus euryale* had a $\delta^{15}N$ value (12.4 $\pm 0.05 \%$) higher than both recent *M. myotis* guano and any value in the ZC core since AD 1970.

There is an overall decreasing trend since AD 1700 in $\delta^{15}N$ of guano, with frequent fluctuations in $\delta^{15}N$ values ($\sim 0.6$ and $2 \%$) particularly between AD 1970 and 2011 (Fig. 5.4). If diet and trophic position are primary controls on $\delta^{15}N$ variation in guano there are two possible explanations for these 2 to 6 year fluctuations in the ZC guano core record: i) the two species have similar diets, but the insect biodiversity/insect availability changed or ii) *M. myotis* has a diet that incorporates a higher percentage of secondary consumers (i.e., carnivorous beetles). It has been documented that bats can change their dietary habits based on availability (Popa-Lisseanu et al., 2007) and this could become necessary if insect availability changes. Although changing climate has been shown to affect herbivorous insect biodiversity and has been recognized in Eastern Europe (Bale et al., 2002; Konvicka et al., 2003), such an occurrence is not documented in Romania. While this does not mean it didn’t occur, the simplest explanation would be that the dominant species of bat in ZC has changed through time and those species
incorporate different proportions of primary and secondary consumer insects into their diets. As noted in Chapter 2, there are observational records of different species roosting in ZC in AD 1952, 2011-2014, and 2015.

![Figure 5.4: δ^15N of guano from Zidită Core (red dots; AD 1970-2011), Rhinolophus euryale (black square; AD 2013), and Myotis myotis (blue rhomb; AD 2015).](image)

The ~0.9 ‰ difference between the AD 2011 ZC guano of *Rhinolophus euryale* and 2015 guano of *M. myotis* could reflect the different dietary preferences that have been documented for the two species in other studies (Chapter 2 of this thesis; Arlettaz, 1995; Pereira et al., 2002; Goiti et al., 2004; Siemers et al., 2011; Miková et al., 2013). The incorporation of more carnivorous insects such as carabid beetles by *M. myotis*, even if this proportion is not markedly different, could explain the ~0.9 ‰ difference. However, the δ^15N value of the AD 2013 *Rhinolophus euryale* guano (12.4 ± 0.05 ‰) does not fit with this interpretation. Even if there were a significant change in insect biodiversity/availability, this value would indicate that the bats were in a higher trophic position, eating organisms such as birds or other bats. While some bats have been shown to eat birds when insects migrate (Popa-Lisseanu et al., 2007), this has not been documented previously for *Rhinolophus euryale* and *M. myotis*. In addition the δ^15N values from
leaves, grasses and insects do not seem to correspond with the results for the AD 2013 guano (8.9 ‰ difference between δ¹⁵N of AD 2013 Rhinolophus euryale guano and insect samples; Fig. 5.3).

Further monitoring work will be required to better evaluate why, how, and when different bat species roost in the Bat Room of ZC. However, the suggestion of a drastic trophic position change by the ∼5-6 ‰ difference between many δ¹⁵N values of guano indicates that this might only have a minor effect on the δ¹⁵N values of guano. The approximate 10 ‰ increase in δ¹⁵N values between initial plant foliage and guano (see previous section) suggests that the δ¹⁵N of AD 2013 Rhinolophus euryale guano is related to a change occurring prior to foliage ingestion.

