Identifying microbes from environmental water samples in a discovery-based learning module

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Abstract

What is the microbe that we are dealing with? Whether it is cholera or anthrax, we want to know the disease-causing microorganism as quickly as possible since prompt identification of the causative organism would help control disease spread - and potentially save lives through provision of appropriate care and medication. But despite the advent of rapid microbial identification tools – particularly those based on mass spectrometry – most undergraduate curricula continue to focus on culture- and nucleic acid-based identification techniques since they are widely used for detecting and identifying microbes in clinical and environmental samples. Mass spectrometry-based methods, however, have increasingly complemented traditional approaches in clinical and research laboratories - but are rarely featured in undergraduate curricula. Motivated by the desire to address the curriculum gap, I developed an inquiry-based laboratory exercise for introducing students to the operating principles and methodology of mass spectrometry-based microbial identification. By requiring students to identify microbes in environmental water samples – a real-life problem with unknown answers – the exercise piqued the students' interest in learning, while helping to stir their curiosity through an interesting field activity where they put on a scientist's hat in solving a mystery. This synopsis article summarizes a piece of published educational research and expands on the discussion of concepts underlying matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)-based microbial identification. For example, the article discusses the relative advantages and disadvantages of the pattern recognition and proteome database search approaches for analyzing mass spectra data. Additionally, the effect of general and tailored sample preparation protocols on identification accuracy is also elaborated. Finally, the pedagogical utility of field- and inquiry-based educational tools is also discussed in greater detail from a post-publication perspective. A full-length synopsis of the work and a structured abstract can be found in the accompanying PDF file, while the original article, "Teaching Microbial Identification with Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) and Bioinformatics Tools," and supplementary material has been published in the *Journal of Microbiology and Biology Education*, Vol. 14, No. 1, pp. 103-

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106, and is available at http://jmbe.asm.org/index.php/jmbe/article/view/494 as an open-access article.

Keywords: ribosomal proteins; biomarker; phylogeny; microbial ecology; taxonomy; educational research; inquiry-based; mass spectrum fingerprinting; microbial typing; scientific method;

Subject areas: microbiology; education; environmental sciences; ecology;

Structured Abstract

Background

Rapid detection and identification of microorganisms is important, for example, in clinical diagnostics and quality control in the food industry. Current methods for identifying microbes rely heavily on cell cultivation or, molecular nucleic acid analysis (e.g., 16S rRNA sequencing). Besides long time-to-result of a few days, culture-based methods are also prone to false-negatives and cultivation-bias since 99% of all known microorganisms have not been cultured under laboratory conditions. Although 16S rRNA analysis is culture-independent - and thus more expeditious than cell culture assays - bias associated with primer selection reduces identification accuracy. In contrast, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged, over the past decade, as a rapid and relatively low-cost microbial identification tool. Specifically, microbes are identified either by matching the mass spectrum of an unknown microorganism with those present in a reference database/library (i.e., the pattern recognition approach) or, via a proteome database search method that relates individual mass peaks to biomarker proteins endowed with species- or strain-specific signatures. Though commercialized, MALDI-TOF MS microbial typing has received relatively little coverage in undergraduate life science curricula compared to culture- and 16S rRNA-based techniques.

Pedagogical tool

To help address the curriculum gap, a simple inquiry-based laboratory exercise for teaching microbial identification via a combined MALDI-TOF MS and proteome database search approach was developed. Specifically, students were engaged in a variety of activities - ranging from sample collection and cell cultivation to mass spectrometric analysis and bioinformatics interrogation of mass spectra data - during identification of microorganisms from an environmental water sample. By encouraging students to use deductive and inductive thinking skills in solving a real-life problem with unknown answers, the educational module helped ignite

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their inquiring minds, while teaching leading-edge mass spectrometry-based microbial identification techniques and concepts.

Potential significance

Collectively, besides introducing the concepts and operating principles of MALDI-TOF MS microbial typing to students, the activity-based educational module also helped students appreciate the connection between textbook description of the scientific method and its application to real-world problem-solving – thereby, demystifying the work of scientists and connecting science to real-life, as well as seeding a science-oriented perspective in thinking about societal issues (many of which are influenced by science and technology).

