Using light stable isotopes to assess stream food web ecology in a general ecology laboratory course

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Abstract

Stable isotopes in natural materials provide a powerful way to study energy flow in many systems and are widely used in fields such as archaeology, ecology, forensics, geochemistry, geology, oceanography, paleoecology and paleoclimatology. Based on the manner in which stable isotopes fractionate in natural systems, they allow scientists to address a wide-array of research topics ranging from tracking climatic shifts, ascertaining organisms' migratory patterns, matching organisms to their diets and/or environments, assessing food web bioenergetics, documenting ecosystem changes through time, measuring soil carbon budgets and soil microbial activity, etc. Students with no prior experience working with stable isotopes successfully met learning objectives by completing the requisite field and laboratory protocols, analyzing data, interpreting results, and communicating their findings in a report modeled after a peer-reviewed scientific journal article. While this activity focused on food web ecology in a stream ecosystem, the method is repeatable, cost-effective, and can be modified relatively easily to evaluate food webs in virtually any other ecosystem.

Keywords

Active Learning; Biological Education; Community Ecology; Experiential Learning; $\delta^{13}C$; $\delta^{15}N$; Light Stable Isotopes

Introduction

Stable isotopes are alternate forms of atoms that differ in the number of neutrons contained within their nuclei. Stable isotopes maintain the same chemical properties of their elements, but differ in their atomic mass (Fry 2006). They are non-radioactive atoms that do not experience radioactive decay (as opposed to radioactive isotopes such as carbon-14/carbon-12 that are commonly used in radiometric dating). Stable isotopes are powerful tools used in many scientific disciplines including community ecology, climate science, fisheries biology, paleoecology, geology, forensic science, archaeology, soil science, and many others. Due to fractionation processes, the partitioning of heavy and light isotopes in natural materials, largely due to mass effects (Sharp 2007), the stable isotope signature in such materials provides clues about energy flow and/or environmental conditions. Thus, stable isotopes are commonly used to track changes in climate (Cerling 1984, Lipp et al. 1991, McDermott 2004, West et al. 2006, Baker et al. 2017), ascertain migratory patterns (Rubenstein et al. 2002, Rubenstein and Hobson 2004, Hobson 2016), match organisms to their environments (Bearhop et al. 2004, Hogan et al. 2014) or diets (Hilderbrand et al. 1996, Richards et al. 2000, Bearhop et al. 2003, Divine et al. 2017, Patterson et al. 2019), assess food web bioenergetics (Hobson et al. 1994, Vander Zanden M. J. et al. 1999, Hershey et al. 2017), document ecosystem and environmental changes through time (O'Reilly et al. 2003, Reynolds et al. 2016, 2017, Whitney et al. 2019), measure soil carbon budgets (Hsieh and Yapp 1999, Bridgham et al. 2006), assess soil microbial activity (Boschker and Middelburg 2002, Steinbeiss et al. 2009), and other topics.

Commonly used light stable isotopes include hydrogen, carbon, oxygen, nitrogen, and sulfur. Stable isotopes are measured as a function of the ratio of the more common isotope to the less common isotope, relative to the same ratio measured in an international standard. This value is multiplied by 1,000, and the results are reported in parts per thousand (permil; ‰). Researchers can examine isotopic ratios, designated as delta values (δ) (see Equation 1),

$$\delta = ((Rx - Rstd)/Rstd) * 1000 \qquad \text{Eq. 1}$$

where R is the ratio of the abundance of the heavy to light isotope (e.g., $^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{18}O/^{16}O$, $^{15}N/^{14}N$, $^{34}S/^{32}S$), x denotes sample, and std is the abbreviation for standard, to answer questions of interest (Peterson and Fry 1987, Fry 2006). For example, the $\delta^{18}O$ value in mollusk shell material is commonly used to constrain seawater temperature conditions at the time of deposition when the isotopic composition of the water is known or can be reliably estimated (Epstein et al. 1953, Wanamaker et al. 2007). Another example is the use of $\delta^{13}C$ and $\delta^{15}N$ values to infer animal diets and evaluate ecosystem trophic structure (Hershey et al. 2017).

Using carbon from a plant as an example, researchers would place a sample in an elemental analyzer along with an international standard for carbon, on the Vienna Pee Dee Belemnite (VDPB) scale. The elemental analyzer combusts the sample producing CO_2, and a mass spectrometer measures the intensities of the rare isotope ($^{13}C^{16}O_2$; mass 45) and the common isotope ($^{12}C^{16}O_2$; mass 44) via Faraday cups, and provides an isotopic ratio based on the relative intensities of mass 45 and mass 44 derived from the plant's tissues. The instruments then repeat the measurements on a number of international isotopic standards with well-constrained $\delta^{13}C$ values and are used to place the samples on the international

isotope scale, VPDB. Organic carbon is almost always isotopically negative. For example, the tissues of plants range from a $\delta^{13}C$ of -10‰ to -29‰ (O'Leary 1988), depending on the kind of plant (C_3, C_4, or CAM) and its environment. This is because plants preferentially incorporate the lighter isotope of carbon, which is the most common in nature (more than 98% of the global carbon pool). The negative value simply shows that there is less of the heavier (rare) isotope relative to the lighter (common) one in the plant's tissues when compared to the standard. This property whereby one isotope of an element is incorporated relatively more than another is called fractionation, and can be utilized to understand the flow of energy through systems, including food web ecology. Generally speaking, lighter isotopes have weaker bonds than heavier isotopes (Sharp 2007), thus it is thermodynamically easier for ^{12}C rather than ^{13}C to be incorporated into the leaf of a plant during photosynthesis.

Organisms within an ecosystem are often grouped into trophic levels that are descriptive of how they attain their energy. Producers (autotrophs) comprise the lowest trophic level of a system and convert light (or chemical) energy into usable forms of chemical energy (e.g., sugars). Consumers (heterotrophs) are organisms that gain energy by consuming producers, and can be classified as primary consumers (herbivores, planktivores), secondary consumers (carnivores), tertiary consumers (sometimes carnivores, sometimes omnivores), and apex predators that feed on primary, secondary, and tertiary consumers if there is enough energy in the system to support that many trophic levels. In most cases, approximately 10% of the energy at one trophic level is incorporated into biomass at the next highest trophic level, although that varies from system to system (Molles and Sher 2019).

Freshwater ecosystems receive organic matter from terrestrial environments that is incorporated as allochthonous material (e.g., dead leaves, fallen branches), otherwise described as course particulate organic matter (CPOM), and impacts energy flow. Organisms called shredders (e.g., crane flies, some caddisflies, some stoneflies, some midges) consume CPOM (Cummins and Klug 1979, Cummins et al. 1989), so there is a large detritus-based component of many freshwater stream food webs. Shredders convert CPOM into fine particulate organic matter (FPOM) that may be consumed by downstream collectors (e.g., some caddisflies, some beetles, some dipterans, some midges). Physical breakdown of CPOM and leaching some solutes can also result in dissolved organic matter (DOM) that can be consumed by zooplankton (e.g., diatoms, water fleas). Freshwater ecosystems also contain autochthonous material (e.g., microbes, plankton, algae, aquatic plants) that originate within the system. Scrapers (e.g., some snails, some caddisflies, some fish) and grazers (e.g., mayflies, some beetles, some snails, suckers) often consume these materials. Of course, there are secondary consumers, tertiary consumers and apex predators within these systems as well (e.g., dragonflies, some midges, insectivorous fishes, piscivorous fishes, birds of prey [such as eagles, cranes, and osprey], and some mammals [such as raccoons, river otters, bears, and humans]). A hypothetical stream food web is diagrammed in Figure 1.

Regardless of whether an ecosystem is terrestrial or aquatic, the flow of carbon between trophic levels produces isotopic enrichment. Biological fractionation occurs initially when plants photosynthesize and incorporate the products of photosynthesis into their tissues. Plant tissues are eaten by grazers, which in turn preferentially incorporate the lighter isotopes from the plant tissues into their own tissues. This continues up through trophic levels in a predictable sequence of enrichment of about 1‰ for $\delta^{13}C$ and 3‰ for $\delta^{15}N$ at each step

(DeNiro and Epstein 1976), and means that the trophic position and diet of an organism is reflected in the isotopic composition of its tissues.

In this exercise, we engaged biology undergraduate students in an active learning experience using stable isotopes to evaluate river food web ecology in a Colorado tailwater fishery. The activity centers on the premise that $\delta^{13}C$ and $\delta^{15}N$ can be quantified and are useful in evaluating organismal trophic positions. The approach was successfully implemented in an upper division ecology laboratory course at Western Colorado University, and trained students to be proficient in the use of stable isotopes, increased their understanding of aquatic ecology, enhanced their data management and analytical skills, and refined their scientific communication skills. While this activity was conducted in the southern Rocky Mountains of Colorado, it can easily be adapted to other ecosystems proximate to other institutions.

