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

Wenfa Ng*†

Department of Chemical and Biomolecular Engineering, National University of Singapore

*Corresponding author, Email: ngwenfa771@hotmail.com

†Present address: Novena, Singapore

Abstract

What is the microbe we are dealing with? Irrespective of cholera or anthrax, we want to know the disease causative microorganism as quickly as possible since prompt identification of the etiological organism would help control disease spread - and potentially save lives through provision of appropriate care and medicine. But despite the promulgation of rapid microbe identification tools (such as 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 they rarely feature in undergraduate curricula. Motivated by the desire to bridge the curriculum gap, I developed an inquiry-based laboratory exercise for introducing students to the operating principles and methodology of mass spectrometry enabled microbe 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 stir their curiosity in science 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 education research and expands on the discussion of concepts underlying matrix-assisted laser desorption/ionization time of flight mass spectrometry MALDI-TOF MS microbe identification. Specifically, the article discusses the relative advantages and disadvantages of the pattern recognition and proteome database search approaches for analyzing mass spectra data. In addition, the effect of different sample preparation protocols on identification accuracy is also discussed in detail. Finally, the pedagogy utility of field and inquiry-based education 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

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.399v2 | CC-BY 4.0 Open Access | rec: 5 Jun 2016, publ: 5 Jun 2016

material is published in the *Journal of Microbiology and Biology Education,* Vol. 14, No. 1, pp. 103-106, and is available an open access article at http://www.asmscience.org/content/journal/jmbe/10.1128/jmbe.v14i1.494.

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

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 food industry. Current methods for identifying microbes rely heavily on cell cultivation, or 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, as 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 microbe 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 through a proteome database search method that relates individual mass peaks to biomarker proteins with species or strain-specific signatures. Though commercialized, MALDI-TOF MS microbe typing has received relatively little coverage in undergraduate life science curricula compared to culture and 16S rRNA-based techniques.

Pedagogy tool

To help bridge the curriculum gap, a simple inquiry-based laboratory exercise for teaching microbe identification using a combined MALDI-TOF MS and proteome database search approach was developed. Specifically, students participated in a variety of activities - ranging from sample collection, cell cultivation, mass spectrometry and bioinformatics analysis of mass spectra data - during identification of microorganisms from an environmental water sample. By encouraging

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.399v2 | CC-BY 4.0 Open Access | rec: 5 Jun 2016, publ: 5 Jun 2016

students to use deductive and inductive thinking skills in solving a real life problem with unknown answers, the education module helped ignite their inquiring minds, while teaching leading edge mass spectrometry-based microbe identification techniques and concepts.

Potential significance

Collectively, besides introducing the concepts and operating principles of MALDI-TOF MS microbe typing to students, the activity oriented education module also helped students appreciate the connection between the scientific method (as explained in the textbook) and its application in real world problem solving. The latter helped demystify the work of scientists and connects scientific research 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 identifying disease causative microbe (i.e., pathogens) since high growth rates of most microbes means that delays in detection and identification would severely hamper efforts aimed at containing their spread, or provide timely and appropriate treatment to infected patients.[1-3] The latter is especially important given the increasing prevalence of multi (MDR), extremely (XDR), and totally (or pan) drug resistant (TDR) microbes.[4] Despite the introduction of numerous techniques for rapid and accurate microbe identification,[1, 5-13] culture (chromogenic agar) and nucleic acid (16S rRNA)[14] approaches remain standard techniques employed in most clinical laboratories around the world - due primarily to their methodological robustness and availability of analytical equipment. However, 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 lack of coverage of emerging methods such as mass spectrometric profiling of species biomarkers. Driven by the desire to help fill the curriculum gap, I developed an inquiry-based education activity (Figure 1) to introduce students to a leading edge mass spectrometry microbe 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?

