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STABLE ISOTOPES ISSUE

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Analytical error in stable isotope ecology

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Abstract The increasing popularity of stable isotope analysis (SIA) as an ecological research tool and the ease of automated analysis have created a knowledge gap between ecologists using SIA and the operators of isotope ratio mass spectrometry (IRMS) equipment. This has led to deterioration in the understanding of IRMS methodology and its proper dissemination in the ecological literature. Of 330 ecological research papers surveyed, 63 (19%) failed to report any form of analytical error associated with IRMS. Of the 267 papers that reported analytical error, there was considerable variation both in the terminology and approach used to quantify and describe error. Internal laboratory standards were often used to determine the analytical error associated with IRMS, so chosen because they are homogenous and have isotopic signatures that do not vary over time. We argue that true ecological samples collected in the field are complex bulk mixtures and often fail to adhere to these two criteria. Hence the analytical error associated with samples is potentially greater than that of standards. A set of standard data run over time with a precision typically reported in the ecological literature (1 standard deviation: 1SD = 0.26‰) was simulated to determine the likelihood of spurious treatment effects depending on timing of analysis. There was a 90% likelihood of detecting a significant difference in the stable nitrogen ratio of a single sample (homogenized bovine liver) run in two time periods when $n > 30$. Minor protocol adjustments, including the submission of blind replicates by researchers, random assignment of sample repeats within a run by analytical labs, and reporting 1SD of a single sample analyzed both within and between runs, will only serve to strengthen the interpretation of true ecological processes by both researchers and reviewers.

Keywords Precision · Accuracy · IRMS · Analytical labs · Standards

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Introduction

Stable isotope analysis (SIA) has become a popular tool among ecologists in elucidating dietary information from plants and animals collected in the field (Peterson and Fry 1987; Jardine et al. 2003). Stable isotope ratios of elements including carbon ($^{13}C/^{12}C$), nitrogen ($^{15}N/^{14}N$), sulfur ($^{34}S/^{32}S$), hydrogen ($^{2}H/^{1}H$), and oxygen ($^{18}O/^{16}O$) can yield information about energy flow, trophodynamics, migration patterns, and metabolic processes. The advent of automated analysis (Fry et al. 1992) in isotope ratio mass spectrometry (IRMS) and the establishment of numerous service labs using the technology have led to a rapid increase in the number of ecological studies applying SIA as a research tool (Kelly 2000). As a result, the knowledge gap between ecologists and the operators of IRMS equipment is growing ever larger. The end product of this ever-widening gap is a deterioration of an understanding of IRMS methodology and its proper dissemination in the literature by ecologists. While some authors extensively describe analytical error (Gleason 1986; Harrington et al. 1998; Ponsard and Arditi 2000; Cloern et al. 2002), some of the landmark papers in stable isotope ecology failed to mention its potential influence on interpretation of results (Chisholm et al. 1982; Peterson et al. 1985; Kline et al. 1993; Cabana and Rasmussen 1996).

The purpose of this paper is threefold: (1) to give a brief overview of IRMS methodology, (2) to illustrate the disparity that exists in the ecological literature when reporting stable isotope analytical error, and highlight the potential contribution of analytical error to spurious

ecological effects using a simulation of standard data, and (3) to make recommendations for a standardized method of quantifying and reporting analytical error in future ecological studies using SIA.

IRMS methodology

This paper will not describe analytical procedures in detail. For more thorough treatments of this subject consult documents such as Preston (1992), Werner and Brand (2001), and Dawson and Brooks (2001).

