INNOVATIONS

Using Stable Isotopes of Carbon and Nitrogen to Evaluate Trophic Interactions in Aquatic Environments

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Abstract: This paper describes a series of laboratory exercises for upper level biology courses, independent research and/or honors programs. Students sampled fish from a local water body with the assistance of a local fish and wildlife agency. Tissue samples from collected fish were utilized to obtain estimates of the stable isotopes $\delta^{13}C$ and $\delta^{15}N$. An open-source mass balance model was utilized to estimate fish diets from isotope values. Isotopes were also used to measure trophic position (TP) and evaluate dietary niche overlap between native and introduced species. It was found that $\delta^{15}N$ concentrations increased with each trophic level starting with benthic algae (lowest) to omnivores, then primary carnivores and ultimately piscivores (highest). Estimates of $\delta^{13}C$ suggested all collected taxa utilized littoral habitats for feeding purposes. Native chain pickerel (*Esox niger*) had the highest $\delta^{15}N$ and TP estimates at 14.4 and 4.11, respectively. Nonnative largemouth bass (*Micropterus salmoides*) $\delta^{15}N$ and TP estimates were 13.56 and 3.86, respectively. Model estimates suggested that chain pickerel and largemouth bass diets were partitioned: pickerel consumed only fish and bass consumed principally invertebrates with some intermittent fish contributions. This lab helped integrate multiple disciplines, provide practical experience, and encourage critical-thinking skills while educating students about trophic ecology.

Key Words: Stable isotopes, trophic position, dietary niche, nonnative species

INTRODUCTION

Current pedagogical theory suggests an active-inquiry based approach to teaching can provide students with essential skills such as observation, hypothesizing, experimentation, analysis and synthesis (National Science Foundation, 1996; National Research Council, 2000). Often, inquiry-based laboratory exercises are theory-based experimentation, rather than practical application of scientific understanding. Many students interested in environmental biology disciplines will eventually accept applied field-based positions where descriptive research is most commonly used. In field-based disciplines such as ecology, conservation biology, environmental biology, limnology, fisheries, and marine and wildlife biology, students often graduate deficient in necessary skills needed to compete in their fields (Millenbah and Millspaugh, 2003). These skills could include sampling techniques, data management, data analysis, statistics, oral and written communication mastery, inquiry and critical thinking. Particularly, the ability to integrate these skills into a single approach has been cited as a major shortcoming in contemporary college graduates (Millenbah and Millspaugh, 2003). A balance between experimental inquiry-based learning and applied/descriptive-based approaches should be considered to provide students more experiences appropriate to their future careers. In particular, an integration of multiple disciplines into one project, as would be common in real-life working scenarios, should be encouraged.

In this paper we describe a series of laboratory exercises for upper-level biology, independent research and/or honors courses. The exercise involves students collecting fish taxa from a local pond, fixing tissue samples, managing large data sets, and reconstructing the local food web through the utilization of carbon and nitrogen isotopes and an open-source isotope model. Students are allowed to focus on particular components of the food web such as trophic structure, feeding behavior, resource partitioning and the potential for competition. At the end of the lab sequence students are familiar with common fish sampling methods, fish identification and ecology, the chemistry of carbon and nitrogen isotopes in the environment, data management, basic statistics, ecological modeling, and applied fishery biology. Students also prepare a term paper regarding their research and present it orally to their class. Methods presented here can be adapted to local conditions, budgets and other limitations. The concepts presented here can also be applied to other

aquatic species as well as to organisms in terrestrial environments.

