INQUIRY & INVESTIGATION

Using Environmental DNA to Connect Lab Science with Field Practice

NABT BioClub RECOMMENDATION

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Abstract

Experiential learning helps students make connections between different skill sets and allows them to engage in a deeper level of inquiry. To enhance the connection between field and laboratory practice for undergraduate students in our wildlife ecology curriculum, we developed an exercise using environmental DNA (eDNA) analysis. eDNA sampling involves extracting and amplifying the DNA from specific organisms from an environmental sample, rather than from the organisms themselves, and has been rapidly adopted by conservation practitioners around the world. In our activity, students collect water samples from a local pond and process them to detect the presence of American bullfrogs. Practicing this procedure not only introduces them to professional skills they may utilize in their careers, but also helps create context for how laboratory science and field work support each other and can be used to connect to larger issues of conservation, environmental studies, or ecology.

Key Words: *eDNA; amphibians; biodiversity sampling; experiential learning.*

Introduction

It is a common refrain in many publications about undergraduate biology education that hands-on and experiential class and laboratory exercises benefit student learning and lead to increased science literacy (Beck & Blummer, 2012; Brownell & Kloser, 2015). The most recent report from the AAAS *Vision and Change* program highlights the successes of incorporating case studies and realistic scenarios into undergraduate courses to provide a more authentic experience of a practicing professional scientist (AAAS, 2015). Additionally, the report calls on instructors to engage in activities that cross fields of inquiry, allowing students to experience the interdisciplinary nature of science.

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Our programming in the Smithsonian-Mason Semester for Conservation Studies (http://smconservation.gmu.edu) is based on this tenet that the field of environmental conservation should be taught as it is practiced, namely, as an integration of many disciplines working together to solve urgent conservation issues. Additionally, we use extensive hands-on learning activities that give students the opportunity to practice professional skills with the tools they will be using in their careers.

We offer several programs of study, but one of the clearest examples of this mission comes from our wildlife ecology curriculum. This curriculum focuses on how ecological theory informs conservation and management practice, and as such there is a strong emphasis on methods of monitoring species or populations from different taxa using traditional field biology methods (e.g., visual encounter surveys, live trapping, camera trapping). Many of our students enter the program looking for experience with these field techniques, having come through undergraduate biology courses that focus mostly on theory or laboratory skills. Most have not made the connection between field and lab studies, and how the two aspects of scientific inquiry are not mutually exclusive and are often both necessary to fully describe a system or address an environmental issue. To explicitly illustrate the connection between the two approaches, we developed a unit on the use of molecular tools to monitor species in the wild, specifically the application of environmental DNA (eDNA) analysis for monitoring aquatic species.

Environmental DNA is a relatively new technique that has found widespread adoption among conservation professionals, even those who do not have a lot of experience with molecular techniques (Lodge et al., 2012). The technique involves extracting DNA from an environmental sample, usually water or soil, and identifying which organisms have been present in the medium by probing the isolated DNA with species-specific

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primers. The technique has enjoyed rapid acceptance from practitioners, who find it more cost effective and accurate than traditional techniques for many cryptic or rare aquatic organisms.

○ Methodology

There are still questions, though, surrounding the interaction between environmental factors and detection, including the longevity of DNA in the environment and how small-scale habitat differences affect detection.

Our protocols for the activity are derived from one of the first published accounts of using eDNA for species monitoring, which used American bullfrogs (*Lithobates catesbeianus*) as a test animal (Ficetola et al., 2008). Bullfrogs are widely distributed, and their obligate association with water throughout their life cycle makes them ideal candidates for eDNA sampling since, if they are present and abundant, their DNA signature will be robust and more detectable in the water body. When we first began using this activity, our goal was merely to see if we could detect the presence or absence of bullfrogs within a local pond using eDNA procedures. In three years of investigation, though, we have observed that detection is lowest near where a stream flows into the pond and highest near an outflow from the pond (Figure 1).

The emergence of this pattern has given us an opportunity to frame the activity in the context of a long-term study to which students contribute data every semester and explore the relationship between field and lab techniques and how they support each other. We specifically test the descriptive hypothesis that we will detect bullfrogs with eDNA sampling at locations near the pond outflow more readily than at the inflow. Our learning objectives for the activity are that students will be able to independently implement both traditional field sampling techniques for amphibians and eDNA sampling, describe the relative advantages and disadvantages of both types of sampling, analyze and troubleshoot molecular data and procedures, and justify the use of particular sampling techniques in specific circumstances based on the data the class collects and the larger, long-term data set.