5.3.4 Anthropic Influence on δ¹⁵N Values of Guano

It has been shown that the amount of available nitrogen in the substrate has a substantial effect on the openness of the N-cycle (Vitousek et al., 1998). During fires, biomass nitrogen is volatilized, resulting in a significant decrease in plant-available N (McGrath et al., 2001). Depending on the amount of burning, losses can be between 50 and 90 % of vegetation-stored nitrogen that will not be returned to the soil except through renewed fixation (Kaufmann et al., 1998). In this case, nitrogen can become a limiting nutrient and a conservative N cycle is produced, resulting in little fractionation and lower δ¹⁵N values of foliage (Davidson et al., 2007). In the ZC guano data, trends towards more negative δ¹⁵N values during periods characterized by agricultural activity, expansion of pastures, and increases in microcharcoal are noticeable (Fig. 5.5B, D, and F) (Forray et al., 2015). An example of this is during a ∼30 year (AD 1880 - 1915) period of expanding farming practices that was recognized by a decrease in tree:grass pollen ratios (Forray et al., 2015). This expansion of grasses was interpreted as being induced by the relative increase in agricultural activity and fire frequency.
The largest change (a decrease of \(~3.6\,\%\) in $\delta^{15}N$ values of the guano prior to 1960 occurs during this time (Fig. 5.5D). The $\delta^{15}N$ values continue to decrease until AD 1920. There is a slight difference between the change in the pollen record (~AD 1911) and rising $\delta^{15}N$ values (AD 1920). But considering the $^{14}C$ dating error, the $\delta^{15}N$ values could indicate the actual position of this boundary. This could also represent a ~10 year lag between the pollen and nitrogen isotopic record, however as the insects are directly sampling the vegetation and pollen is incapable of reaching the guano pile through cave ventilation this is unlikely. Therefore, the nitrogen isotopic data suggest that anthropogenic activity was a large affect on the nitrogen pool of the region until AD 1920. The substantial change in the human influence during this period includes the increase of controlled fires, which leading to substantial nitrogen loss and the resulting decrease of $\delta^{15}N$ values. This effect is also seen at the end of the AD 1785-1840 interval, a period with increased fire frequency (Fig. 5.5B). The vegetation assemblage also indicates a period of landscape openness and increased agricultural activities between AD 1621 and 1785 (Forray et al., 2015), but there is no corresponding decrease in $\delta^{15}N$ values between AD 1700 and 1785 (Fig. 5.5A). However, Forray et al. (2015) also reports an expansion of *Quercus, Fraxinus, Acer, Tilia, and Ulmus* taxa during the local pollen assemblage zone encompassing AD 1508-1785. This indicates that while there was an increase in landscape openness, this was not completed to the degree of preventing forest expansion. Although herbaceous taxa correspond with landscape openness, $\delta^{15}N$ values suggest that the farming practices had a less substantial effect on the nitrogen pool as forest expanded.

When forests are able to recover and nitrogen stocks are restored, rates of nitrification and denitrification increase, resulting in a source pool increasingly enriched in $^{15}N$ (Amundson et al., 2003). This system is described as a leaky N cycle, leading to higher $\delta^{15}N$ values in foliage (Davidson et al., 2007). In general, nitrogen concentration in forest soils and the corresponding
foliage δ^{15}N values increases with forest age (Vitousek et al., 1989; Davidson et al., 2007; Amazonas et al., 2009). This can be seen in the guano record where the few decadal trends towards more positive δ^{15}N values appear to correspond with the timing of forest expansion. Forray et al. (2015) identified a period (AD 1845 - 1880) of major expansion of *F. sylvatica* and *Carpinus betulus* forests (Fig. 5.5C), which coincides with δ^{15}N values of guano over this interval trending towards samples more enriched in ^{15}N. Although charcoal and therefore fires were highest at this time, δ^{15}N values indicate that forest expansion likely had a greater effect on the regional nitrogen pool. A similar event occurred between AD 1915 and 1966, when an increase in forest taxa *Quercus, Fraxinus, Acer, Tilia,* and *Ulmus,* and decrease in *F. sylvatica* and *Carpinus betulus,* indicates a warmer/drier climate (Fig. 5.5E). During this latter period, the decrease in cereals and other herbs may indicate a decline in

Figure 5.5: δ^{15}N (red), δ^{13}C (blue), and charcoal percentage (black) of bulk guano. Green shading represents the timing of recognized forest expansion/decrease in agricultural activity; white shading represents the timing of deforestation/increases in farming practices (adapted from Forray et al., 2015). See text for further information.
human activities (agriculture and grazing) that would have allowed for the nitrogen pool to recover and result in higher $\delta^{15}$N values in foliage and subsequently in guano. Both of these periods of forest expansion are contemporaneous with decreases in herbaceous taxa and an increasing trend in $\delta^{15}$N values that is typical of an aging forest. The decrease in the burning of forest and/or farming practices allowed for the expansion and recovery of the nitrogen pool.