Researchers typically sample plant and animal tissues from organisms collected in the field over space and time, with the intention of gaining an understanding of some ecological process or condition. Tissue types can range from fine particulate organic matter to leaves and twigs to muscle and bone. Samples are dried and ground to a fine powder-like consistency to achieve homogeneity. Small aliquots (milligram amounts) of powdered tissue are then converted to a gaseous state in a sealed system, and the resultant gases delivered to a mass spectrometer. Gases are ionized and the mass to charge ratio measured, yielding a raw ratio (R) of the heavy (e.g. ^{13}C) to light (e.g. ^{12}C) isotope in a sample. These raw ratios are then converted to delta values, in parts per thousand, using the formula: $\delta X = [(R_{sample}/R_{standard}) - 1] \times 1{,}000$, where X is the heavier isotope. $R_{standard}$ refers to the raw ratio of an internationally accepted standard gas for the isotope being analyzed (Coplen et al. 1983; Coplen 1994). Combustion of certified solid standards available from the International Atomic Energy Agency (e.g. IAEA-N1, IAEA-N2) and the National Institute of Standards and Technology (e.g. NBS-16, NBS-17) is now commonly used to correct delta values to an international scale (Werner and Brand 2001). This allows comparison across labs and studies (Haines and Montague 1979; Coplen and Kendall 1982; Hobson and Schell 1998). Due to the limited availability of IAEA and NBS standards, analytical labs are encouraged to develop their own internal lab standards (ILSs) that can be used to calibrate samples within an analytical run. These ILSs must generate consistent delta values to be acceptable as reference materials. Measured values of IAEA standards or ILSs within each run are plotted against their expected values to generate a calibration curve (e.g. two-point correction line for $\delta^{13}C$ with ~5 analyses per point, Fig. 1). This calibration curve is then used to correct delta values of the samples analyzed to a scale consistent with studies from other laboratories.

Each step in the analytical process potentially generates a small amount of error, and the degree of error can change over time depending on analytical conditions and equipment function. With the proliferation of labs using IRMS in the past 20 years, there is considerable variability in the quality assurance procedures employed from lab to lab. Furthermore, the error inherent at various steps of SIA is often under-appreciated by ecologists who are unfamiliar with the procedure, and is reflected by the wide range of approaches used in reporting. This paper will attempt to demonstrate this lack of understanding and its possible implications.

Methods

To determine the range of analytical error reporting in the literature, we surveyed 330 ecological research papers with original data from journals including Acta Oecologica (1), The American Midland Naturalist (1), The American Naturalist (1), Aquatic Ecology (1), The Auk (4), Canadian Journal of Fisheries and Aquatic Sciences (63), Canadian Journal of Zoology (12), Chemical Geology (3), The Condor (4), Coral Reefs (1), Deep-Sea Research (2), Diseases of Aquatic Organisms (1), Ecological Research (1), Ecology (20), Ecology Letters (1), Ecology of Freshwater Fish (4), Ecosystems (2), Environmental Biology of Fishes (2), Environmental Pollution (1), Environmental Science and Technology (1), Environmental Toxicology and Chemistry (3), Estuarine and Coastal Marine Science (1), Estuarine, Coastal and Shelf Science (3), Fisheries Management and Ecology (3), Fisheries Oceanography

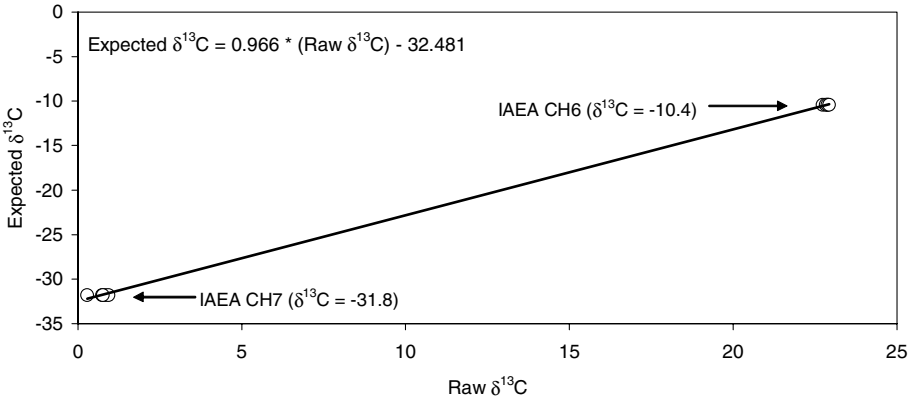

Fig. 1 A typical two-point calibration curve for $\delta^{13}C$ using standards obtained from the International Atomic Energy Agency (IAEA CH6 & CH7). Expected values are compared to observed (raw) values to generate the curve, which is then used to correct the samples