The entire eDNA activity spans 2.5–3 days and begins with field exercises. We bring the students to the field site (Figure 1), and introduce and demonstrate traditional field sampling techniques for amphibians, including drift fences, minnow traps, and visual encounter surveys (Figure 2). We set up the drift fence in the field, demonstrating that the fence should be oriented parallel to the bank of the pond to intercept any animals attempting to enter the pond (Figure 2A). The day before the visit to the field site, faculty set up a minnow trap for demonstration purposes that is retrieved in the field (Figure 2B). Any captured organisms are observed in a pan with water from the pond before being returned to the water. The use of a net to sample aquatic vegetation is demonstrated as a method of visual encounter survey (Figure 2C).

Following these demonstrations by faculty, students are given the coordinates of an assigned sampling station (Figure 1), a GPS unit, a net, and a pan. They navigate to their station and conduct a visual encounter survey of the aquatic vegetation at that site by taking three samples with the net, and observing and recording the number and diversity of fauna they collect. We rarely encounter adult bullfrogs using these methods, but do capture many tadpoles and salamanders. Students do not share equipment between stations to reduce risk of spreading biological material, and we only visit one pond on the field trip. Additionally, all field equipment is rinsed with a 10 percent bleach solution before being stored to reduce the risk of spreading pathogens between sites.

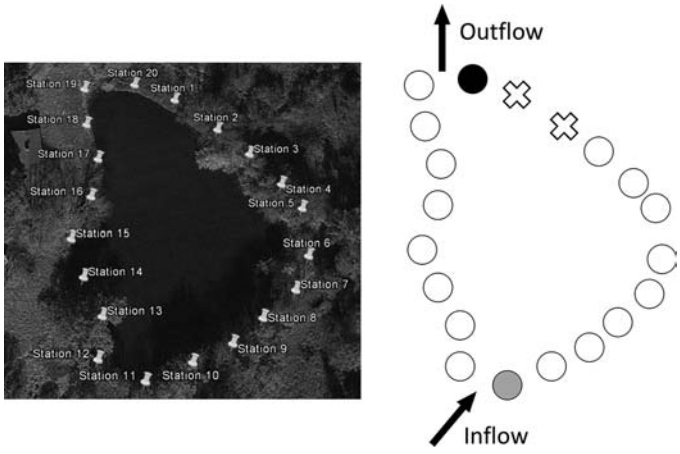

Figure 1. Sampling station locations at our local pond are depicted on the left. There are 20 stations arrayed around the border of the pond, each ~10 m from the next. The figure on the right is the output of a Hot Spot Analysis (Getis-Ord Gi*) run in ArcMap (Environmental Systems Research Institute (ESRI), Release 10.2, Redlands, CA) on bullfrog detection data collected in four semesters from fall 2014, 2015, and 2016, and summer 2016. The black circle indicates a 90% confidence in a higher detection probability than random at that station; the gray circle indicates a 90% confidence in a lower detection probability than random at the station; and the white circles indicate no significant pattern in detection. The black arrows indicate water flow into and out of the pond. There was insufficient data available for stations 1 and 2 in the analysis (white Xs).

Figure 2. Examples of traditional field sampling techniques for amphibians. Drift fence **(A)** and minnow trap **(B)** deployment are demonstrated by faculty. Students conduct visual encounter surveys using samples collected via nets from aquatic vegetation **(C)**.

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The day after sampling the pond using traditional field methods, we return and collect water samples to process using eDNA techniques. Each student is given a GPS, a plastic graduated cylinder, a 50 mL centrifuge tube containing a solution of 1.5 mL 3M sodium acetate (ThermoFisher Scientific, #S210) and 20 mL of 100 percent ethanol (Pharmco-Aaper, Brookfield, CT; #E200), and a clipboard with a data sheet (Figure 3).

Students navigate to the same pre-determined station they used the day before and collect a 15 mL water sample that they add to the centrifuge tube. The sodium acetate helps protect the DNA from degrading; these samples are stable at room temperature for several hours or even days. On the data sheet, students note habitat features in the area near where they collected the sample. Each student has their own equipment to reduce the risk of mixing DNA between stations.