Beyond AD 1960, the $\delta^{15}$N of guano approach the lowest values in the core (Fig. 5.6). Over this interval there are frequent fluctuations in tree taxa, likely in response to deforestation (Forray et al., 2015). Results indicate that between AD 1968 and 1978 there was a more open landscape and low fire frequency (low microcharcoal percentages) (Fig. 5.6A). With the notable increase in cereals, *Secale, Zea, Plantago lanceolata,* and *Rumex,* this decade represents a time of extensive

![Figure 5.6: $\delta^{15}$N (red) and $\delta^{13}$C (blue) values and charcoal percentage (black) of bulk guano from AD 1960 to 2012. A. Dramatic increase in agricultural activities; B. Increase in controlled fires with no significant forest expansion during this period.](image)

Farming activity (Forray et al., 2015). An increase in fires between AD 1983 and 1999 may also be related to farming practices (Fig. 5.6B). A switch from manure ($\delta^{15}$N = ~10-25 ‰; Fig. 8.5) based to synthetic fertilizer ($\delta^{15}$N = ~0 ‰) since AD 1965 might have contributed to the significant decrease in $\delta^{15}$N values, however historical records indicate that synthetic fertilizer is seldom used
in the Mada region and therefore fertilizer application is not likely to have a role in the trends in δ¹⁵N (Forray, pers. comm.). The combination of these significant anthropogenic effects and the lack of allowance of forest recovery appear to have an influence on the drastic change to the region’s nitrogen pool.

Although the δ¹⁵N values appear to correspond with the timing of changes to the vegetational regime there is room for skepticism. Without significantly more measurements of soil and vegetation δ¹⁵N over a long time scale, it is not possible to unequivocally demonstrate how quickly N cycling of the region would respond to decadal trends in anthropogenic activities. In addition, some studies have shown that amount of nitrogen lost through volatilization during fires is similar to the abundance in the burnt vegetation that is reintroduced to the system, resulting in little fractionation (Cook, 2001; Aranibar et al., 2003). Due to the fact that the most important factor in determining the isotopic composition of ecosystem is the δ¹⁵N value of nitrogen leaving the system (Amundson and Baisden, 2000; Brenner et al., 2001), this could significantly diminish the effect fire frequency has on N cycling.

**5.3.5 Climate-related Variations of δ¹⁵N Values**

There is a well-documented relationship between decreasing values of δ¹⁵N in foliage and soil with increasing precipitation (Austin and Vitousek, 1998; Handley, 1999; Robinson, 2001; Amundson, 2003; Swap et al., 2004; Cheng et al., 2009), as well as increasing mean annual temperature (MAT; Martinelli et al., 1999; Amundson et al., 2003). This holds true on a global scale as δ¹⁵N values of soil and plants in temperate ecosystems are typically lower than that of more arid locations (Handley, 1999; Cheng et al., 2009; Amundson et al., 2003). Our understanding of the mechanisms behind this relationship is still in its infancy, however, Amundson et al. (2003) made some general conclusions. When precipitation is higher (high N availability), there is an increase in
the loss of $^{15}$N-depleted forms (NO$_3^-$, N$_2$O, etc.) of nitrogen, whereas nitrogen is more efficiently conserved and recycled under cold/wet conditions. Nitrogen loss occurs through denitrification, NH$_3$ volatilization and leaching of nitrate and the soil N pool and plants that access it become more enriched in $^{15}$N as a result (Amundson et al., 2003; Ma et al., 2012). This generally agrees with the conclusions of Austin and Vitousek (1998) that the isotopic composition and forms of nitrogen loss changes as MAP increases. While $\delta^{15}$N values in both C$_3$ and C$_4$ plants are sensitive to changes in precipitation amount and temperature, this sensitivity is higher in C$_3$ plants (-0.6 ± 0.07 ‰/100 mm) than in C$_4$ plants (-0.3 ± 0.08 ‰/100 mm) (Ma et al., 2012).