Synopsis

Time is of the essence in microbial identification (especially in the clinic) since the high growth rates of most microbes means that delays in detecting and identifying disease-causing microorganisms would severely hamper efforts aimed at containing their spread, or provide timely and appropriate treatment to infected patients - failure of which may result in morbidity or even mortality.[1-3] The latter is particularly important given the increasing prevalence of multi- (MDR), extremely- (XDR), and totally-drug resistant (TDR) microbes.[4] Despite the advent of numerous techniques for rapid and accurate identification of microbes,[1, 5-13] culture-based and molecular nucleic acid (16S rRNA)[14]-based approaches remain standard techniques employed in most clinical laboratories around the world - due primarily to their methodological robustness and availability of analytical equipment. Nevertheless, the commercialization, in recent years, of mass spectrometry instruments (and accompanying bioinformatics software) capable of identifying microbes with high sensitivity and accuracy may usher in a paradigm shift in the field[1, 15-17] – as demonstrated by increasing adoption of the technique in both research and clinical laboratories.[18, 19] Unfortunately, the situation is not mirrored on the education front where the focus remains on conventional identification techniques - with a lack of coverage of emerging methods such as those based on mass spectrometric profiling of species biomarkers. Driven by the desire to help fill the curriculum gap, I developed an inquiry-based activity-focused education tool (Figure 1) for introducing students to a leading-edge mass spectrometric microbial identification technique (i.e., matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), (Ng, 2013)[20] where students gain practical and theoretical knowledge of the analytical instrument and associated bioinformatics tools, while employing the scientific method in solving a real-world problem with unknown answers: what are the microbes present in an environmental water sample?

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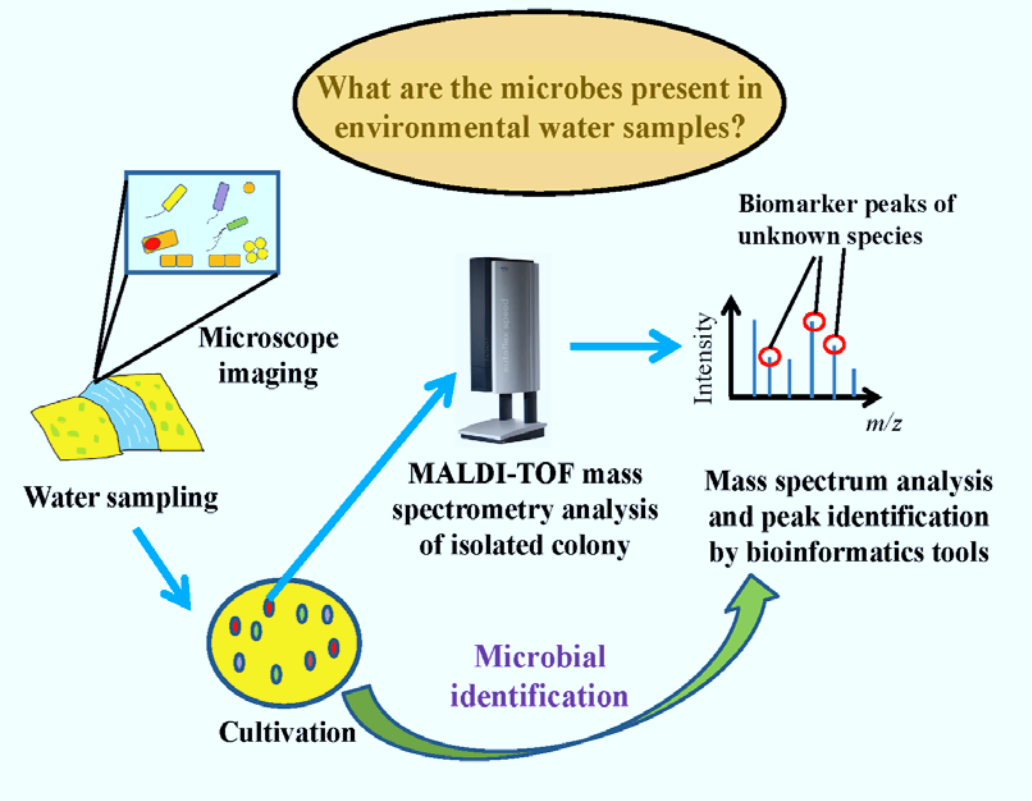

Figure 1: Schematic diagram outlining the major steps of the inquiry-based educational tool. From water sample collection and characterization of microbial cells via microscopy, to cell cultivation and mass spectrometry analysis, students had the opportunity of participating in all aspects of the discovery process in identifying unknown microbes from an environmental water sample. (Adapted from Figure 1 in Ng (2013), *JMBE*, Vol. 14, No. 1, pp. 103-106)