Isotope Ecology

Students were first educated on the concepts of food web and isotope ecology. Ratios of the stable isotopes of carbon 13/12 and nitrogen 15/14 can be used to estimate organism diet and trophic position (Cabana and Rasmussen, 1996; Vander Zanden and Rasmussen, 1999; Vander Zanden et al., 1999; Vander Zanden et al., 2000; Fry 2006). These isotopes exist in the environment at relatively predictable ratios: ^{12}C exists at about 98.9% while ^{13}C is approximately 1.1% and ^{14}N is 99.6% while ^{15}N is about 0.4% (Fry 2006). The lighter isotope ^{14}N is more readily processed through organic tissue while the heavier isotope ^{15}N tends to bio-accumulate (Vander Zanden and Rasmussen, 1999). When compared to a two-point normalized standard curve, $\delta^{13}C/\delta^{12}C$ in muscle tissue is similar to that of the prey consumed. The ratio of $\delta^{15}N/\delta^{14}N$ becomes enriched with $\delta^{15}N$ from prey to predator at a rate of approximately 3-4‰ (DeNiro and Epstein, 1981; Cabana and Rasmussen, 1994; Vander Zanden and Rasmussen, 1999). Since these ratios represent carbon and nitrogen accumulation in muscle tissue, $\delta^{13}C/\delta^{12}C$ and $\delta^{15}N/\delta^{14}N$ represent a time-integrated estimate of organism diet (Fry 2006; Christensen and Moore, 2009). Plants and algae fractionate carbon differently from each other, and are expressed through the food web differently, indicating various potential sources of carbon and spatial feeding locations of predators (Vander Zanden and Rasmussen, 1999). For example, pelagic algae (phytoplankton) and benthic algae (periphyton) in lakes fractionate carbon differently, leading to spatial food web differentiation in lakes (Vander Zanden and Rasmussen, 1999). However, nitrogen fractionation is relatively constant and indicates trophic position (Vander Zanden and Rasmussen, 1999). Therefore, when used together, nitrogen and carbon isotopes can give a time-integrated, spatial and trophic estimate of feeding behavior, energy flow and food web dynamics. A fish that is $\delta^{13}C$ depleted in a lake, for example, would likely be feeding in pelagic (open water) while a $\delta^{13}C$ enriched fish would likely be feeding in littoral (near shore) habitats (Vander Zanden and Rasmussen, 1999). A fish that is $\delta^{15}N$ enriched is likely a top-predator, consuming other fish species while a fish with low $\delta^{15}N$ is likely consuming zooplankton or is an omnivore (Vander Zanden and Rasmussen, 1999).

A number of mass-balance isotope mixing models have been developed to assist researchers in estimating potential diet sources using stable isotopes. We used an open-source model provided by the Environmental Protection Agency (EPA) (Phillips and Gregg, 2003). Values of $\delta^{13}C$ and $\delta^{15}N$ were inputted for both predator and potential prey sources. The model then used an algorithmic approach to estimate a range of diet possibilities that sum to the isotopic signature of the predator. The model can estimate a range (0-100%) of diet possibilities for up to 10 prey sources for each predator. However, previous knowledge of potential diet possibilities must exist so inappropriate comparisons are not made.

METHODS

This exercise required four 3-hour lab periods to complete. However, the labs can be adjusted to meet faculty and student needs or any possible limitations. Students were first given material to read regarding the ecology of the common fish species found in Pequot Pond, Westfield, MA. They also read material regarding lake and fishery management, invasive species and food web dynamics. Students were then encouraged to make well defined objectives. Some objectives included: create an isotopic map depicting possible feeding relationships among fish species, determine the trophic position of fish and invertebrates, use the multiple-source mixing model to estimate diets of largemouth bass (*Micropterus salmoides*) and chain pickerel (*Esox niger*), measure the level of resource partitioning between largemouth bass (an introduced species) and chain pickerel (a native species).

Lab One: Fish Sampling

During lab one, students collected fish taxa from Pequot Pond with the Massachusetts Department of Fish and Wildlife (MDFW). Arrangements were made with MDFW several weeks in advance. Fish were collected using an electrofishing john boat fitted with a 5000 watt generator. Electricity was applied to the water causing galvanotaxis (forced swimming) in fish. Students then netted the fish as they swam towards the boat. Because we are a small teaching university, funds were not available for expensive field sampling equipment such as electrofishing boats. However, state fish and wildlife agencies, if asked, are often willing to assist colleges and universities with field trips. This provided students with exposure to working professionals and real-world sampling techniques. Also, the MDFW utilized their own collection permits and sampling authority. If we had conducted the sampling ourselves, we would have been required to apply for a collection permit through the MDFW well in advance. Since fish are vertebrates, it was necessary to request authorization through our institution's animal care-use committee.

Other sampling methodologies could also be applied that would not require the assistance of fish and wildlife agencies, as long as the proper permits were acquired. These methods include gillnets, beach seines, cast nets and/or minnow traps. Each of these methods can be effective for a variety of near shore (littoral) lake, river and stream fish species. It may also be possible to focus on macroinvertebrate

collections that include species from different trophic levels and that are spatially distributed throughout a lake or pond. Using macroinvertebrates would eliminate the need for collection permits and IACUC authorization.

Once netted using the electofishing technique, fish were placed in a live-well where they recovered quickly. Four fish of each species and of similar lengths were removed from the live well for further analysis. The rest of the fish were identified by students and released back into the pond.