Student Learning Outcomes (SLOs)

Students will:

(1) demonstrate proficiency in sampling aquatic organisms using different techniques including drift nets, kick nets, angling, and plankton tows (optional).
(2) classify aquatic organisms to biological order (e.g., Amphipoda, Annelida, Ephemeroptera, Plecoptera) or family (e.g., Chironomidae, Salmonidae).
(3) designate the trophic level for sampled aquatic organisms (e.g., producers, primary consumers, secondary consumers, tertiary consumers, apex predators, detritus).
(4) demonstrate safe laboratory practices (wearing appropriate personal protective equipment while washing samples under a fume hood), and avoiding cross-contamination of samples while preparing them for submission.
(5) analyze data in the open-source statistical software program R, reassess *a priori* assumptions, and draw conclusions based on the data.
(6) communicate their findings in a scientific report modeled after a peer-reviewed journal article that includes background literature, a description of the methods employed, the outcomes of statistical analyses, graphical representations of the data, discussion/conclusions, and a literature cited section.

Optional (could be assessed via a pre-laboratory worksheet, but were not when this exercise was implemented):

(7) define common terminology used in stream ecology: allochthonous/autochthonous material; course particulate organic matter (CPOM)/fine particulate organic matter (FPOM)/dissolved organic matter (DOM); shredders/collectors/grazers/scrapers.
(8) define common terminology used in stable isotopes analyses: delta values, permil, fractionation, elemental analyzer, mass spectrometry.

In this laboratory exercise, we reconfirmed the applicability of stable isotopes to stream food web ecology as a learning tool. Our approach was as follows: (1) sample aquatic organisms using a variety of techniques, (2) use safe laboratory techniques to wash and prepare samples for analysis, (3) analyze stable isotope data using open-source statistical software, (4) reassess *a priori* trophic level designations and draw conclusions.

Procedures

Site Selection

Aquatic organisms were sampled from the catch-and-release area on the Taylor River (Figure 2), just below Taylor Park Reservoir, approximately 14 miles northeast of Almont, CO, USA. This site was chosen because (1) it is a tailwater fishery with relatively constant seasonal water temperatures and is accessible year-round (we conducted sampling in February when most other water bodies in the area were frozen over), (2) it is not a highly productive ecosystem, so is relatively depauperate and capturing a high proportion of its biodiversity was likely, (3) it is in close proximity (~31 miles) to Western Colorado University campus where students engaging in the exercise were enrolled, (4) it is a popular sport fishery that many Western Colorado University students are familiar with and excited to learn more about.

Field Sampling

Because we sought to collect organisms from several trophic levels, we employed an array of sampling methods (SLO1). These are outlined as follows (see also Supplemental File 1):

Angling
A self-selected group of five students with their own fishing equipment and valid Colorado fishing licenses attempted to catch trout (the presumed apex predator of the system) using artificial flies and lures. If successful, they were instructed to take a small fin clip (approximately 1 cm x 0.5 cm), which grows back, then immediately release the fish back into the river. Students were instructed to use forceps to handle fin clips so as not to risk contaminating the sample with oils from their fingers, then to place the fin clip into an appropriately labeled plastic bag. While it is has been demonstrated that different fish tissues (e.g., muscle, fin, liver, gonad) exhibit variability in isotopic signatures (Jardine et al. 2005), we opted for the least intrusive, non-lethal method for acquiring tissue.

Macroinvertebrate sampling
The remaining students worked in teams to set three drift nets downstream of the sampling area, where they were left to collect materials carried by the current for the duration of the sampling activity (~1 hour). Once the drift nets were deployed, pairs of students sampled aquatic macroinvertebrates using kick nets. The technique for sampling with kick nets is fairly simple: students were instructed to place the net perpendicular to the flow of the river, then kick along the bottom of the river so that rocks overturned and the current carried any dislodged macroinvertebrates into the net. This activity was conducted for 30 seconds per sampling unit, after which the sample was transferred from the kick net to a sorting tray. Macroinvertebrates, algae and detritus were sorted according to taxonomic group (e.g., Plecoptera, Ephemeroptera, Chironomidae; SLO2), then removed using forceps and placed into individually labeled plastic bags. Each pair of students repeated these steps three times, but organisms were pooled (within student working groups) by their taxonomy (i.e., each group

pooled all their stoneflies into one bag, all their mayflies into another, algae into a third, detritus into a fourth, and so on). When kick net sampling was completed, drift nets were checked and samples were collected from them and sorted in the same manner. Samples collected from kick nets and drift nets were kept separate. All plastic bags containing biological samples were packed in snow to keep samples cold during transport back to the laboratory. Upon return to the institution, samples were frozen at -20°C for one week, after which they were thawed and further sorted (see *Laboratory Techniques* below). Cold storage of samples is only necessary if they will not be processed within the same laboratory period.

Plankton sampling (optional)

The sun set and temperatures dropped too quickly to sample using plankton tows while in the field in February, but this method could easily be added to the exercise at different times of year, or even by assigning a subset of students to conduct plankton tows instead of sampling with kick nets. Groups of 2-3 students could conduct oblique plankton tows wherein a plankton tow net is pulled through the water at the same depth for a known distance (see Supplemental File 1). This allows students to calculate the total volume of the water sampled. Once the tow is complete, students use a wash bottle to wash any plankton that is clinging to the sides of the net into the collection jar. These samples need to be sorted in the laboratory under a dissecting microscope.

Laboratory Techniques

Sorting

Samples were already sorted by trophic level as best as students could ascertain in the field (see *Macroinvertebrate Sampling* above). However, it was critically important for the success of the exercise for them to refine sample identifications to ensure that samples were isolated from those of other trophic levels. This required students to pick through samples under dissecting microscopes and make accurate identifications (SLO2) and preliminary trophic level designations (SLO3). Prior to sorting, students were instructed to wear gloves when handling specimens and reminded of the importance of not touching the samples bare-handed so as not to contaminate them with oils from their skin.

Students used plastic forceps and weigh boats (again, taking care not to touch either bare-handed) to sort samples into the following categories: detritus (sticks, leaves, etc.), algae, midges, mayflies, stoneflies, amphipod crustaceans, fish fin clips. Each sample was then assigned a unique identification code that included a group number, a tentative trophic level, and a sample number (SLO4; Table 1). Weigh boats used in sorting, and glass Petri dishes used for washing (see below) were labeled accordingly using a permanent marker.

Prior conversations with an aquatic ecologist familiar with the system (Dr. Kevin Alexander, Western Colorado University, personal communication with DDH) affirmed that the sampling area was relatively depauperate. Therefore, taxonomic identifications were able to be very broad. For example, stoneflies can be categorized as predators, shredders, collectors, and grazers in various areas, depending on the species that occupy those areas, but in this system all stoneflies were likely to be grazers.

Washing

Samples were placed into labelled glass Petri dishes according to biological classification/presumed trophic level. Students donned gloves and safety goggles, then transferred Petri dishes containing samples to a fume hood where they were repeatedly washed with a 2:1 chloroform:methanol solution, taking care to keep track of sample IDs during the process (SLO4).

The washing procedure was as follows: (1) Students transferred 10 mL of the 2:1 chloroform:methanol solution into a labelled Petri dish containing a sample using a glass transfer pipette. (2) Using metal tweezers that had been pre-washed with the solution (the wash solution reacts with plastic tools, hence the need to use metal and glass, respectively), students gently agitated the samples for 30 seconds to remove lipids and other surface contaminants (e.g., dirt, pollen) according to previously published methods (Wassenaar and Hobson 1998). (3) Students then transferred the dirty wash solution to a glass waste container in the fume hood using the same glass transfer pipette, and then discarded the dirty pipette into an appropriately labelled broken glass waste container. (4) Using clean glass transfer pipettes for each subsequent wash, students washed the samples twice more, agitating the sample for 30 seconds each time.

Drying

All samples were left to dry in the Petri dishes under the fume hood (a dust free environment) for three days. Once dry, the instructor transferred the samples to a low-temperature drying oven, where they were dried for 48 hours at 50-60°C (anywhere from 24-48 hours should suffice). Dried samples were then transferred by the instructor to 1-dram glass vials, sealed, labelled, and shipped to the Stable Isotope Paleo Environments Research Group (SIPERG) Laboratory at Iowa State University for further processing. Some samples were pooled at the instructor's discretion, after checking students' taxonomic identifications, to ensure that adequate amounts of samples (0.5-1.0 g of dried tissue) were submitted as well as to reduce costs (see Table 1).

Iowa State University SIPERG Stable Isotope Laboratory

Once received, samples were placed into tin capsules and immediately sealed. Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotopes analyses were conducted on a Costech Elemental Analyzer attached to a Finnigan Delta Plus XL mass spectrometer in continuous flow mode. $\delta^{13}C$ was corrected according to the international VPDB standard, and $\delta^{15}N$ was corrected via isotopic reference materials (Air). Corrections were made using a regression method, and results reported in permil (‰). Percent concentration (%) was calculated using the peak intensity of the sample against well-characterized (C:N) acetanilide standards. Analytical uncertainty at 1σ was ±0.11‰ for C and ±0.09‰ for N.

Instructions for sorting and processing samples in the laboratory were provided to students in a handout (see Supplemental File 2).