Capable of ionizing large biomolecules without inducing molecular fragmentation, and detecting the resulting singly charged molecular ions via a time-of-flight (TOF) detector, MALDI-TOF MS affords rapid (minutes per sample)[17, 21-23] identification of a variety of microbes (such as bacteria, spores, mycobacteria, fungi, and viruses) from various samples - down to the species level with high accuracy and sensitivity.[1, 15, 24-26] Strain and isolate level discrimination is also possible[26-28] – but the achievable accuracy is less than that at species and genus level.[15] In general, accuracy of MALDI-TOF MS microbial identification progressively decreases from the genus to species and sub-species level, but active research is underway to improve strain-level identification; for example, by developing better sample preparation strategies,[29] culture conditions,[30] new matrixes for facilitating ionization of biomolecules,[31] and data analysis algorithms capable of discerning small mass spectra differences of closely-related microbes.[32] Of greater importance from a clinical perspective is MALDI-TOF MS's capability of delivering comparable performance in genus- and species-level identification relative to conventional

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biochemical, nucleic acid and culture-based techniques - but at lower cost per sample;[27, 33] thus, opening up prospects for its clinical application in complementing conventional techniques. Specifically, since MALDI-TOF MS is capable of identifying most (but not all isolates), it could be used in earlier parts of an identification workflow; thereby, leaving more challenging microbes to more time-consuming and laborious techniques for confirmatory identification.[19] Currently, strains belonging to *Shigella*, pneumococci and viridian streptococci cannot be reliably identified by MALDI-TOF MS microbial typing.[19]

Though MALDI-TOF MS requires a culture step for generating sufficient cells to allow accurate analysis,[1] the total cell quantity required is small (~10^3 cells/mL). Thus, the high sensitivity (and low detection limit) of MALDI-TOF MS potentially allows the direct identification, upon isolation of sufficient number of cells, of difficult-to-culture species such as those employing anaerobic metabolisms, are slow-growing, or which require special growth conditions.[34] Additionally, microbes typically exist as communities comprising different species and strains – thus, any microbial identification technique must be capable of identifying individual species from a mixed population. To this end, various studies have demonstrated the utility of MALDI-TOF MS in discriminating microbes from populations comprising multiple species and strains.[35, 36] Besides its use in identifying microbes, another emerging application of MALDI-TOF MS is in determining their antibiotic susceptibility[15, 19, 24, 37, 38] – which holds potential for expediting the detection of antibiotic-resistant strains compared to conventional culture-based techniques.

Sample preparation is critical to achieving accurate and high sensitivity detection of target analytes in various analytical approaches – and depending on the analytical technique, may be the most complex and time-consuming aspect of the process. For intact-cell (also known as whole-cell) MALDI-TOF MS, sample preparation is easy and typically involves smearing small amount of cell sample (for example, from an isolated colony on an agar plate) onto a MALDI target plate, mixing-in an organic matrix (for facilitating ionization of biomolecules), and placing the target plate in a mass spectrometer (for laser-induced ionization).[1, 23, 24] Additionally, a protein-extraction protocol can be included for enhancing identification accuracy; specifically, better quality mass spectra are obtained by treating cells with hydrolytic enzymes such as trypsin or lysozyme prior to MALDI analysis.[1, 15, 19, 23, 24] Moreover, immunomagnetic and affinity-based separation of cell extracts also help improve mass spectra quality and, by extension, analytical sensitivity.[15] MALDI-TOF MS also allows multiple samples to be simultaneously analyzed; thereby, increasing sample throughput while reducing analysis time. And, most important, unlike methods based on polymerase chain reaction (PCR), prior knowledge of the microbe's identity is not required.[1]

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Though sample preparation and culture conditions are known to affect MALDI-TOF MS's ability at differentiating between closely-related bacterial species and strains,[39] the issue does not impact on the screening of microbes at the genus level. Moreover, delineation of the effect of various culture and sample preparation conditions on identification accuracy has facilitated the development of dedicated protocols for discriminating specific groups of closely-related microbes – which would find use after an initial screen (using standard techniques) has revealed an isolate's putative genus and, in some cases, preliminary species identity.[39] Such tiered and complementary use of standard and dedicated protocols for pinpointing microbial isolate provenance would help improve the resolution (and confidence) of sub-species level microbial identification.