With respect to guano studies, Wurster et al. (2007) also found a linear relationship between $\delta^{13}$C and $\delta^{15}$N in sites affected by the North American Monsoon System, but concluded that $\delta^{15}$N was not a reliable indicator of climate. In the subsequent work of Wurster et al. (2010) the authors found an increase in solvent-extracted guano $\delta^{15}$N values that correlated with lower $\delta^{13}$C values and attributed these results to aridity. As described previously, $\delta^{13}$C values in guano in ZC are interpreted as a proxy of water availability (Forray et al., 2015). Therefore, the relationship between $\delta^{15}$N and precipitation can be observed through comparison to the $\delta^{13}$C record, which represents variations between wet and dry periods. Statistical analysis demonstrates that there is a significant negative relationship between $\delta^{15}$N and $\delta^{13}$C of bulk guano (p of <0.005; Fig. 5.7). This indicates that during wetter conditions (more negative $\delta^{13}$C), $\delta^{15}$N values trend towards less positive values. Notably, this is the opposite of the relationship the aforementioned studies have recognized between precipitation amount and the $\delta^{15}$N of soil and plant foliage.

Although at present there is not a concrete explanation for this relationship as related to guano, visually the relationship for ZC is clear (Fig. 5.8). Unfortunately, due to the uncertainty and resolution of the age model we cannot precisely directly compare the $\delta^{15}$N record to the instrumental records
of nearby weather stations. However, the guano samples of known age at AD 2012, 2013 and 2015 can be compared to precipitation data. Meteorological data from the Sebeș, Romania station indicates that the total precipitation in 2013 (538.6 mm) was higher than in 2012 (446.5 mm) and 2015 (January-present: 264.3 mm) (Table 5.2; Romanian National Meteorological Agency).

However, since bats are foraging between April and September (when guano accumulates), it is
unlikely that any rain received during the winter of any given year is contributing to the isotopic signature of guano deposited during that year. Therefore, it is worth considering that the precipitation from the previous year’s winter months may contribute to N availability during the subsequent warmer months when plant growth is highest. The total precipitation between October 2011-August 2012 (393.1 mm) and October 2014-August-2015 (394.2 mm) are significantly lower than the precipitation received between October 2012-August 2013 (492.0 mm) (Table 5.2). When bats were foraging in 2012 and 2015 the intervals can be described as drier in comparison to 2013. The drier intervals correspond with lower δ¹⁵N values in 2012 (7.0 ‰) and 2015 (7.9 ‰), where as in 2013 when there was significantly more precipitation the δ¹⁵N value of guano was higher (12.4 ‰). This agrees with relationship expressed by the δ¹⁵N and δ¹³C records of the ZC core.

Table 5.1: Precipitation data from Sebeș, Romania weather station and ZC guano δ¹⁵N.

<table>
<thead>
<tr>
<th>Year</th>
<th>Total Precipitation (mm)</th>
<th>Total Precipitation (mm) previous winter-August</th>
<th>Guano δ¹⁵N</th>
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<td>413.1</td>
<td>502.8</td>
<td>----</td>
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<td>492.0</td>
<td>12.4 ‰</td>
</tr>
<tr>
<td>2014</td>
<td>524.7</td>
<td>547.1</td>
<td>----</td>
</tr>
<tr>
<td>2015</td>
<td>264.3*</td>
<td>394.2</td>
<td>7.9 ‰</td>
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</table>

* January - August

The stress of warmer and drier conditions is the main cause for a disruption from the steady state N cycling system that Amundson et al. (2003) associated with δ¹⁵N changes in foliage and soil. However, the mechanisms involved in this region are likely different based on the opposing relationship between δ¹⁵N and δ¹³C represented in the guano record. Even so, from these results it would appear that climate is a primary control of δ¹⁵N in guano.
CHAPTER SIX:
CONCLUSIONS