Data Analyses

Statistical analyses and graphing were conducted in R v3.2.2 (R Core Team 2015) using the packages ggplot2 v.2.1.0 (Wickham et al. 2009) and mgcv v.1.8 (Wood 2006). Students completed an R tutorial covering basic statistics earlier in the semester. Students were assigned to conduct numerous tasks in an R notebook tutorial (Supplemental File 3) including loading the data, creating a dual isotopes plot, plotting histograms, assessing distributions, removing outliers, plotting a pairwise Euclidean distance matrix, conducting cluster analyses, and interpreting figures (SLO5). Pairs of students worked through the tutorial together, and turned in completed versions with properly annotated answers to questions that were then graded by the instructor. Because these students were naïve to stable isotope analyses, these data analyses were kept relatively simple (but a more sophisticated tutorial for more advanced students who have successfully completed the introductory exercise is included; Supplemental File 4).

When data analyses were completed (the third day of the exercise, on personal computers that were available in the teaching laboratory), students were assigned to write a paper modeled after a peer-reviewed scientific manuscript (SLO6) including an introduction that included background literature, complete methods, a summary of the results (including graphical representations of their data), conclusions, and a literature cited section. Students were instructed to format these according to guidelines for the journal Ecology. This paper was then graded by the instructor.

A separate advanced R tutorial was tested by 1 undergraduate student who had previously completed the introductory exercise. This tutorial used package dplyr v.0.8.2 (Wickham et al. 2009) to filter and summarize data. The advanced tutorial also included the development of Bayesian isotope mixing models using package simmr v.0.4.1 (Parnell 2019). The student agreed to act as a tester for the advanced exercise, and worked through it individually, with limited guidance from the instructor. Tasks in the advanced R notebook exercise included loading the data, extracting elements of a data frame via indexing, subsetting data frames and matrices, conversion of data frame objects to matrices or vectors, building a simmr object, running and interpreting mixing models, creating dual isotope and box and whisker plots, and estimating diet proportions of target taxa. The student turned in a completed version with properly annotated answers to questions which were then evaluated by the instructor.

Results

Sampling and Stable Isotopes

A general ecology laboratory course with 21 registered students obtained 26 biological samples from the catch-and-release area of the Taylor River below Taylor Park Reservoir, CO, in February, 2019 (Table 1). These biological samples spanned a wide taxonomic breadth, and could be categorized under multiple trophic levels. One sample was omitted from further analysis because it did not meet the minimum size requirements, and others collected by different groups were pooled to ensure minimum size requirements were met as well as to reduce costs. Thus, a total of 19 samples were submitted for stable isotopes analyses at ISU's

SIPERG Laboratory, and those results are provided herein (Table 2). In brief, corrected $\delta^{13}C$ ranged from -17.54‰ to -29.51‰, and corrected $\delta^{15}N$ ranged from 8.41‰ to 49.26‰ (Table 2).

Data Analyses

Students following a well-annotated R notebook tutorial (Supplemental File 3) produced several graphical representations of the data. These included dual isotope plots (Figure 3), frequency histograms (Figure 4), distance matrices (Figure 5) and cluster plots (Figure 6). Students were able to manage data, generate graphical representations of their data, and answer questions pertinent to the analytical steps they were taking with high levels of success (Table 3).

SLO Results

Student success rates were substantial for each of the student learning outcomes for a general ecology laboratory course (Table 3). While students did not achieve 100% success on all six SLOs, they did on four of the six. For the two SLOs where students achieved <100% success, students attained a 92.3% success rate on SLO3, and an 82.2% success rate on SLO6 (Table 3). Similarly, one student familiar with the exercise successfully completed the advanced R exercise with a 100% success rate, and while that sample size is small and unlikely to be representative of an entire class, we are confident that students could achieve high levels of success in completing that exercise as well, either in a more advanced class, or as a follow-up exercise in a general ecology class.

Discussion

Stable Isotopes Results

Stable isotopes are useful for assessing organismal trophic levels (Hobson et al. 1994, Vander Zanden M. J. et al. 1999, Hershey et al. 2017), and to match organisms to their diets (Hilderbrand et al. 1996, Richards et al. 2000, Bearhop et al. 2003, Divine et al. 2017, Patterson et al. 2019). At the conclusion of this exercise, students were able to reassess the *a priori* assumptions they made while categorizing the biological samples they obtained. While many of the samples were confirmed to have the trophic status they were initially thought to hold, not all were. For example, one group of students caught a leech, which they presumed to be a secondary consumer (based on the common assumption that leeches are parasites). However, the Euclidean distance matrix they produced revealed the leech's isotopic signature to be most similar to primary producers rather than to consumers (Figure 5). Moreover, cluster analysis placed the leech in a group with producers, an herbivore, and a detritivore (Figure 6). Hence, the students reassessed their *a priori* assumption and concluded that the leech was likely a free-living non-parasitic species that fed on plant or algal material (a conclusion that is corroborated by a Bayesian isotope mixing model in the advanced R exercise; Supplemental File 4.

Both $\delta^{13}C$ and $\delta^{15}N$ in living tissues vary widely across space and time. Plants, and aquatic plants in particular, are often regarded as problematic in food web studies because of a high degree of unexplained variability in their isotopic signatures (Chappuis et al. 2017). In general, plants at high elevation tend to have higher $\delta^{13}C$ values in their tissues relative to low-

elevation plants (Körner et al. 1988). This is due not only to the effects of lower growing season temperatures on isotopic fractionation, but also to the signal produced by low internal to external partial pressure ($pC0_2$) ratios (Körner et al. 1988). In aquatic environments, however, the signal appears to be driven largely by pH and dissolved inorganic carbon (DIC) (Chappuis et al. 2017), which may be highly variable throughout the year and from location to location. Plants incorporate the DIC into their tissues and in turn serve as the ultimate source of organic carbon for the vast majority of the rest of the organisms. The absolute $\delta^{13}C$ values of higher trophic level organisms may be shifted relative to other locations, seasons, and elevations because of the original DIC signature on which the system is based. For this reason, ecosystems cannot be directly compared without careful site selection and controls. Care should therefore be taken when instructing students to reference appropriate literature. Students must be asked to compare offsets in the isotopic signatures between trophic levels in their study and others, not the absolute values, unless differences between locations are made an explicit goal of the exercise.

The adage "You are what you eat plus a few permil" (DeNiro and Epstein 1976) has deservedly become a rule of thumb in ecosystem studies. The cumulative effect of biological isotopic fractionation is to produce trophic levels that are enriched approximately 3‰ in $\delta^{15}N$ from producers to grazers, grazers to secondary consumers, and so forth, and enriched approximately 1‰ in $\delta^{13}C$ from one level to the next in terrestrial systems (see Post 2002 for a thorough review of the subject). $\delta^{13}C$ enrichment is smaller in freshwater systems, averaging 0.2‰ (France and Peters 1997). The present food web shows evidence of nitrogen enrichment with increasing trophic level, as expected, but unexpectedly shows carbon depletion between one trophic level to the next. The negative offset between the $\delta^{13}C$ of the trout and its apparent primary food source, stoneflies, is likely to be due to time lags in tissue turnover, exacerbated by harsh winter conditions at high elevation. Fish fins are understood to have long tissue turnover times in the absence of damage, and therefore reflect diet of several months to more than a year prior to sampling, depending on the fish's growth rate (Busst and Britton 2018). Work by Thomas and Crowther (2015) would predict that the stoneflies which made up the trout's diet likely have a much faster tissue turnover time than the trout, but also have more severely reduced metabolic activity in the winter months. Stoneflies sampled in the present study were likely representative of the previous fall's food supply. This "time averaging" effect is an established, though often overlooked, consideration (O'Reilly et al. 2002), and one which should be introduced to students fully. Allowing students to interact with complexity and unexpected results in a real-world context is a critical component of scientific training.

Assessment

This exercise focused on student learning over three laboratory periods via a well-organized series of field- and laboratory-based experiential learning exercises wherein students collected biological samples using a variety of methods, applied safe and effective laboratory procedures to process samples, analyzed data, interpreted results, and communicated their findings.

The exercise included six student learning outcomes. SLOs 1-4 allowed students to develop new field- and laboratory-based skills. SLO5 facilitated increased confidence in students pertaining to their ability to manage and analyze data using R statistical software. SLO6 enhanced students' scientific communication abilities. Performance assessments revealed

that success rates for all six SLOs were ≥ 82.2% (Table 3). The advanced exercise was completed by an undergraduate student tester with 100% success. Therefore, students demonstrated very high levels of learning.

Educational Impact

Students were able to function as "real" scientists and were introduced to new field and laboratory techniques, demonstrating proficiency in the required skills to complete the exercise. While this exercise did have six SLOs, the focus was on student learning via the *process* of these field and laboratory activities. This exercise provided a quality educational experience that connected students to their environment in a unique way, two important components of experiential learning (Kolb 1984; Katula and Threnhauser 1999; Kolb and Kolb 2005). Moreover, the exercise raised the students' ecological consciousness, which may translate into improved societal, ecological, and environmental understanding (Hill et al. 2004). In addition to students performing well and achieving high levels of success on the six SLOs, at the conclusion of the exercise, several students communicated verbally with the instructor that it was their favorite exercise of the semester.