Fish selected for isotopic analysis were pithed and a small muscle sample the size of a quarter (1-4 g wet-weight) was removed from the left dorsal side of the fish just anterior to the dorsal fin (Christensen and Moore, 2009). These tissue samples were rinsed with distilled water and then placed separately in small plastic vials, labeled and frozen (Christensen and Moore, 2009). Tissue samples were collected within an hour after fish were captured.

Macroinvertebrates were collected throughout the littoral zone of the pond using D-style invertebrate nets. We had four invertebrate sampling locations that included vegetated, organic muck, sand and gravel zones. These locations were indicative of the different habitat types common to the littoral region of Pequot Pond. Sampling effort in each of these regions was approximately 30 minutes. Each macroinvertebrate species was identified and then stored in small labeled plastic vials and frozen. We attempted to collect 1-4 g (wet weight) for each invertebrate species. Due to their small size, multiple invertebrate samples were collected for each vial. The entire invertebrate was used for analysis.

Lab Two: Tissue Preparation

During the next lab, all the fish and macroinvertebrate samples were thawed. All skin from fish was removed while the entire macroinvertebrate was used (Christensen and Moore 2009). Samples were then re-rinsed and placed in a drying oven for 48 hr at 75°C (Christensen and Moore, 2009). Once the samples were dried, a mortar and pestle were used to homogenize each individual sample separately until it reached a floury consistency. Due to their small size, macroinvertebrates were pooled to ensure adequate dry weight (at least 1 mg). Samples were labeled in small air tight vials and shipped to the Washington State University (WSU), Biology Department, Isotope Core Laboratory for processing. We contacted the WSU Isotope Core Laboratory at the beginning of the semester to set up a billing account and then contacted them again several days in advance before sending the samples.

At the WSU Isotope Core Laboratory stable isotope analysis of ^{13}C and ^{15}N was performed for each sample using a continuous flow isotope ratio mass spectrophotometer (Delta Plus XP, Thermofinnigan, Bremen; Brenna et al., 1997). Delta notation (δ) was utilized to express the deviation of ^{13}C and ^{15}N from a standard material utilized in the lab. The international standards Vienna Pee Dee Belemite (VPDB) for ^{13}C and atmospheric nitrogen for ^{15}N were used. All values were expressed in parts per thousand (‰) utilizing the following equation where R equals $\delta^{13}C/\delta^{12}C$ or $\delta^{15}N/\delta^{14}N$:

$$\delta^{13}C \text{ or } \delta^{15}N = (R_{sample} / R_{standard} - 1) * 1000$$

The WSU Isotope Core Laboratory performed all of the analysis and sent a Microsoft Excel file with the results. The file was easy to understand and manage. The cost per sample, which included both $\delta^{13}C$ and $\delta^{15}N$ together, was $8.20 and the delay from shipping to return was about three weeks. While we waited for the samples to be returned, students worked on other, non-related exercises regarding aquatic biology. This time could also be utilized to further educate students about isotopic analysis and trophic ecology. Due to a limited budget we pooled fish and invertebrate samples of the same species. Although this is a fundamental problem in scientific investigation because no standard error could be determined and any useful statistical analysis was greatly reduced, the results still provided students with a useful picture of trophic interaction. A laboratory fee or allotted departmental budget could eliminate this shortcoming and pay for more isotope lab analyses.

Labs Three and Four: Data Analysis and Modeling

The trophic position was calculated for each fish species and invertebrate species sampled following the method described in Table 1 (Vander Zanden et al., 2000). This method is unique in that it not only determines the appropriate trophic level, but also considers variation within each level (Vander Zanden et al., 2000).

We used the model IsoSource (Phillips and Gregg, 2003) to determine the range of possible diet proportions (0-100%) from our data (Figure 1). IsoSource is an algorithmic mass-balance model that uses the mixture of prey (source) isotopic signatures to determine all possible diet combinations that sum to the isotopic signature of the predator (consumer). Trophic fractionation needs to be accounted for by subtracting 3-4‰ from the $\delta^{15}N$ of the predator (Phillips and Gregg, 2003). The output is simply an

Table 1. Formulae used in the calculation of the trophic position with largemouth bass and chain pickerel as examples (Vander Zanden, 1999). The $\delta^{15}N$ baseline was the lowest recorded primary heterotroph in our samples, which was the common freshwater snail ($\delta^{15}N$ = 7.24 ‰).