After collecting the samples, we return to the lab and extract the DNA from the water using the commercially available QIAGEN DNA mini kit (#51304, QIAGEN, Valencia, CA) used in many research laboratories. After mixing the samples by inverting the 50 mL centrifuge tubes, students pull out a 200 µL subsample, which is then processed using the extraction procedure provided in the kit. Even though a very small volume of sample is used in the lab procedure, it is important to collect a large volume in the field because the eDNA signature is very dilute. Sample processing amounts to a series of initial reagents and incubations that extract the DNA and inactivate proteins in the sample, and then a series of spins and washes using a benchtop centrifuge to clean contaminants out of the sample and concentrate the DNA, which is stored on ice.

In addition to the chemicals provided in the kit, faculty must provide 100 percent ethanol, deionized water, 2 mL microcentrifuge tubes, micropipettors and tips, a centrifuge able to spin microcentrifuge tubes at 6,000 and 25,000 *g* (8,000–15,000 rpm), a vortex mixer, a digital dry bath incubator able to reach 56°C, a thermocycler, gel rig system, and an imaging system. Throughout these protocols, we highlight that our methodologies are derived from the primary literature (Ficetola et al., 2008), and we are using the same tools that researchers would use. Students must complete lab notes for the procedures they conduct, though we do provide them with the reagents and written instructions provided with the QIAGEN kit, much as if they were graduate students using this procedure for their thesis research (see Online Supplementary Material).

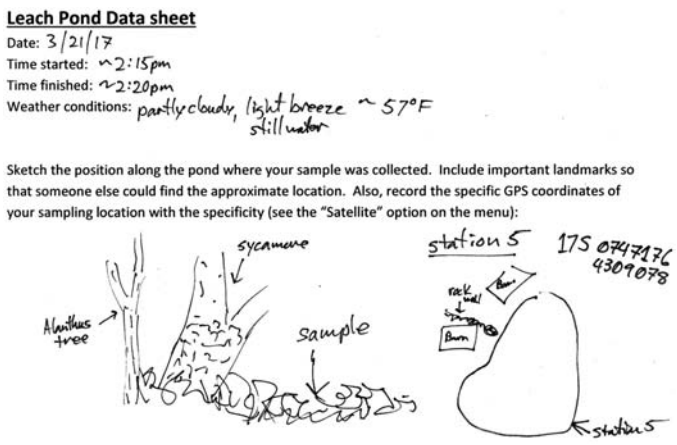

Figure 3. Example of field data sheet.

Once the students isolate the DNA, they amplify sections of DNA specific to our target organism using the polymerase chain reaction (PCR) with primers and protocols outlined in Ficetola et al. (2008). The PCR reaction is standardized to 50 µL. We obtain Express Oligos primers (5′-GCCAACGGAGCATCATTC-3′ and 5′-ATAAAGGTAGGAGCCGTAGT-3′) from Eurofins Genomics (Louisville, KY), without modifications, using salt-free purification and a 10 nmol scale. Each reaction contains 3 µl of each primer, both with a final concentration of 1 µM. We use 25 µL of AmpliTaq Gold Master Mix (#4398876, ThermoFisher Scientific, Waltham, MA) in each reaction, as well as 2 µL of 360 GC Enhancer (#4398876, ThermoFisher Scientific, Waltham, MA, USA) provided with the Master Mix. Students add 17 µL of either DNA sample or deionized water to each reaction, but the Master Mix is the last reagent added to each reaction. Each student prepares four PCR reactions: one negative control without DNA (deionized water instead of sample), one positive control using a sample we know contains bullfrog DNA, and two replicates of their own sample. Immediately after adding the Master Mix, the samples are amplified using a BioRad C1000 Touch Thermal Cycler (Hercules, CA) thermocycler, using the temperature and time parameters described in Ficetola et al. (2008).