Isotopic analysis of vegetation, insect remains, and guano from 2015 indicate that there is an approximate 10 ‰ increase in $\delta^{15}$N values from the initial plant to guano. More intensive sampling within the foraging range of the bats will be required to prove this conclusively, but this value offers the first approximation of the fractionation that occurs along the plant-insect-bat-guano pathway. %N, %C, and C:N ratio results indicate that guano from AD 1700 to 2011 is appropriate for interpretation of $\delta^{15}$N values as primary, and largely unaffected by diagenesis within the guano. The significantly higher $\delta^{15}$N value of the *Rhinolophus euryale* guano from AD 2013 in comparison to guano from AD 2012 (*Rhinolophus euryale*) and 2015 (*Myotis myotis*) suggest that diet is not a significant contributor to the variations in ZC $\delta^{15}$N. There is potential for *Myotis myotis* and *Rhinolophus euryale* to have slightly different diets with respect to the incorporation of carnivorous insects, however it is unlikely, for example, that this difference would result in the $\sim$5 ‰ decrease in $\delta^{15}$N values from AD 1970.

Increasing trends of $\delta^{15}$N in guano occur contemporaneously with periods of known expansion of forests and decreasing fire frequency and human activity, suggesting a conversion to a “leaky” N cycle within the region. Likewise when $\delta^{15}$N values decrease there is an increase in herbaceous pollen and charcoal, which signifies events of deforestation, increased fire frequency, and anthropogenic influence. Trends toward lower $\delta^{15}$N values during these events suggests a more conservative N cycle is reached in which where less nitrogen is lost from the soil nitrogen pool due to denitrification, ammonia volatilization or leaching. At AD 1845-1880
high fire frequency coincides with expansion of a *F. sylvatica* and *Carpinus betulus* forest. The increasing trend δ¹⁵N indicates that forest expansion may have a greater effect on the regional nitrogen pool. This could be explained by burnt vegetation reintroducing nitrogen into the system and compensating for the nitrogen loss that occurs through volatilization.

While the timing of anthropogenic related changes to the vegetational assemblage appears to coincide with specific events in the δ¹⁵N of guano, statistical results indicate that climatic forces (specifically moisture availability) may have a more significant effect. Data from ZC core indicate that as precipitation increases, δ¹⁵N values trends toward higher values and δ¹³C values decrease. While dating error prevents the direct comparison of the nitrogen isotopic composition of guano to any instrumental record, known age samples from AD 2012, 2013, and 2015 agree with this relationship. This negative relationship is the opposite of the one documented in numerous studies of precipitation amount and vegetation/soil δ¹⁵N values. Future research will be needed to explore the mechanisms behind why the δ¹⁵N of foliage in proximity to ZC decrease when moisture availability is high. However, from these results it appears that climate has a first order control on the N cycling dynamics of the region that is sampled by bats of Zidită Cave. There is also sufficient evidence that suggests vegetational shifts and fire frequency related to both natural and anthropogenic activities applies at least a secondary control on δ¹⁵N values represented in bat guano. Additional work will be required to explore and model these two signals, but it is evident that δ¹⁵N bat guano has further use as a paleoenvironmental and paleoclimate record beyond a simple confirmation of trophic position.
REFERENCES


APPENDIX A:

DATA
Table A.1: Isotopic and elemental results for the Ziditā guano core.