Instructors interested in conducting similar activities in their own courses can view necessary supplies and costs (Supplemental File 5). Much of the necessary equipment (e.g., waders, kick nets, drift nets, dissecting scopes, fume hood, drying oven, etc.) was already available for use at WCU, and glassware, consumables, and per sample costs of stable isotopes analysis on the mass spectrometer did present an 'up front' cost that warrants consideration. This exercise was conducted for a cost of approximately $750 in Spring Semester 2019, but subsequent activities would be less expensive (or could include more samples) with the reusable materials already purchased. As stated at the end of the introduction, this activity could easily be tailored to other ecosystems proximate to other educational institutions.

Data Accessibility Statement

The field sampling methods handout provided to students (Supplemental File 1), laboratory methods handout provided to students (Supplemental File 2), introductory and advanced R tutorials (Supplemental Files 3 and 4, respectively), instructor keys (Supplemental Files 3Key and 4Key, respectively), materials list and budget (Supplemental File 5), a software helpfile for instructors (Supplemental File 6), and a spreadsheet of raw data used in this exercise in Microsoft Excel format (Supplemental File 7 [2019_EA_Houston_class project.xlsx]; See also Table 2) are available via public GitHub repository: https://github.com/hannahcarroll/Aquatic-isotopes-public

Disclosure Statement

No potential conflict of interest was reported by the authors.

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Table 1. Biological samples students acquired from the catch-and-release area of the Taylor River, below Taylor Park Reservoir, CO. Samples were assigned a taxonomic classification, categorized by trophic level, and assigned a unique sample identification number.

Sample #	Group #	Presumed Trophic Level (by Students)	Biological Classification	Sample ID	Common Name
1	1	Secondary consumer	Annelida	G1PAANS1	Leech
2	1	Grazer	Amphipoda	G1GRAMS2	Scud[1]
3	1	Grazer	Plecoptera	G1GRPLS3	Stonefly
4	1	Grazer	Ephemeroptera	G1GREPS4	Mayfly
5	1	Collector	Chironomidae	G1COCHS5	Midge[2]
6	1	Detritus	Unidentified Plant	G1DEDES6	Twig
7	2	Detritus	Unidentified Plant	G2DEDES1	Twig
8	2	Grazer	Chironomidae	G2GRCHS2	Midge
9	2	Grazer	Amphipoda	G2GRAMS3	Scud[1]
10	2	Grazer	Ephemeroptera	G2GREPS4	Mayfly
11	2	Grazer	Plecoptera	G2GRPLS5	Stonefly
12	2	Producer	Algae	G2PRALS6	Algae[3]
13	3	Shredder	Plecoptera	G3SHPLS1	Stonefly
14	3	Collector	Chironomidae	G3COCHS2	Midge[4]
15	3	Shredder	Ephemeroptera	G3SHEPS3	Mayfly
16	3	Producer	Algae	G3PRALS4	Algae
17	3	Detritivore	Amphipoda	G3DEAMS5	Scud[1]
18	3	Apex Predator	Salmonidae	G3APSAS6	Brown Trout
19	4	Producer	Algae	G4PRALS1	Algae[3]
20	4	Grazer	Ephemeroptera	G4GREPS2	Mayfly[5]
21	4	Detritus	Unidentified Plant	G4DEDES3	Twig[6]
22	4	Grazer	Chironomidae	G4GRCHS4	Midge[4]
23	5	Detritus	Unidentified Plant	G5DEDES1	Twig[6]
24	5	Producer	Algae	G5PRALS2	Algae
25	5	Shredder	Trichoptera	G5SHTRS3	Caddisfly
26	5	Grazer	Chironomidae	G5GRCHS4	Midge[5]

[1] Amphipod samples that were pooled by the instructor after drying to ensure that the minimum quantity requirement was met

[2] Sample included a single midge that did not meet the minimum size requirements, but also appeared to be a different species from other samples, so this sample was omitted from further analysis

[3] Algal samples that were pooled by the instructor after drying to ensure that the minimum quantity requirement was met

[4] Midge samples that were pooled by the instructor after drying to ensure that the minimum quantity requirement was met

[5] Misidentified samples – these samples were the same species of stonefly, not midges or mayflies, and were pooled by the instructor after drying

[6] Detritus samples that were pooled by the instructor after drying to ensure that the minimum quantity requirement was met

Table 2. Corrected $\delta^{13}C$ (VPDB) and $\delta^{15}N$ (air) values for 19 biological samples collected by students for this laboratory exercise. $\delta^{13}C$ values are negative as a result of fractionation. Percent concentrations of C and N, taxonomic identifiers, and presumed trophic levels are also provided.

Sample ID	Identifier	Trophic Level	$\delta^{13}C$	$\delta^{15}N$	%C	%N
G1PAANS1	Leech	Secondary Consumer	-23.10	8.41	40.30	9.19
G1GRAMS2, G2GRAMS3, G3DEAMS5 pooled	Amphipods	Detritivore	-19.37	8.80	40.25	8.10
G1GRPLS3	Stoneflies	Herbivore/Detritivore	-26.58	9.42	50.19	10.32
G1GREPS4	Mayflies	Herbivore	-24.38	8.07	48.65	10.43
G1DEDES6	Wood	Detritus	-18.87	19.80	49.35	0.92
G4DEDES3, G5DEDES1 pooled	Wood	Detritus	-23.15	10.46	44.40	1.83
G5PRALS2	Algae	Primary Producer	-24.38	9.06	22.42	3.05
G5SHTRS3	Caddis	Detritivore	-29.51	8.40	47.34	9.08
G4GREPS2, G5GRCHS4 pooled	Stoneflies	Herbivore/Detritivore	-27.25	8.88	48.78	10.10
G2DEDES1	Wood	Detritus	-17.54	49.26	50.21	0.18
G2GRCHS2	Midges	Detritivore	-26.01	8.50	49.56	9.67
G2GREPS4	Mayflies	Herbivore	-26.20	7.41	45.50	9.20
G2GRPLS5	Stoneflies	Herbivore/Detritivore	-26.78	10.51	47.04	10.78
G2PRALS6, G4PRALS1 pooled	Algae	Primary Producer	-22.58	8.62	21.69	2.75
G3COCHS2, G4GRCHS4 pooled	Midge	Detritivore	-25.87	6.94	37.59	7.83
G3SHPLS1	Stoneflies	Herbivore/Detritivore	-27.25	9.62	49.34	10.35
G3SHEPS3	Mayflies	Herbivore	-26.43	7.68	49.37	9.75
G3PRALS4	Algae	Primary Producer	-22.75	8.50	16.44	2.22
G3APSAS6	Trout	Apex Predator	-27.48	11.33	29.98	8.89

Table 3. Results of student learning outcomes.

SLO #	Assessment Method	Success Rate
SLO1	Successful capture of a variety of aquatic organisms by students	100%
SLO2	Determination of broad taxonomic categories by students, corrected when necessary by the instructor (see Table 1)	92.3%
SLO3	Tentative assignment of samples to a trophic level by students, with justification to the instructor for why (these were reassessed later in the R tutorial)	100%
SLO4	Laboratory activities supervised by the instructor and/or teaching assistant	100%
SLO5	Graded R tutorial with numerous questions answered (including a bonus question; Supplemental File 1)	104%
SLO6	Graded laboratory report modeled after a peer-reviewed scientific article	82.2%
SLO7	Graded pre-laboratory worksheets covering stream ecology terminology	Not assessed
SLO8	Graded pre-laboratory worksheets covering stable isotopes terminology	Not assessed

Figure 1. A graphical representation of a generic aquatic food web depicting energy flow across multiple trophic levels (modified after Merritt and Cummins 1996). Organisms are not drawn to scale. Abbreviations are as follows: CPOM – coarse particulate organic matter; DOM – dissolved organic matter; FPOM – fine particulate organic matter.