$$TP_{fish} = [(\delta^{15}N_{fish} - \delta^{15}N_{baseline})/3.4] + 2$$

$$TP_{largemouth\ bass} = [(13.56 - 7.24)/3.4] + 2 = 3.86$$

$$TP_{chain\ pickerel} = [(14.4 - 7.24)/3.4] + 2 = 4.11$$

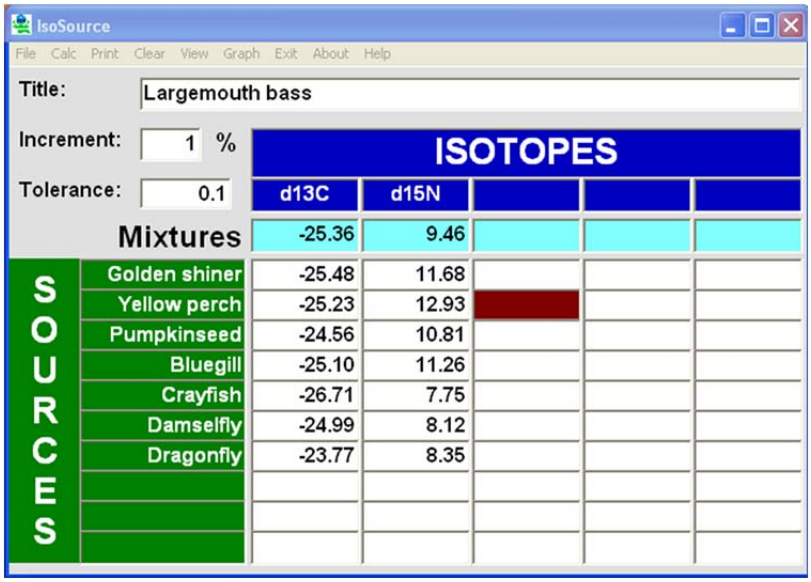

Fig. 1. Example of the multiple source mixing model IsoSource. The display illustrates potential sources (prey) of largemouth bass in Pequot Pond, MA. The mixtures represent the $\delta^{13}C$ and adjusted $\delta^{15}N$ of the largemouth bass. The $\delta^{15}N$ was adjusted down by 3.5 ‰ to account for fractionation (Phillips and Gregg 2003). The range of results was presented in 1% increments with an error tolerance of 10%, the minimum allowed

estimate and the entire range of possibilities should be presented since multiple combinations may have the same probability of occurrence (Phillips and Gregg, 2003). The model is open-sourced and can be accessed through the Environmental Protection Agency website (U.S. EPA, 2012).

We plotted the adjusted predator isotopic signature and the signatures of all possible prey. Since trophic fractionation was subtracted from the predator, all valid prey signatures should be located around the predator (Phillips and Gregg, 2003). We then created mixing-polygons around the predator to indicate all possible prey sources. All the sources should be within the polygon. When the predator signature is nearest the line connecting two prey sources a "constrained solution" exists (Phillips and Gregg, 2003). IsoSource output will display a bell-shaped curve indicating that the two prey sources contribute most to the predator diet. A predator signature more toward the middle of the polygon suggests a "diffuse" contribution of prey (Phillips and Gregg, 2003). Model output for a diffuse solution would consist of incomplete curves. Because muscle turnover rates in most fishes can be relatively slow, isotopic estimates were indicative of late summer feeding behavior (MacAvoy et al., 2001).

RESULTS AND DISCUSSION

Students analyzed data based on their defined objectives. For example, trophic and spatial position was determined for all sampled fish and invertebrate species (Table 2 and Figure 2). We found in Pequot Pond that chain pickerel had the highest trophic position estimate, suggesting a piscivorous (eats other

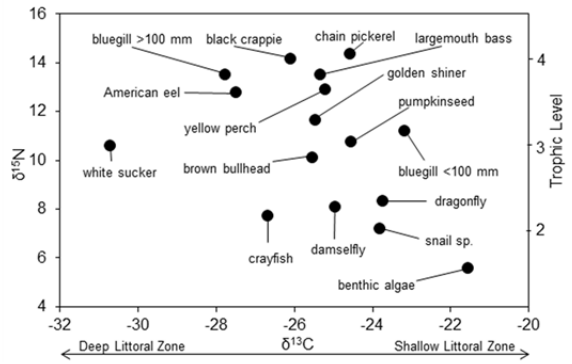

Fig. 2. Late summer isotopic signatures and trophic position of fishes, invertebrates and benthic algae in Pequot Pond, MA. Stable isotope estimates for bluegill > 100mm were unusually high suggesting sample contamination.

fish) diet. The brown bullhead (*Ameiurus nebulosus*) and white sucker (*Catostomus commersonii*) had the lowest trophic position estimates, suggesting omnivory. The white sucker, bluegill (*Lepomis macrochirus*) and American eel (*Anguilla rostrata*) had depleted $\delta^{13}C$ values, indicating deeper littoral habitat utilization than other fish species. Invertebrates all had lower trophic positions than fish. The common snail was the lowest recorded heterotroph. Benthic algae, the base of the food web had the lowest $\delta^{15}N$ estimate.