(1), Fisheries Research (1), Fisheries Science (4), Fishery Bulletin (2), Freshwater Biology (11), Functional Ecology (2), Geobios (1), Geochimica et Cosmochimica Acta (8), Hydrobiologia (7), ICES Journal of Marine Science (3), Isotopes in Environmental and Health Studies (1), Journal of Animal Ecology (2), Journal of Applied Ecology (2), Journal of Ecology (1), Journal of Experimental Botany (1), Journal of Experimental Marine Biology and Ecology (4), Journal of Fish Biology (5), Journal of Marine Systems (1), Journal of the North American Benthological Society (6), Journal of Wildlife Management (1), Limnology and Oceanography (29), Marine and Freshwater Research (1), Marine Biology (3), Marine Ecology Progress Series (21), Nature (8), Naturwissenschaften (2), Oceanologica Acta (1), Oecologia (28), Oikos (4), Organic Geochemistry (1), Pacific Science (1), Proceedings of the National Academy of Sciences of the U.S.A. (1), Progress in Oceanography (2), Rapid Communications in Mass Spectrometry (5), Regulated Rivers: Research and Management (1), Science (15), Science of the Total Environment (1), Transactions of the American Fisheries Society (6).

To test for the possibility of spurious treatment effects owing to analytical error, we ran a Monte Carlo simulation (MATLAB, Mathworks Inc., Natick, MA, USA) using stable nitrogen data from a single internal laboratory standard (homogenized bovine liver) run in two sample periods in 2003 (designated time periods A and B) at the Stable Isotopes in Nature Laboratory, University of New Brunswick. The delta value of the liver standard was corrected each time it was run using IAEA standards (mean ± 1 standard deviation [1SD]) N1 ($\delta^{15}N = 0.4 \pm 0.2‰$) and N2 ($\delta^{15}N = 20.3 \pm 0.3‰$) using a two-point calibration as described above (Fig. 1). The Monte Carlo simulation estimated the probability of detecting a significant difference in the bovine liver standard between the two time periods (*t* test for means, $P < 0.05$) by using randomly selected data points with varying sample sizes (Fig. 2) and conducting 1,000 repetitions.

Results

Of the 330 papers surveyed, 63 (19%) failed to report any form of analytical error associated with IRMS. Of the 267 papers that reported analytical error, there was considerable variation in the terminology used to describe error (Table 1). Of those papers that reported error, only 93 (35%) reported error with data from samples, while the remainder used data from standards or did not provide enough detail to judge the procedure.

Mean and 1SD for the laboratory standard (bovine liver) over the entire period ($n = 98$) was $7.31 \pm 0.26‰$ (Fig. 2). This level of precision is typically reported in the ecological literature (Peterson 1999). However, samples designated time period A (mean ± 1SD = $7.23 \pm 0.21‰$) were shifted slightly in a negative direction relative to samples designated time period B (mean ± 1SD = $7.44 \pm 0.29‰$) (Fig. 2). Probabilities generated by the Monte Carlo simulation showed a greater than 90% likelihood of detecting a significant difference between the two sampling periods when the sample size exceeded 30 (Fig. 3).

Discussion

In the majority of cases, the differences associated with treatment effects (e.g. sites, seasons, tissues, etc.) in a given study will far outweigh the error generated by IRMS (Peterson 1999). However, when subtle ecological effects are being measured (Cabana and Rasmussen 1994; Marra et al. 1998; MacAvoy et al. 2001; Morinville and Rasmussen 2003), the importance of analytical error may become significant (Lancaster and Waldron 2001).

Internal and commercially available laboratory standards are used specifically because they are easily homogenized and provide consistent isotope ratio measurements over time (Coplen et al. 1983). In reality, ecological samples are not necessarily as homogenous,

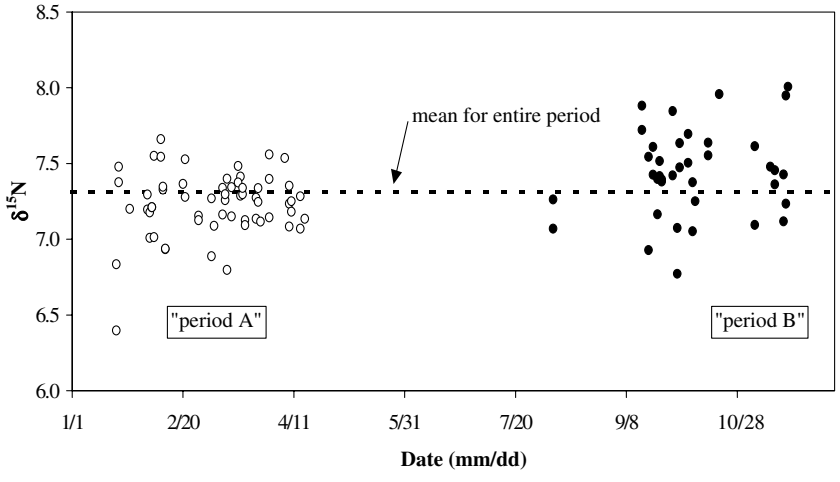

Fig. 2 Stable nitrogen data used for Monte Carlo simulation. Each data point represents a measurement of a single homogenous bovine liver standard (*open circles* time period A, *solid circles* time period B)