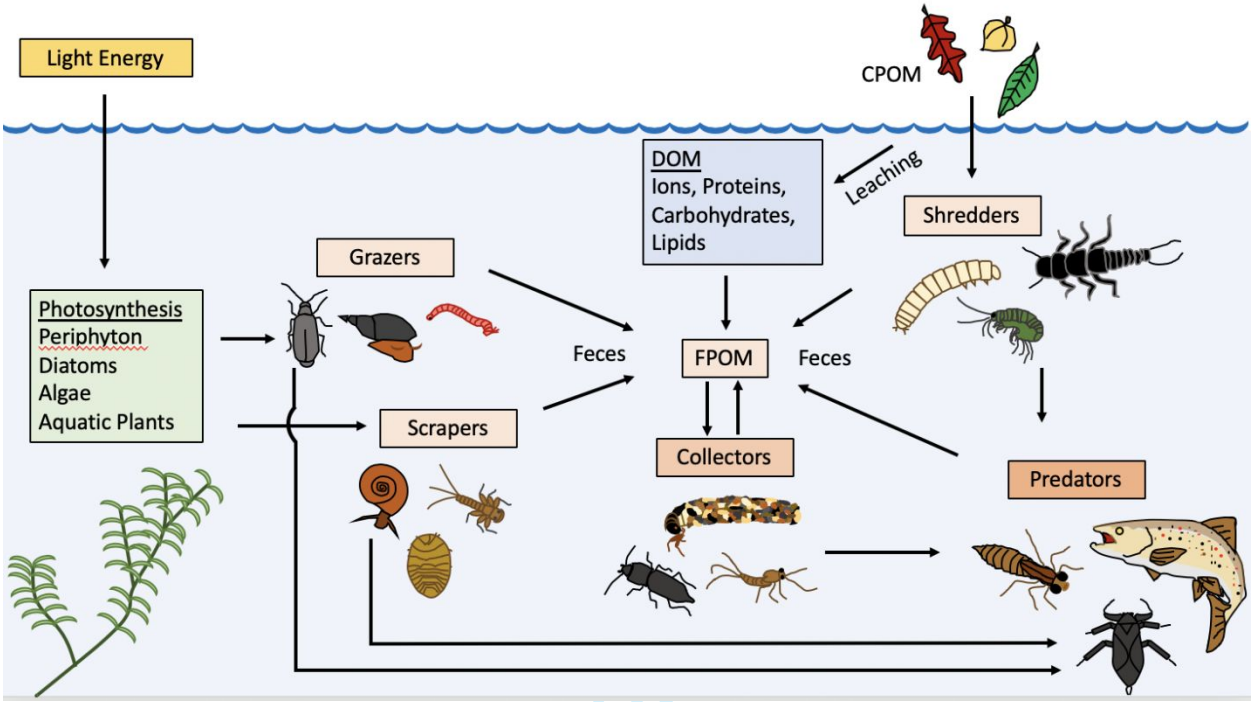

Figure 2. Students sampling aquatic organisms from the catch-and-release section of the Taylor River, CO. Top left: Students receiving instruction (from DDH) on how to deploy drift nets. Top right and bottom left: Groups of students picking through kick net samples and sorting organisms by taxonomic group and/or presumed trophic level. Bottom right: Students pulling drift nets to conclude the sampling exercise.

Figure 3. Student example of dual isotopes plots. Left panel: Prior to removal of outliers. Right panel: Outliers removed.

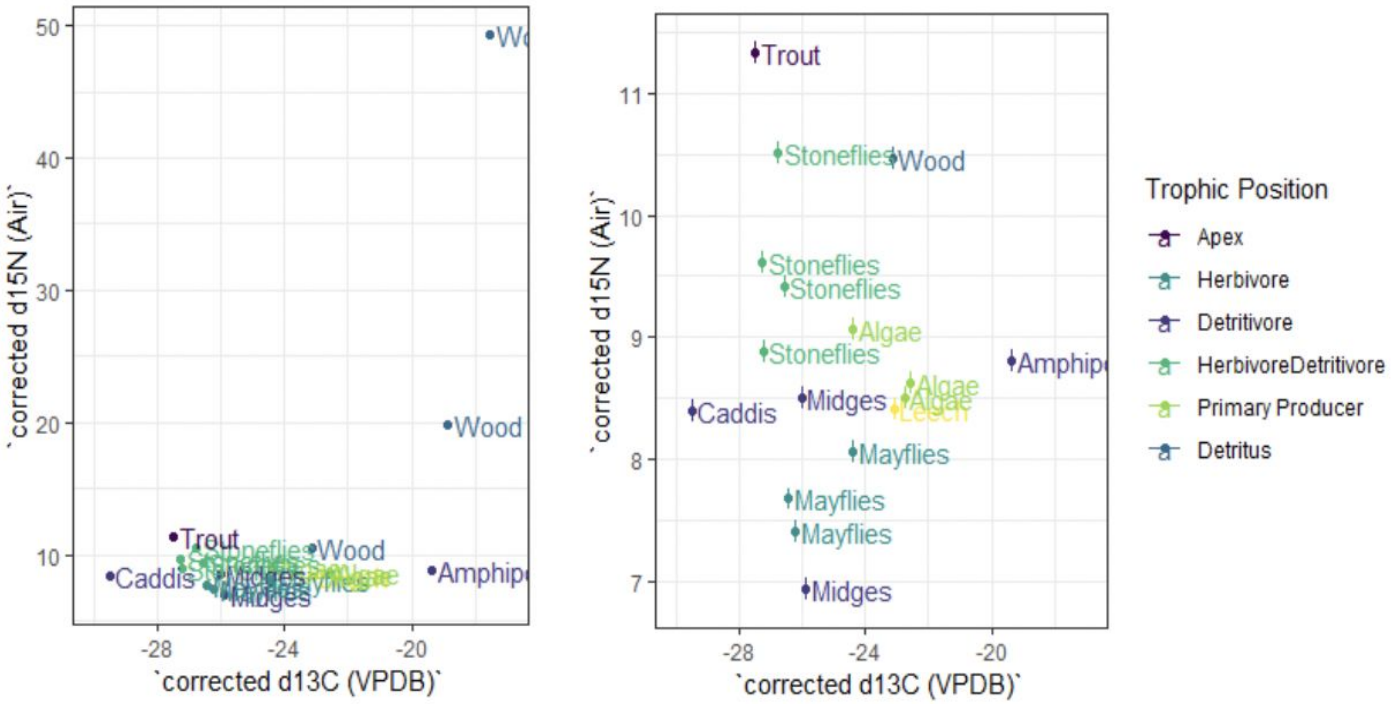

Figure 4. Student example of histograms using isotope data. Top panel: Full data set; Middle panel: One outlier removed; Bottom panel: Second outlier removed. Students were asked to describe the distributions, justify cutoff points for the removal of outliers, and write lines of code to remove outliers in the R tutorial. Removal of outliers was justified by the abnormal isotopic signal of two wood (detritus) samples that may have been caused by samples not being thoroughly cleaned and dried, or were contaminated by microbial activity.

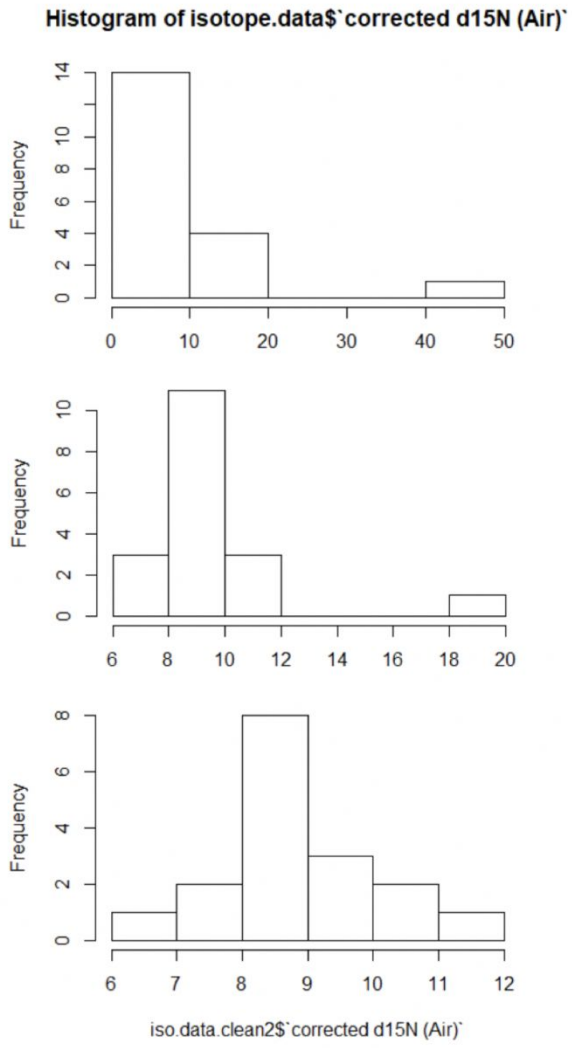

Figure 5. Student example of a pairwise Euclidean distance matrix comparing various trophic categories. Color coding ranges from blue (completely similar) to dark orange (highly dissimilar). Students received instruction on how to interpret these pairwise differences, then were assigned to reassess their preliminary trophic level designations.