MALDI-TOF MS determination of microbial provenance depends on the existence of unique sets of biomarkers in each microbe, which upon mass spectrometric profiling, yields a species- or strain-specific mass spectrum fingerprint comprising mass peaks at particular mass/charge (*m/z*) ratios.[23] Such biomarkers include ribosomal proteins, unique metabolites (for example, toxins), or signalling molecules secreted by particular species and strains. Besides using ribosomal proteins as biomarkers for species identification, MALDI-TOF MS is also capable of discriminating different strains of Shiga toxin secreting *E. coli* via profiling the subtype of toxins produced.[40] Specifically, by combining top-down proteomics approaches with MALDI-TOF MS operated in the tandem MS/MS mode, sequence-specific fragment ions indicative of subtypes of Shiga toxin are generated – thereby allowing the differentiation of different strains of pathogenic *E. coli* based not on more conventional ribosomal protein biomarkers but by the strain-specific toxin secreted.[40] Use of ribosomal proteins would not be useful in this instance since they would show high degree of similarity across the different strains.

Nevertheless, ribosomal proteins are good biomarkers for tracking microbial phylogeny across the tree of life given their high relative abundance[41] (which affords ease of detection) and high degree of conservation across species (where small differences afford the discrimination of different microbes and, more important, allows the comparison of their evolutionary relationships on the same basis). Thus, the remaining discussion will focus on ribosomal protein biomarkers for MALDI-TOF MS microbial typing. Specifically, mutations in ribosomal protein genes translate into changes in amino acid composition and sequence, and, in turn, mass differences detectable by most commercial MALDI-TOF mass spectrometers, which would help generate an isolate-specific mass spectrum. In essence, given their important physiological role in mediating protein translation and presence in almost all species across the three domains of life, ribosomal proteins are ideally suited for serving as molecular time-keepers of evolution, where extent of sequence differences is a good measure of evolutionary distance and relatedness. Thus, a given microbe can be identified by comparing the similarity of its MALDI-TOF mass

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spectrum with those of known microorganisms catalogued in a mass spectra reference library, in an approach known as pattern recognition (or mass spectrum fingerprinting).[42] In a simulation study, for example, a variant of the pattern recognition approach is shown to be useful for discriminating between pairs of microbes at the species and strain level using, as input, a single mass spectrum comprising mass peaks from both species, for comparing with mass spectra of other microbes from a reference library.[16] Collectively, the described method holds promise for use in differentiating various microbial species and strains present in complex microbial consortia.

Though conceptually feasible, the pattern recognition approach runs into difficulties associated with the high cost of developing the requisite reference library either in-house or via procurement from a commercial vendor. An alternative approach (i.e., proteome database search)[43, 44] exists for circumventing the problem by tapping on publicly available genomic and proteomic information of diverse microbes for annotating mass peaks and thus, microbial provenance (Figure 2). Specifically, the method attempts to assign each peak in the mass spectrum to a particular biomolecule using information (such as molecular identities, sequences and molecular weight) present in genome and proteome databases.