We emphasize at this point that we cannot predict the outcome of the PCR. Acknowledging the uncertainty here illustrates the real experience of doing research: these are real samples, so you can prepare and follow plans and protocols, but ultimately cannot control the data. After completing the PCR, we process the samples using gel electrophoresis (#3487–5000, DNA Plus gel system, USA Scientific, Ocala, FL; and #1645050, PowerPac Basic, BioRad) and determine which samples detected bullfrog DNA. We use a 2 percent agarose (#50090, NuSieve 3:1 Agarose, Lonza, Alpharetta, GA) gel with incorporated Gel Red fluorescent DNA stain (#41003, Biotium, Freemont, CA). The samples run on the gel for 1 hr at 95 V, and we visualize the DNA using a Enduro GDS gel documentation system (Labnet, Edison, NJ) UV light source with an integrated camera.

The activity concludes with a discussion of the results and how the class's data compares with that from previous semesters. We address our original hypothesis—eDNA is more frequently detected near the outflow vs. the inflow of the pond—and explore how the current class's data fits into this framework. Students are asked to pose fundamental hypotheses to explain why this pattern exists, such as the influence of flowing water on shaping the habitat in those areas, which could affect the presence of bullfrogs at particular stations. Bullfrogs are algal feeders as tadpoles, so flowing water may remove resources from the inlet and deposit them near the outflow, which could account for the differential detection. By examining whether bullfrog individuals were found at these sites using the traditional techniques, we can assess the likelihood of this scenario. Another alternative explanation is that frogs are present in the inflow, but the movement of water in the area removes the DNA signal, which could also account for the increased detection near the outflow as it accumulates there. On exams following this activity, we ask students to describe how they would test these hypotheses, using both field and lab techniques, to evaluate their understanding of the procedures and how they can be applied.

We also discuss any inconsistencies in the data, and as a class attempt to troubleshoot any unusual findings. Each class contributes to refining the protocol for the next group, just as professionals

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would do in optimizing any protocol. For example, when we first began this activity, we unintentionally delayed putting the samples into the thermocycler after adding the Master Mix, and the samples incubated at room temperature for almost 20 minutes. As a result, our amplifications were a random assortment of different-sized pieces of DNA because of the unregulated amplification occurring outside of the conditions in the thermocycler. Although these results were not what we expected, this circumstance did provide an opportunity to walk through the logic of troubleshooting our protocol and results, and led to a refinement of our procedure, where the Master Mix is always added immediately before the samples are placed in the thermocycler.

Finally, we discuss in class the broader implications and applications of eDNA sampling. As mentioned previously, we rarely capture adult bullfrogs when doing our traditional sampling in the field, even in the summer and fall when they are prevalent, but always find tadpoles. However, green frog (*Lithobates clamitans*) and bullfrog tadpoles can be difficult, if not impossible, to differentiate in the field, and both species are present at our sample site. If green frog primers were to be included in this activity, this could show how eDNA is a simple way to assess whether one or both species are present at these sites, in the absence of a morphological marker for differentiating the tadpoles. We also discuss examples from the scientific literature where eDNA has been applied. For instance, Goldberg et al. (2014) used eDNA sampling to determine the distribution of American bullfrogs at Fort Huachuca, Arizona, where bullfrogs are considered an invasive species. eDNA sampling allowed those scientists to cover a wider area using fewer staff and less time than traditional methods of sampling. As a final evaluation, students must submit a short paper describing how they would apply eDNA to investigate a system or question of their own choosing. They must clearly state the question and hypothesis they would test as well as provide a rough outline of the methodology. They must also justify why a molecular technique like eDNA is more efficient than traditional sampling techniques in their chosen system. This assignment requires outside research on the part of the student to sufficiently describe how they would successfully apply the technique, and students are evaluated based on the thoroughness and specificity of their response.

○ Implications

The actual procedures for processing the samples use robust molecular techniques that are familiar to most biology majors, but the activity applies them in a new context to determine important information about the presence or distribution of species. For students who have a grounding in laboratory science, utilizing this technique helps them build on their experience and connect their findings to field practice as well. In post-course evaluations, students rate this activity very highly in terms of usefulness to their professional development: 4.5 out of 5 on a scale of helpfulness, where 4 is "much help" and 5 is "great help" (n = 44 students over four semesters). Many students express their excitement that the results of this activity are unknown, unlike other labs where the results are predetermined based on the sample you are given. Most importantly, students comment that before doing this activity, they did not realize how techniques learned in a laboratory setting (e.g., DNA extraction and PCR) could be applied to a field question.