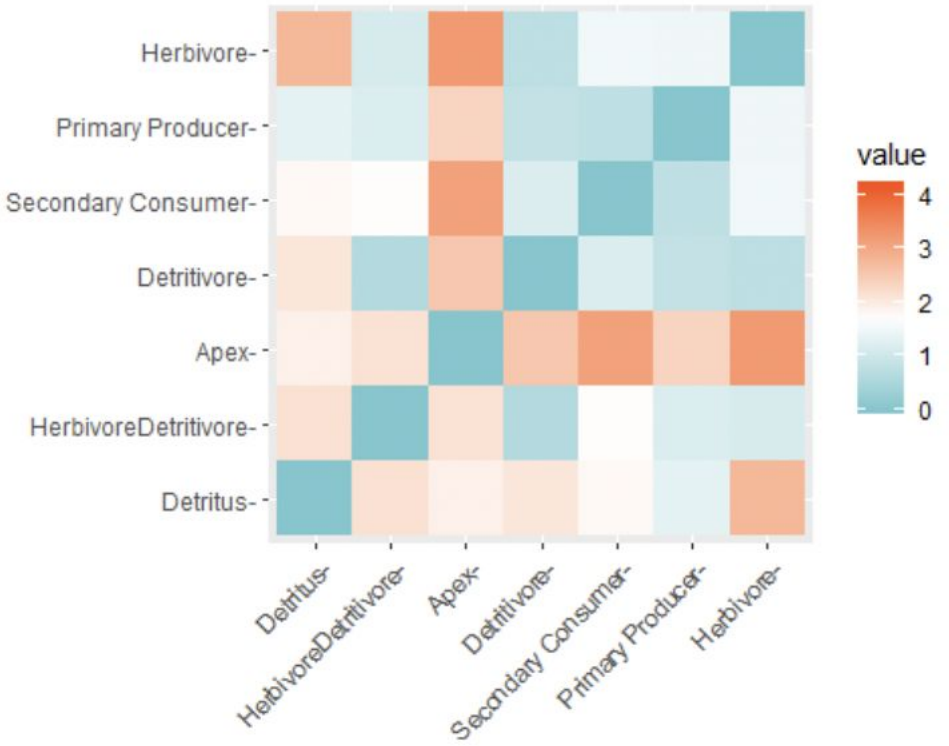

Figure 6. Student example of a cluster plot grouping samples together based on corrected $\delta^{13}C$ and $\delta^{15}N$ values. Students were instructed to produce numerous plots, using different numbers of clusters, then select the number of clusters they felt best represented the data and explain why in the R notebook tutorial and in their laboratory report.

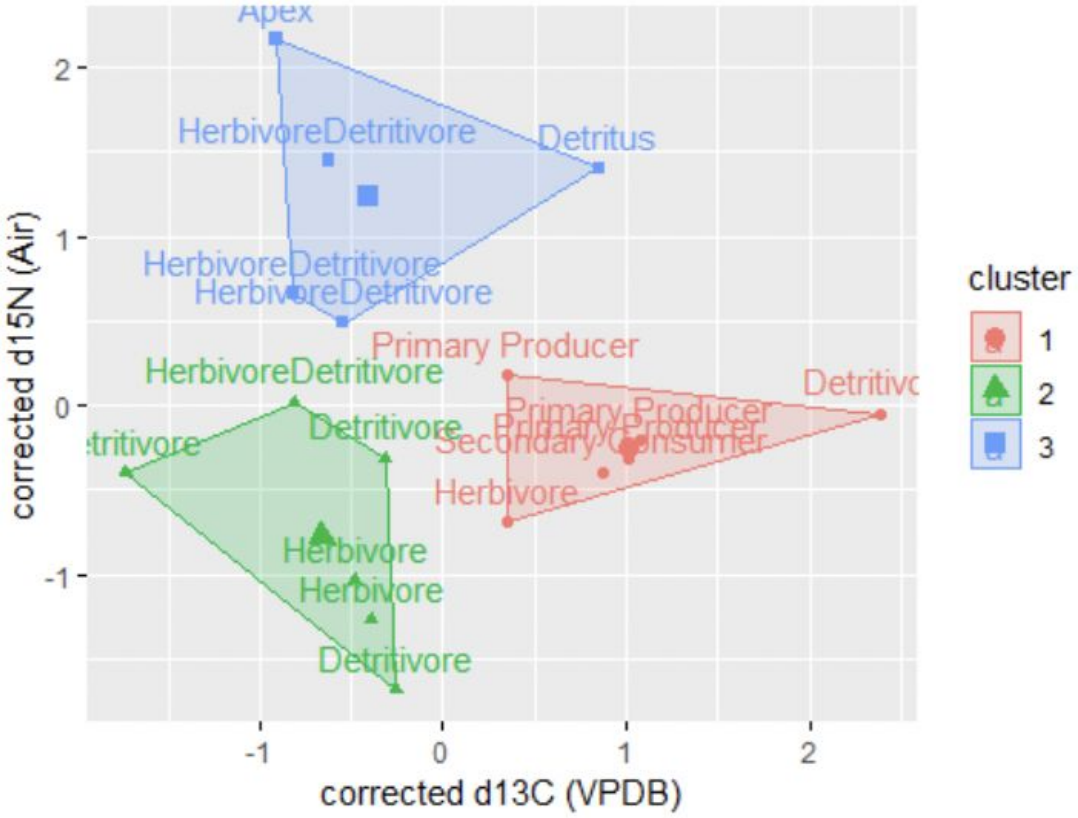

Figure Captions

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Biology 302: Ecology Laboratory
Using Stable Isotopes to Assess Food Web Ecology in a Tailwater Fishery

Stable Isotopes

Stable isotopes are alternative forms of atoms that differ in the number of neutrons contained in their nuclei. Stable isotopes maintain the same chemical properties of their elements, but differ in their atomic mass. These are non-radioactive atoms (as opposed to those that experience radioactive decay with a certain half-life, such as carbon-14 to carbon-12 used for radiometric dating), that are used by ecologists, climatologists, fisheries biologists, paleoecologists, soils scientists, and many others. Stable isotopes are commonly used to track changes in climate, track migratory patterns of birds, butterflies, mammals, and more, match organisms to their environments and/or diets (e.g., breeding birds, anadromous fishes), assess food web bioenergetics, document ecosystem changes through time, measure soil carbon budgets and soil microbial activity, etc. Some commonly used stable isotopes include hydrogen ($^{2}H/^{1}H$), carbon ($^{13}C/^{12}C$), oxygen ($^{18}O/^{16}O$), and nitrogen ($^{15}N/^{14}N$). Researchers often examine isotopic ratios, designated as **delta values**, to answer their questions of interest (Peterson and Fry 1987). For example, $\delta^{18}O$ is a measure of ^{18}O and ^{16}O stable isotope ratios, and is commonly used to assess hydrographic properties of sediment carbonates, such as salinity and temperature, to estimate microenvironmental conditions at the time of sediment deposition (Wanamaker et al. 2007). Another example is the use of $\delta^{13}C$ and $\delta^{15}N$ to infer animal diets and evaluate ecosystem trophic structure (Hershey et al. 2017).

Stable isotopes are measured as a function of the ratio of the more common isotope to the less common isotope, relative to same ratio measured in a global standard. This value is multiplied by 1,000, and the results are therefore reported in parts per thousand, called **permil** or **per mil**, which is represented by the symbol **‰**. Note that this is different from a percent sign, %, which is shorthand for parts per hundred (per *cent*, from the Latin *centum*, meaning 100).

Using carbon from a plant as an example, we would place our sample in an **elemental analyzer** along with a sample of the global standard for carbon, Vienna Pee Dee Belemnite (VDPB). The elemental analyzer combusts the sample, and a **mass spectrometer** counts the number of atoms of the rare isotope (^{13}C) and the number of atoms of the common isotope (^{12}C) in the plant's tissues. The instruments then repeat the measurements on our standard. We calculate the isotopic signature of our sample from those counts. Organic carbon (the carbon produced by living organisms) is almost always isotopically negative. For example, the tissues of plants range from a $\delta^{13}C$ of -10‰ to -24‰, depending on the kind of plant and its environment. This is because plants preferentially use the lighter isotope of carbon, which is the most common in nature (more than 98% of the global carbon pool). The negative value simply shows that there is less of the heavier (rare) isotope relative to the lighter (common) one in the plant's tissues when compared to the standard. We will use the property whereby one isotope of an element is used more frequently than another, called **fractionation**, to understand a food web in one of our local streams.

Trophic Levels

Organisms need energy to fuel the myriad metabolic processes inside their cells. Producers can convert light (or thermal) energy into usable chemical energy, whereas consumers must obtain energy through their diets. Organisms can be grouped into **trophic levels** that are descriptive of how they attain their energy. The lowest trophic level consists of the **producers** (or autotrophs) within a system (i.e., plants and algae). The next level up is made up of **primary consumers** (or heterotrophs) in the system (i.e., herbivores, planktivores) that gain energy by consuming producers. **Secondary consumers** (i.e., carnivores) comprise the third trophic level, and attain energy by consuming primary consumers. Depending on the ecosystem, there can be **tertiary consumers** (sometimes carnivores, sometimes omnivores), and **apex predators** that feed on primary, secondary and tertiary consumers if there is enough energy in the system to support that many trophic levels. A general rule of thumb is that approximately 10% of the energy at one trophic level makes it to the next highest trophic level, although that varies from system to system (Molles and Sher 2019).

We will examine stable isotopes among trophic levels in a tailwater fishery (the catch-and-release section of the Taylor River, just below Taylor Park Reservoir). Freshwater aquatic ecosystems can sometimes be slightly different in terms of energy flow because some materials from the terrestrial environment are incorporated as **allochthonous material** (e.g., dead leaves, fallen branches/trees, otherwise known as **course particulate organic matter** or **CPOM**) that are consumed by **shredders** (e.g., crane flies, some caddisflies, some stoneflies, some midges), so there is a large **detritus**-based component to many freshwater stream food webs. Shredders convert CPOM into **FPOM (fine particulate organic matter)** that may be consumed by downstream **collectors** (e.g., some caddisflies, some beetles, some dipterans, some midges). Physical breakdown of CPOM can also result in **DOM (dissolved organic matter)** that can be consumed by zooplankton (e.g., diatoms, water fleas). Freshwater ecosystems also contain **autochtonous material** (e.g., microbes, plankton, algae) that originate within the stream. **Scrapers** (e.g., some snails, some caddisflies, some midges, some fish) and **grazers** (e.g., mayflies, some beetles, some snails, suckers water fleas) often consume these materials. Of course, there are secondary consumers, tertiary consumers, and apex predators within these ecosystems as well (e.g., dragonflies, some midges, insectivorous fishes, piscivorous fishes, birds of prey [such as eagles, cranes and osprey], and some mammals). A hypothetical freshwater stream food web is diagramed in Fig. 1. The organisms we will see should be different than these, but the same groups (shredders, collectors, scrapers, predators) should be represented.