IsoSource model outputs suggested some diet overlap between introduced largemouth bass and native chain pickerel. However, there appeared to be some distinct diet partitioning between the two species. Crayfish were the principal diet constituent for largemouth bass while chain pickerel diet was primarily pumpkinseed (*Lepomis gibbosus*) (Figures 3 and 4). These results suggested resource partitioning between a native and introduced predatory fish in Pequot Pond. Largemouth bass diets also consisted of more invertebrates while chain pickerel diet estimates were entirely other fish species. These estimates are consistent with current scientific understanding for these two species (Hartel et al., 2002; Wydoski and Whitney, 2003).

Students could also use these data to explore management implications of nonnative species, quantify energy flow through food webs, and predict potential shifts due to disturbances. Hypotheses for future research could also be explored. Students used these data and results to complete a paper and oral presentation that explored potential impacts of nonnative fish introductions to a local food web. Students further hypothesized regarding management techniques that could minimize nonnative fish influence on food web dynamics.

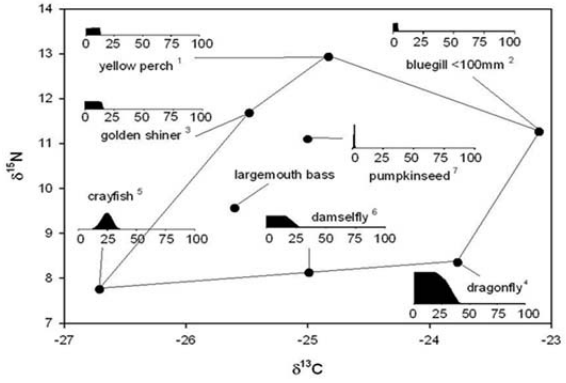

Fig. 3. Mixing polygon that depicts the most probable diet composition of largemouth bass based on outputs from the model IsoSource. Bell-shaped curves depict the most probable diet contribution while incomplete curves represent a more intermittent diet contribution. Diet contributions were: 1. yellow perch 0-16%; 2. bluegill <100mm 0-2%; 3. golden shiner 0-12% ; 4. dragonfly 0-43%; 5. crayfish 13-35%; 6. damselfly 0-27%; 7. pumpkinseed 0-5%. Largemouth bass $\delta^{15}N$ signatures were adjusted down by 3.5 ‰ to account for trophic fractionation (Phillips and Gregg 2003).

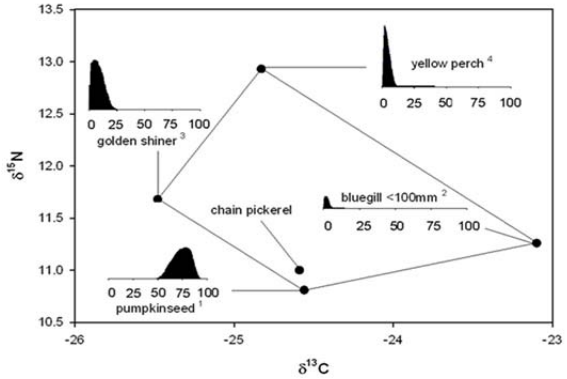

Fig. 4. Mixing polygon that depicts the most probable diet composition of chain pickerel based on outputs from the model IsoSource. Bell-shaped curves depict the most probable diet contribution while incomplete curves represent a more intermittent diet contribution. Diet contributions were: 1. pumpkinseed 48-96%; 2. bluegill <100mm 0-15%; 3. golden shiner 0-25%; 4. yellow perch 0-41%. Chain pickerel $\delta^{15}N$ signatures were adjusted down by 3.5 ‰ to account for trophic fractionation (Phillips and Gregg 2003).

Working with MDFW personnel was particularly rewarding in that several students made contacts that may become beneficial in their careers. Also, interacting with working professionals gave students an opportunity to learn about the practical, real-world perspective of biological work. After completing the lab series students felt confident they could develop similar projects that would address food web dynamics in other systems. In particular, students developed an appreciation of the multi-disciplinary approach often used in biology and the importance of critical thinking skills.

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