<table>
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<th>Sample ID</th>
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<td>4.5</td>
<td>11.0</td>
<td>2.83</td>
</tr>
<tr>
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<td>110</td>
<td>-17.6</td>
<td>4.5</td>
<td>11.0</td>
<td>2.83</td>
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<tr>
<td>PZ-110</td>
<td>112</td>
<td>-17.4</td>
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<td>PZ-112</td>
<td>114</td>
<td>-17.2</td>
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<td>116</td>
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<td>2.83</td>
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<tr>
<td>PZ-116</td>
<td>118</td>
<td>-16.8</td>
<td>4.5</td>
<td>11.0</td>
<td>2.83</td>
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<tr>
<td>PZ-118</td>
<td>120</td>
<td>-16.6</td>
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<td>2.83</td>
</tr>
<tr>
<td>PZ-120</td>
<td>122</td>
<td>-16.4</td>
<td>4.5</td>
<td>11.0</td>
<td>2.83</td>
</tr>
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</table>
Table A.2: Radiocarbon ages, the calibrated years AD and the dates used for construction of the age-depth model of the Zidită Cave guano core. Reprinted from Quaternary Science Reviews, Vol. 127, Forray et al., A Late Holocene environmental history of a bat guano deposit from Romania: an isotopic, pollen and microcharcoal study, pages 141-154, Copyright (2015), with permission from Elsevier.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lab no.</th>
<th>Depth (cm)</th>
<th>(^{14}C) Age ((^{14}C) yrs BP)/remarks</th>
<th>(^{14}C) activity (pMC)</th>
<th>Calibrated years AD</th>
<th>Date used AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PZ1</td>
<td>Poz-51928</td>
<td>4–6</td>
<td>Modern</td>
<td>108.39 ± 0.38</td>
<td>AD 2000–2004 (95.2%) 2σ</td>
<td>2002</td>
</tr>
<tr>
<td>PZ2</td>
<td>Poz-51933</td>
<td>32–34</td>
<td>Modern</td>
<td>110.53 ± 0.39</td>
<td>AD 1995–2000 (97.8%) 2σ</td>
<td>1998</td>
</tr>
<tr>
<td>PZ3</td>
<td>Poz-51935</td>
<td>47–49</td>
<td>Modern</td>
<td>120.07 ± 0.37</td>
<td>AD 1984–1986 (68%) 2σ</td>
<td>1985</td>
</tr>
<tr>
<td>PZ4</td>
<td>Poz-51936</td>
<td>57.5–60</td>
<td>Modern</td>
<td>130.57 ± 0.42</td>
<td>AD 1978–1979 (91.1%) 2σ</td>
<td>1979</td>
</tr>
<tr>
<td>PZ5</td>
<td>Poz-51937</td>
<td>73–75</td>
<td>Modern</td>
<td>137.22 ± 0.39</td>
<td>AD 1974–1976 (84.3%) 2σ</td>
<td>1975</td>
</tr>
<tr>
<td>PZ6</td>
<td>Poz-51938</td>
<td>79–81</td>
<td>Modern</td>
<td>147.74 ± 0.42</td>
<td>AD 1971–1973 (93.1%) 2σ</td>
<td>1972</td>
</tr>
<tr>
<td>PZ7</td>
<td>Poz-51939r</td>
<td>85–87</td>
<td>Modern</td>
<td>155.31 ± 0.33</td>
<td>AD 1971–1973 (94.3%) 2σ</td>
<td>1971</td>
</tr>
<tr>
<td>PZ8r</td>
<td>Poz-51940r</td>
<td>98–100</td>
<td>Modern</td>
<td>193.31 ± 0.35</td>
<td>AD 1964–1965 (94.3%) 2σ</td>
<td>1964</td>
</tr>
<tr>
<td>PZ10</td>
<td>Poz-51929</td>
<td>119–120.5</td>
<td>110 ± 30 BP</td>
<td>–</td>
<td>–</td>
<td>1805</td>
</tr>
<tr>
<td>PZ11r</td>
<td>Poz-51930r</td>
<td>127–129</td>
<td>337 ± 30 BP</td>
<td>–</td>
<td>–</td>
<td>1540</td>
</tr>
<tr>
<td>PZ12</td>
<td>Poz-51931</td>
<td>133–136</td>
<td>435 ± 30 BP</td>
<td>–</td>
<td>–</td>
<td>1450</td>
</tr>
<tr>
<td>PZ13r</td>
<td>Poz-51932r</td>
<td>146–148</td>
<td>913 ± 30 BP</td>
<td>–</td>
<td>–</td>
<td>1080</td>
</tr>
</tbody>
</table>

Fig. 4. Stratigraphy of the guano core showing the radiocarbon samples (white rectangles) and their calibrated ages. Transitions between different layers are generally more diffuse than illustrated.
APPENDIX B:

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