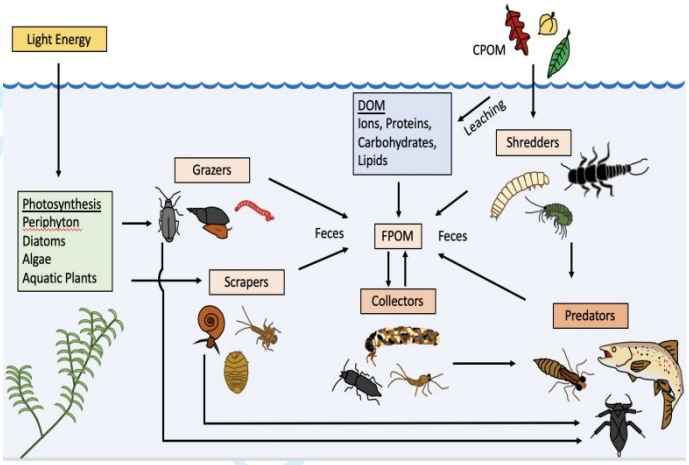

Fig. 1. A graphical representation of a typical stream food web depicting energy flow across multiple trophic levels (modified after Merritt and Cummins 1996)

Sampling Techniques

Because we seek to collect organisms from several trophic levels, we will need to employ an array of sampling methods. These are outlined as follows:

Plankton sampling

We will use a plankton tow net to conduct oblique plankton tows (Fig. 2). These nets have a conical shape with mesh sides that allows outflow of water while keeping plankton within the screen. The focal point of this conical net is a collection jar that accumulates plankton. It is typically important to standardize the distance of the tow, to be able to calculate the volume of water sampled. We will try a meter at first, knowing that it may require longer tows if we do not get much plankton. It is also important to try and keep the net at the same depth for the duration of the tow to sample the same plankton community for the duration of the tow. Once the tow is complete, wash any plankton that is clinging to the sides of the net down into the collection jar with a wash bottle. These samples will need to be sorted in the laboratory.

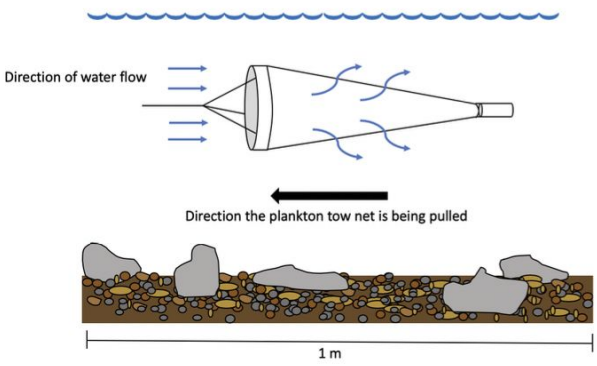

Fig. 2. Depiction of an oblique plankton tow

Macroinvertebrate sampling

We will sample aquatic macroinvertebrates using different techniques. The first is the use of a kick net. The technique is relatively simple: Place the net perpendicular to the flow of the river, then kick your feet along the bottom, so that rocks are overturned and the current will carry any dislodged macroinvertebrates into the mouth of the net (Fig. 3). Do this for 30 seconds, then transfer the sample into a plastic tray where it can be picked through and the macroinvertebrates removed using forceps (it is important not to touch these bare-handed so oils do not contaminate the samples). Sort the macroinvertebrates into categories (grazers, scrapers, shredders, predators).

Fig. 3. Kick net technique
fba.org.uk

The other method we will use to sample aquatic macroinvertebrates is a drift net. The idea is similar to that of a kick net, with the difference being that these nets are set in the current, and checked after some amount of time without necessarily kicking the substrate in front of them. Some scientists check these every hour for a 24 hour period, then repeat for different days or seasons, but we will set them when we arrive at the site, and take them down when we are finished with the other sampling. Macroinvertebrates will be collected

and sorted as described above. There is a good chance that we will get algae, aquatic plants, or even CPOM off the drift nets as well.

Fish sampling

A subset of the class will spend their time angling for trout in an attempt to catch an apex predator from this system for our analysis. This is a catch and release area, only artificial flies and lures may be used, and anglers must have a valid Colorado fishing license. If successful, we will take a fin clip (they regrow) and release the fish back into the river.

We will need to store our samples in properly labeled Ziploc bags for transportation to Western (on ice), where they will be frozen until we are able to sort samples in the laboratory.

Laboratory Techniques

Sorting samples

Because we are evaluating food web dynamics, it is important that our samples be properly sorted into the major categories of a stream food web: detritus, producers, primary consumers, secondary consumers, tertiary consumers, and apex predators. Once sorted, each category should be placed in a separate Ziploc or Whirl-Pak bag and frozen until they can be processed further. More instructions on how to prepare samples will be provided at a later date.

Literature Cited

Hershey, AE, RM Northington, JC Finlay, BJ Peterson (2017) Stable isotopes in stream food webs. *Methods in Stream Ecology Volume 2: Ecosystem Function,* pp 3-20.

Merritt, R., and K. Cummins (1996) An introduction to the aquatic insects of North America. 3rd edition. Kendall/Hunt Publishing Company, Dubuque, IA.

Molles, MC, Jr., AA Sher (2019) Ecology Concepts and Applications. McGraw Hill Higher Education. 592 pages.

Peterson, BJ, B Fry (1987) Stable isotopes in ecological studies. *Ann. Rev. Ecol. Syst.* 18:293-320.

Wanamaker, AD, Jr., KJ Kreutz, HW Borns Jr., DS Introne, S Feindel, S Funder, PD Rawson, BJ Barber (2007) Experimental determination of salinity, temperature, growth, and metabolic effects on shell isotope chemistry of *Mytilus edulis* collected from Maine and Greenland. *Paleoceanography* 22:PA2217

Biology 302: Ecology Laboratory
Stable Isotopes: Sample Preparation in the Laboratory

In today's laboratory exercise, we will be preparing the samples we obtained last week for stable isotopes analysis.

IT IS IMPORTANT TO WEAR GLOVES AND NEVER TOUCH THESE SAMPLES OR ANY LAB SUPPLIES WITH YOUR BARE HANDS. Oils from your hands could contaminate the samples, and result in skewed values.

Sorting

You already sorted samples by trophic level as best as you could in the field, but it is critically important to the success of this study to ensure that samples are isolated from those of other trophic levels. This will require picking through samples under a dissecting scope and making accurate identifications.

Using plastic forceps and weigh boats (do not touch either the sample or the boats bare-handed), sort samples into the following categories:

- Detritus (sticks, leaves, etc.)
- Algae (will likely have diatoms attached, look under scope to determine)
- Midges
- Mayflies
- Stoneflies
- Mysis shrimp
- Fish fin clips

Not all groups will have representatives of all these categories, but separate what you did get into these categories. Our goal is to include 0.5-1.0 g of dried tissue, after washing and drying (about the size of a painted lady butterfly wing), so it is important to get as much of some of these samples (e.g., midges) as possible.

LABEL EACH SAMPLE AS INSTRUCTED!!
A proper label should include a group number, a trophic level, and a sample number. These will be assigned in lab. Write these on the weigh boats you sort samples onto using a sharpie, and again on lab tape that you will attach to the glass Petri dishes when you wash the samples.

Washing

THE WASHING STEPS MUST BE CONDUCTED UNDER A FUME HOOD!! DO NOT REMOVE THE CHLOROFORM:METHANOL SOLUTION FROM THE HOOD UNDER ANY CIRCUMSTANCES. BREATHING CLOROFORM FUMES CAN CAUSE UNCONSCIOUSNESS (OR EVEN DEATH).

GLOVES AND SAFETY GOGGLES ARE NECESSARY.

NOTE: The chloroform:methanol wash solution will MELT PLASTIC. Do not use plastic tools of any kind. It will also remove Sharpie and dissolve lab tape adhesive. Be very careful not to spill the solution!