Table 1 Terminology used to describe error associated with isotope ratio mass spectrometry (IRMS) and its frequency of occurrence in 267 ecological research papers

Term	Frequency of occurrence	Percent
Precision	104	39
Reproducibility	36	13
Error	30	11
Accuracy	3	1
Agreement	2	< 1
Variability	2	< 1
Repeatability	1	< 1
Replication	1	< 1
Uncertainty	1	< 1

Eighty-seven of 267 cases (33%) did not use a specific term to describe the error associated with IRMS, or stated explicitly the method of calculation (i.e. "standard error for *x* replicate analyses was...."); some authors used more than one term to describe error

particularly those with multiple constituents (e.g. pelleted feed, DeNiro and Epstein 1978; T. Jardine, unpublished data) or those that may vary spatially (e.g. feathers or scales, T. Jardine, unpublished data). The simulation reported in this paper for a single sample over time showed that the high levels of precision previously reported may not be sufficient to guard against spurious treatment effects (Lancaster and Waldron 2001). While this disparity between two time periods is not common in IRMS (Werner and Brand 2001, T. Jardine, personal observation), the exercise illustrates the *potential* for such spurious effects when researchers are unaware of the limitations of the analysis procedure. Due to the overall complexity of IRMS, instruments are routinely reconfigured and subjected to maintenance operations. Hence providing information about the precision of a lab over an extended time period can obscure more subtle differences in analytical performance during specified times.

Uncertainty associated with IRMS analytical error can be better quantified and the resolution of disparity in its reporting can be achieved by incorporating the recommendations described below.

Recommendations

For ecologists using SIA

1. When reporting analytical error in primary research papers, provide measures of both precision and accuracy (Stephenson and Lyon 1982; Schelske and Hodell 1995; Doucett et al. 1999), so that reviewers may judge the confidence with which to assign analytical data. Accuracy should be reported by comparing measured values (mean ± 1SD) for commercially available standards (e.g. acetanilide) that are calibrated alongside the samples with IAEA standards or ILSs (i.e. these should be independent measures of accuracy). These standards have been distributed and tested by various labs with accepted values; an expansion of this approach will require further inter-laboratory comparisons ("ring tests") to generate accepted values for new standards (See recommendation 7 for analytical labs below) and publication of their values. Precision should be reported by providing measured values for standards (precision across runs during the time period samples were analyzed), sample repeats (precision within runs) and for a single sample analyzed every time samples are run (precision across runs), again with mean ± 1SD.
2. Submit blind replicates to ensure quality control (Keough et al. 1996). Analytical labs routinely rerun samples when optimal conditions are not met. Blind replicates provide more information on (and a stricter test of) the performance of their equipment over time.
3. Request information from the lab on procedures used to ensure a high degree of data quality, such as the use of IAEA standards or ILSs to calibrate the data, and the number of replicate samples analyzed within and across runs.
4. Under certain conditions, run all samples in duplicate (e.g. France and Steedman 1996; Kling et al. 1992; Forsberg et al. 1993; Focken and Becker 1998). While this may seem redundant and be impossible due to budgetary constraints, it is a method preferred by