Environmental DNA as a sampling tool is still being refined, so there are opportunities for students to be involved in the development of the technique in very real ways. Issues of efficiency of detection, the relationship between population size and eDNA signal detection, and how eDNA spreads in bodies of water are still being actively researched and could be incorporated into curricula at many levels. For example, students could also collect water quality and chemistry measurements when they collect their water samples, and determine whether there are correlations in detection with factors such as temperature, pH, or alkalinity. They could even experimentally manipulate these factors on collected samples back in the lab. They could also work on ways to optimize the sample collection and processing procedure for different species. Bullfrogs are well-studied in this regard, but there are many other aquatic and semi-aquatic amphibians that have not been thoroughly studied, but could benefit from using this type of tool, such as mole salamanders that are only present in water bodies for short periods of time in the spring when they are mating. If eDNA could be successfully applied to detect the presence of these species, it could help us understand their distributions better and possibly detect population declines sooner, in time to take preventative action.

Another benefit to students from this activity is the development of professional skills. Because the activity is structured using the real tools of a practicing molecular biologist, students are able to state on their resumes that they have experience with these particular kits and techniques. For students who have never worked in a laboratory setting, it helps them develop confidence that they need not be expert to be proficient at these skills, and molecular techniques can be integrated into studies in many different areas, such as species monitoring, landscape ecology, wildlife management, or even law enforcement.

Beyond the collection of the data, though, this activity also serves to demonstrate how scientific practice can move forward. By using published protocols and adapting them to our specific needs, students get experience with trouble-shooting standardized procedures in a new circumstance. Also, because our data are collected periodically in the same location over time, we are able to see long-term patterns developing. This emphasizes to students how biodiversity monitoring is an ongoing endeavor that requires persistence and time to detect patterns—a realization that will become important to them as they go into their careers. This activity provides a clear example of how applying different skill sets to problems in conservation can lead to more efficient practice, which ultimately could lead to better solutions for pressing conservation issues.

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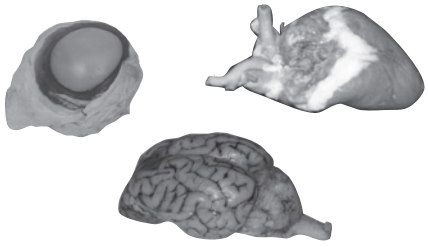

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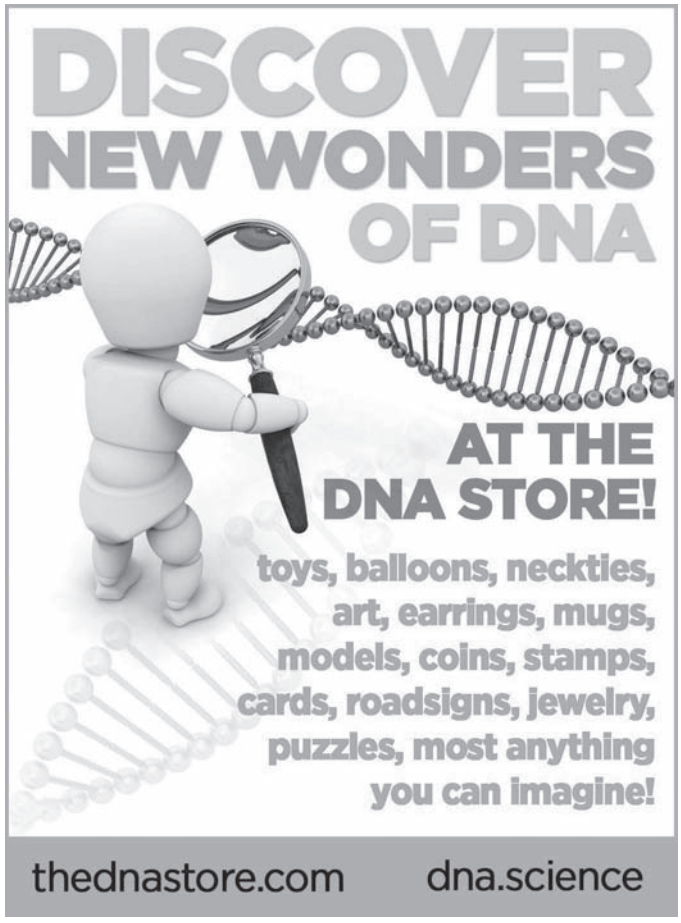

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