- Place samples into glass Petri dishes, according to trophic level. Petri dishes will be marked into quadrants, and each group will be responsible for ensuring that their sample is placed into one of these and labelled appropriately, taking care to only place samples belonging to the same trophic level onto the same Petri dish (i.e., only midges go with midges, only algae goes with algae, etc.). Write your sample ID numbers on lab tape and attach them to each of your quadrants ON THE OUTSIDE OF THE PETRI DISH. **If you have questions, ask! We must be able to track our samples through this process.**
- Wash samples in a 2:1 chloroform:methanol solution – Transfer 10 ml of this solution into the glass Petri dish containing the sample using a glass transfer pipette.
- Using metal tweezers, gently agitate the samples for 30 seconds to remove lipids and other surface contaminants (e.g., dirt, pollen) according to previously published methods (Wassenaar and Hobson 1998). (Plastic tweezers will melt onto the samples and ruin them.)
- Using a glass transfer pipette, remove the wash solution and pipette it into the waste container in the fume hood.
- Using a CLEAN glass transfer pipette, repeat the washing steps twice more, gently agitating the sample for 30 seconds each time.
- Dispose of as much of the cleaning solution as you can pipette off the samples in the waste container in the fume hood after each wash.
- Dispose of all used glass pipettes in the broken glass waste.

Drying

All samples must air dry on glass Petri dishes in the fume hood (a dust free environment) for a minimum of three days.

Once dry, samples will be transferred by your instructor to a low-temperature drying oven, where they will be dried for 24-48 hours at 50-60°C. Hence, the reason why properly labelling your samples is so important.

Dried samples will then be transferred to 1-dram glass vials and shipped to the Stable Isotope Paleo Environments Research Group (SIPERG) Laboratory at Iowa State University for further processing. Metal spatulas will be used to scrape samples into the glass vials, and these must be washed with ethanol and dried with a kim-wipe between samples.

ISU SIPERG Laboratory

Once received, samples will be placed into tin capsules and immediately sealed. Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotopes analyses will be conducted on a Costech EA attached to a Finnigan MAT Delta Plus SL. $\delta^{13}C$ will be corrected to a Vienna Pee Dee Belemnite (VPDB) standard, and $\delta^{15}N$ will be corrected to an air standard.

Statistical Analysis

Statistical analyses and graphing will be conducted in R v3.2.2 (R Core Team 2015) using packages ggplot2 v.2.1.0 (Wickham 2009) and mgcv v.1.8 (Wood 2006).

Literature Cited

R Core Team. 2015. R: A language and environment for statistical computing.

Wassenaar, L. I., and K. A. Hobson. 1998. Natal Origins of Migratory Monarch Butterflies At Wintering Colonies in Mexico : New Isotopic Evidence. PNAS **95**: 4.

Wickham, H. 2009. ggplot2: elegant graphics for data analysis. New York, NY: Springer-Verlag.

Wood, S. N. 2006. Generalized Additive Models: An Introduction with R, C. and Hall [ed.]. CRC.

Using light stable isotopes to assess stream food web ecology in a general ecology laboratory course

Hannah M. Carroll[a], Derek D. Houston[b], Suzanne Ankerstjerne[c], and Alan D. Wanamaker, Jr.[c]

[a] Department of Ecology Evolution and Organismal Biology, Iowa State University, Ames, IA, USA; [b] Department of Natural and Environmental Sciences, Western Colorado University, Gunnison, CO, USA; [c] Department of Geological and Atmospheric Sciences, Iowa State University, Ames, IA, USA

Supplementary Information: List of Supplies and Budget Information

Field Sampling

- Waders (or Hip Boots)
- Kick Nets
- Drift Nets
- Plankton Tow Net
- Disposable Plastic Forceps
- Polyethylene Larval Sorting Trays (9 ¾ x 7 ¾ x 1 ½")
- Ziploc Sandwich Bags (or Whirl-Pak bags)
- Permanent Markers
- Angling Equipment (students' personal gear; valid fishing licenses; beach seines and/or minnow traps would be acceptable substitutes in some systems)
- Scissors or a One-hole Paper Punch (for taking fin clips)

Laboratory

- Stainless Steel Featherweight Entomology Forceps (disposable plastic forceps will melt in the wash solution)
- Plastic Sorting Trays
- Disposable Glass Pasteur Pipettes (2 mL capacity)
- Natural Rubber Dropper Bulbs (2 mL capacity; for use with disposable pipettes)
- Kim-wipes
- Nitrile Gloves (all sizes)
- Safety Goggles
- Stainless Steel Micro Scoop Laboratory Spatulas
- Plastic Square Weigh Boats (Medium)
- Glass Petri Dishes (60 mm; but other sizes would suffice)
- 2:1 Chloroform:Methanol Wash Solution (use 95% CH_3OH; keep in 1 L glass bottle in fume hood)
- 1 L Glass Bottle (waste container for wash solution disposal; keep in fume hood)
- Broken Glass Waste Container (for used pipettes)
- Standard Fume Hood
- 1 Dram Glass Vials (w/cap)
- Filter Paper (optional, if plankton tows conducted)
- Funnel (optional, if plankton tows conducted)
- Glass Erlenmeyer Filtering Flask (optional, for vacuum filtration of plankton samples)
- Low Temperature Drying Oven

Budget (Prices Paid Spring Semester, 2019)

Item	# Purchased	Price
Plastic Forceps (Disposable)[e]	100	$19.49
Stainless Steel Featherweight Forceps	6 @ $6.99 ea.	$41.94
Glass Pasteur Pipettes (2 mL capacity)[e]	250	$24.33
Natural Rubber Dropper Bulbs (2 mL)[e]	72	$24.99
Kim-wipe [e]	6 packs of 280 11x21 cm wipes	$19.30
Nitrile Gloves (S, M, L, XL)[e]	100 count box @ $8.95-$9.95 ea.	$37.80
Safety Goggles[r]	6 pairs @ $9.85 ea.	$59.10
Plastic Weight Boats (M)[e]	500	$17.49
1-dram Glass Vials (w/caps)[e]	144	$34.99
Glass Petri Dishes (60 x 15 mm)[r]	6 orders of 5 @ $13.79 ea.	$82.74
Ziploc Sandwich Bags[e]	152 count	$4.66
Stainless Steel Micro Scoop Lab Spatulas[r]	6 pack @ $6.99	$6.99
Filter Paper (12.5 cm)[e]	100 pack @ $10.49	$10.49
Funnel[r]	3 @ $9.75 ea.	$29.25
ISU SIPERG Stable Isotopes Analyzed	19 samples @ $15.00 ea.	$285.00
WCU Van Rental Fees (2 15-passenger vans)	124 miles (62 ea.) @ $0.53/mile	$65.72
Items Already Available For Use at WCU		
Waders[r]	N/A	--
Kick Nets[r]	N/A	--
Drift Nets[r]	N/A	--
Plankton Tow Net[r]	N/A	--
Polyethylene Sorting Trays[r]	N/A	--
Scissors (Stainless Steel)[r]	N/A	--
Fume Hood[r]	N/A	--
Low Temperature Drying Oven[r]	N/A	--
Broken Glass Waste Container	N/A	--
2 1-L Glass Bottles	N/A	--
Chloroform:Methanol Solution	N/A	--
Sharpie Permanent Markers[r]	N/A	--
Angling Equipment	N/A	--
Glass Erlenmeyer Filtering Flask[r]	N/A	--
TOTAL BUDGET		**$744.79**

[e] Extra materials left over for use in future laboratory exercises

[r] Reusable materials that will not need to be reordered for future laboratory exercises unless they are broken or otherwise damaged beyond use

Software Helpfile

Hannah M Carroll

September 12, 2019

Supplementary Information - Software Helpfile

The authors claim no affiliation with or authorship of any software package used in these exercises. The information below is provided solely to assist those new to teaching in R/RStudio.

Software Requirements

Introductory exercise:

R Version 3.2.2 or later (3.5.1 or later preferred)
RStudio Version 1.1.456 or later

R packages
readxl 1.1.0
ggplot2 3.2.0
cluster 2.0.7.1
factoextra 1.0.5
tRophicPosition 0.7.7
viridis 0.5.1

Advanced exercise:

R Version 3.5.1 or later
RStudio Version 1.1.456 or later
JAGS version 4.3.0 or later

R packages
rjags 4.8
simmr 0.4.1
dplyr 0.8.2
ggplot2 3.2.0

- Note: Some versions of R may produce an error during package installation stating that the package rtools must be installed. This message can be ignored.

Installation

Each exercise contains code to install the R packages for that exercise. This may take a substantial amount of time. In particular, dplyr installation can take up to an hour or more on slow connections. If this is a concern, please ensure that dplyr has been pre-installed on each machine on which these exercises are to be used. The code will check for and skip installation of any packages which are already installed.

JAGS vs rjags

JAGS (Just Another Gibbs Sampler) is a standalone software program which was developed to analyze Bayesian hierarchical models using Markov Chain Monte Carlo (MCMC) simulation. It must be installed on each computer on which the *advanced* exercise will be used. It is not needed for the introductory exercise. The R package rjags is your interface to JAGS. Package rjags solves isotope mixing models using the framework provided by JAGS. The exercise has been tested on JAGS version 4.3.0 using rjags version 4.8. Find the JAGS readme at http://mcmc-jags.sourceforge.net/ and rjags documentation at https://cran.r-project.org/web/packages/rjags/rjags.pdf

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Contact information

Bug reports should be submitted to Hannah Carroll via e-mail at carroll.hannah.m@gmail.com or via a GitHub issue report at the project repository: https://github.com/hannahcarroll/Aquatic-isotopes