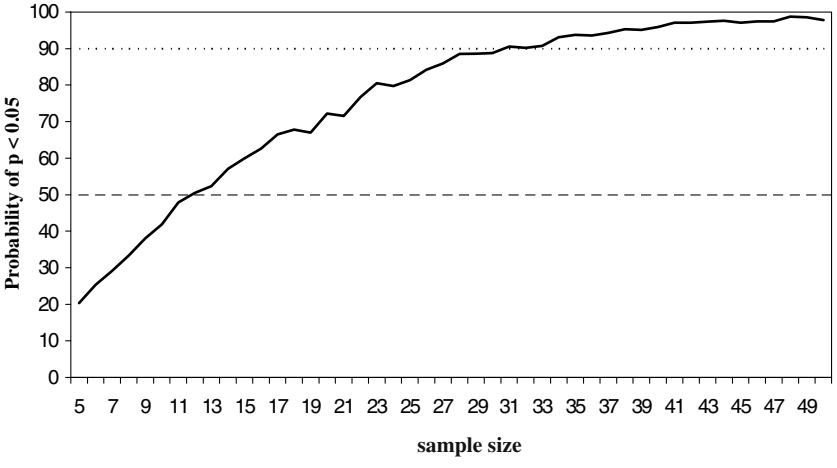

Fig. 3 Probability (*solid line*) of detecting a significant difference (*t* test, $P < 0.05$) for stable nitrogen ratios of a single homogenized bovine liver sample run in two time periods (period A and period B). Probabilities were generated using a Monte Carlo simulation with 5–50 randomly selected data points and 1,000 repetitions. *Hatched lines* are included for reference and indicate 50% and 90% probabilities

many researchers, particularly when the number of samples to be analyzed is small (e.g. <10), or when samples are used that may not be homogeneous (e.g. pooled stream invertebrates). Typically, differences in duplicate measurements greater than $\sim 0.5‰$ (depending on isotope of interest) require a third analysis to "break the tie" (Kline and Willette 2002; Hirons et al. 2001), although some researchers have more strict criteria (Milner et al. 2000).

For analytical labs

1. Use internal standards that are applicable to the sample matrix being analyzed. For example, cod (*Gadhus morhua*) or walleye (*Stizostedion vitreum*) standards were used when analyzing fish tissue (Pinnegar and Polunin 1999; Doucett et al. 1999), while an elephant tooth enamel standard was used when running aquatic mammal tooth enamel apatite (Klementz and Koch 2001). Standards used to calibrate the data (IAEA standards or ILSs) should also bracket the range of isotopic values expected from samples.
2. Advise clients to include a single reference sample every time the client's samples are run (e.g. Gleason 1986) to provide measurements of precision across runs over time.
3. When running replicates of a client's sample within a run, do not place them next to each other in sequence. Output data from IRMS instruments may drift with time (Merritt and Hayes 1994; Anders Ohlsson and Wallmark 1999), hence it is more probable that a deviation from correct values will be detected if repeated samples are run in different sections of an analytical sequence.
4. Include standards that cover the range of percent atomic composition of samples. For example, a range of sample types (periphyton, detritus, seston, zooplankton, shellfish) run by Post (2002) was accompanied by a trout standard (55% carbon, 12% nitrogen) and a cabbage standard (43% carbon, 3% nitrogen).
5. Constrain the weights of samples such that the amplitudes of sample peaks are within a small range; the ideal weight will vary depending on the percent composition of the elements being measured and the sensitivity of the instrument. Alternatively, run standards at a series of different weights so that the effects of linearity can be calculated; this allows for a correction to be made for samples with varying amplitudes.
6. Provide clients with data for standards (both commercially available materials and ILSs) that are run alongside unknowns during the time period the client's samples are analyzed.
7. Continue to develop certified reference materials (commercially available and ILSs) for organic samples and conduct blind inter-lab comparisons ("ring tests"), particularly for those elements (sulfur, oxygen, and hydrogen) that have seen limited analyses.

Special note: coefficient of variation (CV)

Although relatively uncommon in stable isotope ecology error reporting, the CV (a relative measure of variability, expressed as the standard deviation divided by the mean, Zar 1984) has recently been employed to describe variability in stable isotope data (Bunn et al. 1995; Pinnegar and Polunin 1999; Lancaster and Waldron 2001; Johnson et al. 2002). Due to both positive and negative values and the possibility of a zero mean (and hence a CV of infinity) in stable isotope data, CV is not a strong indicator of the relative amount of variability. CV is therefore discouraged as it may provide misleading information about the variability of a set of samples.

Conclusion

While SIA is a relatively new analytical tool in ecology, the potential for gaining a greater understanding of ecological processes through SIA is large. A rigorous treatment of reporting the results and a better understanding of IRMS methodology and analytical error will only serve to improve this field of science. The recommendations in this paper are not an exhaustive list of potential improvements, and further ideas are welcome and encouraged.

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