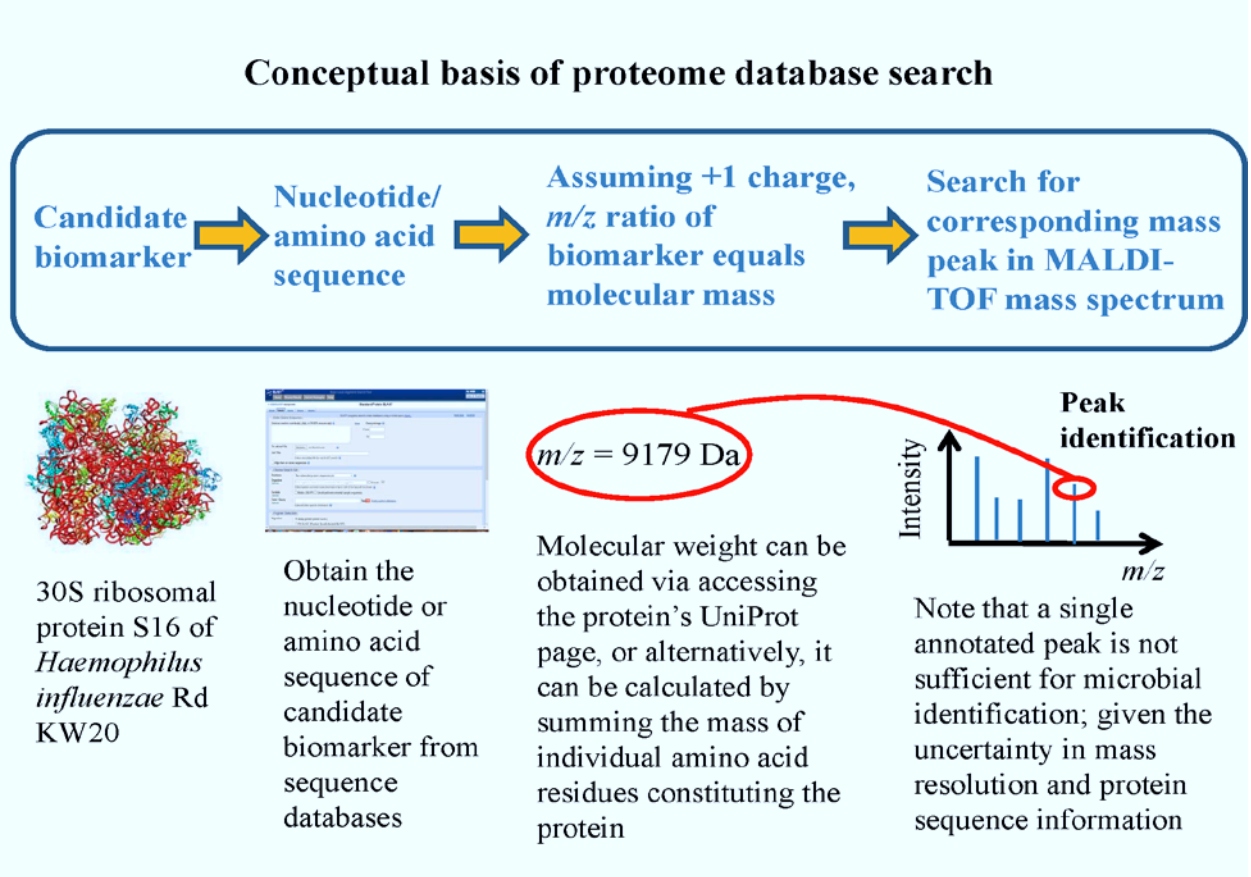

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Figure 2: Conceptual basis of proteome database search in identifying the provenance of mass peaks and microbes. Specifically, after the MALDI-TOF mass spectrum of a microbial isolate is obtained, possible candidate biomarkers (e.g., ribosomal proteins) are proposed and their nucleotide/amino acid sequence retrieved from relevant sequence databases. With the amino acid sequence in hand, molecular weight of the biomarker can be obtained directly from the protein's UniProt page, or calculated by summing the individual mass of amino acid residues constituting the protein. Since MALDI-TOF MS generates singly charged non-fragmented molecular ions, the mass/charge (*m/z*) ratio of the candidate biomarker equals its molecular mass. Finally, alignment of calculated *m/z* with a mass peak position (within a small margin of error given differing mass resolution of different instruments) associates a particular mass peak with a putative biomarker. Typically, positive microbial identification requires the annotation of at least a few mass peaks – with the goal of identifying as many of the biomarker peaks as possible.

Given the phylogenetic importance of ribosomal proteins, annotation of ribosomal protein peaks would significantly increases the chances and reliability of identifying a microbe – thus, a major focus of the proteome database search approach is the positive identification of peaks belonging to ribosomal proteins. Although multiple biomolecules with closely-similar molecular mass could potentially be assigned to a specific mass peak, greater cellular abundance of ribosomal proteins meant that the probability of other biomolecules accounting for a given peak (particularly those of high molecular weight) is low since ribosomal proteins are typically of high molecular mass. As mentioned, utility of ribosomal proteins as molecular chronometers arises from their high degree of conservation across species on various branches of the phylogenetic tree, where only small sequence differences in selected sections of ribosomal protein genes (known as hypervariable regions) separate distinct species and strains. Nevertheless, since most commercial mass spectrometers have sufficient resolving power for discriminating between ribosomal proteins that differ in just one amino acid, the proteome database search approach is capable of discriminating between closely-related species. Finally, although only about 2000 complete and a similar number of draft microbial genomes are available in public databases,[45, 46] anticipated improvement in sequencing speed accompanied by declining cost - through the promulgation of new sequencing technologies - will likely provide a vastly expanded compendium of genome and proteome information of microbes in the near future; a resource that will undoubtedly facilitate the use of proteome database search approaches for MALDI-TOF MS microbial typing.

Compared to more conventional approaches, mass spectrometry-based microbial typing techniques in general, and MALDI-TOF MS in particular, are generally not covered in undergraduate life sciences curricula. Additionally, although exercises designed for teaching bacterial identification exist, the focus has been on testing students' ability at devising a

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sequence of biochemical and culture assays for identifying an unknown microbe – through progressive elimination of candidate species and narrowing of search space – with the goal of using as few tests and in the shortest time possible.[47] This is significantly different from the current case where bioinformatics tools are used in searching genomic and proteomic databases for annotating peaks in mass spectra. But, for bridging the curriculum gap, what educational tools would be most effective in introducing the necessary concepts and practical experience to students, while infusing an element of fun and igniting students' curiosity? Accumulating evidence from educational research indicates that inquiry-based exercises with practical experiential components (which fall under the general category of active learning tools) are useful for increasing students' motivation towards learning and enhancing content mastery.[48-54] In particular, acquisition of conceptual understanding and problem-solving skills often go beyond algorithmic exercises; for example, students need to feel a sense of challenge in working on an interesting problem, which would motivate them to go the extra mile in thinking about various solution strategies.[51] Finally, casting a glance towards the future, the next generation of students would likely judge the quality of teaching and the education that they receive via yardsticks such as the extent of critical thinking and active learning incorporated in educational exercises (e.g., homework, laboratory classes, field work, etc.).[51] Thus, further highlighting the importance of active learning educational tools in stimulating interest in the subject matter and motivating students to learn.

Drawing from experiences documented in the educational literature, I designed a learning module for guiding students in identifying microbes in environmental water samples using a combined MALDI-TOF MS and proteome database search approach.[20] By solving a research problem with unknown answers, students put into practice the scientific method that they learnt (via flowcharts) in textbooks but seldom employ in problem-solving.[53] In addition, students were exposed to the complete suite of techniques and practical skills - ranging from collection of water samples and cell culture, to mass spectrometry analysis and bioinformatics search tools. Suitable either as a standalone laboratory module or, as a complement to a microbiology or bioinformatics class, the described activity helped, in a small way, to acquaint students with how scientists conduct field work – and thus, bring science closer to daily life. Specifically, students got to experience the excitement inherent in exploring the natural world through the rarely used educational tool of field work, while being equipped with technical and thinking skills useful either in industry or graduate school. More important, the experience also helps students realize that science is not an esoteric activity secluded in university labs – but rather, is a way of life where a logical sequence of questions guides investigation and solution of real-world problems. By illuminating the close connection between science and the world around us, such inquiry-based educational tools would also help increase students' interest in science that, in turn, hopefully translates into higher retention of students in STEM (science, technology, engineering, and mathematics) fields.[55-57] With a strong interest in science and technology seeded in

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university, there is a higher chance that students will continue to stay engaged with science – for example, by participating in the wide variety of citizen science projects available.[58] Even if students do not pursue a scientific career after graduation, or find employment in science- and technology-oriented jobs, the analytical, logical, and critical thinking skills cultivated would enable them to disentangle the myriad interacting factors typical of problems in a modern economy, as well as assess the scientific evidence surrounding important societal issues such as climate change and genetically modified food. Modular in design, elements of the exercise can be easily tailored to suit the particular needs and objectives of individual cohorts and classes; for example, determining the set of microbes present on plants' leaves[59, 60] - instead of in water samples - would also achieve similar educational outcomes. Finally, tips on implementing the exercise as well as some background information on MALDI-TOF MS are also available in the article and supplementary information.

To conclude, a laboratory exercise combining active inquiry by students during identification of microbes in water samples, with practical training in cell culture, mass spectrometer operation, and bioinformatics tools, was developed for teaching mass spectrometry-based microbial identification. Imbued with a fun element for motivating students to learn, the activity put students through the paces in using deductive and inductive thinking for solving a real-world problem with unknown answers, and, in the process, gaining a better appreciation of how scientists work, while, at the same time, igniting their inquiring minds and cultivating an interest in science and engineering.

The article and supplementary material is available at *Journal of Microbiology and Biology Education*, Vol. 14, No. 1, pp. 103-106, http://jmbe.asm.org/index.php/jmbe/article/view/494 as an open-access article.

Conflict of Interest

This synopsis describes a published paper written by the author.

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