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.399v2 | CC-BY 4.0 Open Access | rec: 5 Jun 2016, publ: 5 Jun 2016

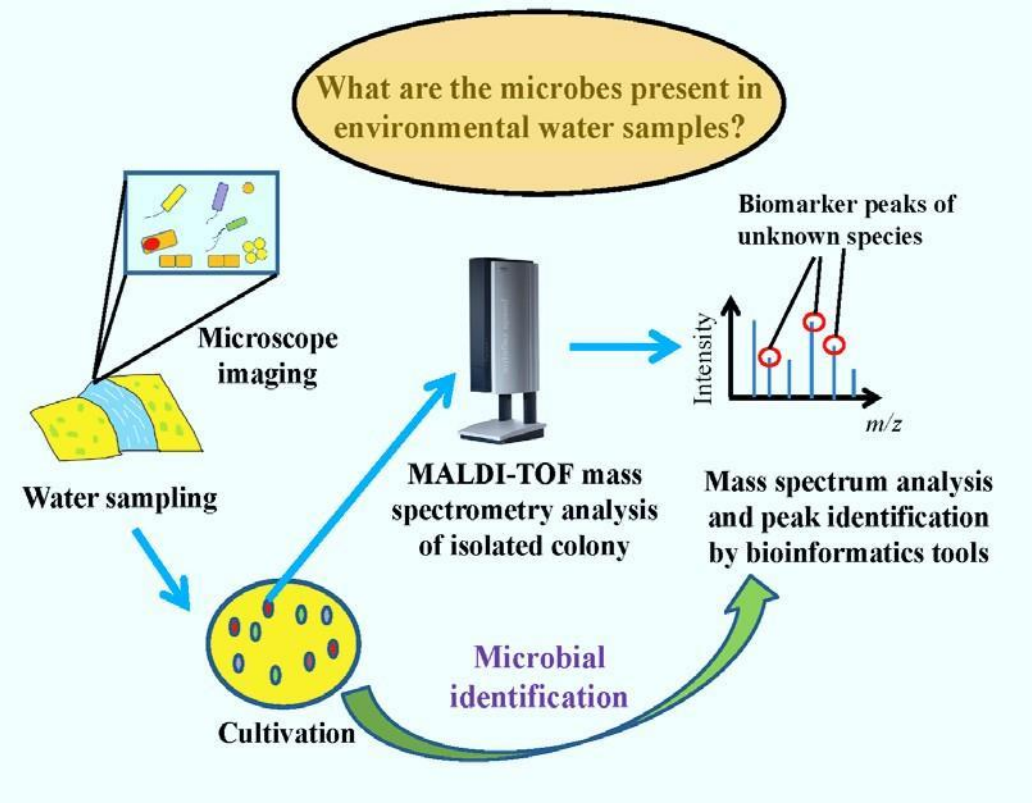


Figure 1: Schematic diagram outlining the major steps of the inquiry focused education activity. From water sample collection and characterization of microbes by microscopy, to cell cultivation and mass spectrometry analysis, students had the opportunity of participating in all aspects of the discovery 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 through a time of flight (TOF) detector, MALDI-TOF MS enables rapid (minutes per sample)[17, 21-23] identification of a variety of microbes such as bacteria, spores, mycobacteria, fungi, and viruses from various samples. Species level identification with high accuracy and sensitivity is possible with the technique.[1, 15, 24-26] Strain and isolate level discrimination is also possible[26-28] – but the accuracy is less than that at species and genus level.[15] In general, accuracy of MALDI-TOF MS microbe 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 compared to

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.399v2 | CC-BY 4.0 Open Access | rec: 5 Jun 2016, publ: 5 Jun 2016

conventional biochemical, nucleic acid, biochemical and culture-based methods, but at lower cost per sample.[27, 33] Current application potential of MALDI-TOF MS microbe identification is in complementing conventional techniques in clinics and research. Specifically, MALDI-TOF MS is capable of identifying most (but not all isolates); thus, it could be used in earlier parts of an identification workflow and leave more challenging identification problems to confirmatory techniques, which are more laborious and time-consuming.[19] In general, strains belonging to *Shigella*, pneumococci and viridian streptococci are difficult to be reliably identified by MALDI-TOF MS identification.[19]

Compared to 16S rRNA sequencing, which is culture independent, MALDI-TOF MS requires a culture step for generating sufficient cells to allow accurate analysis,[1] but the total cell quantity required is small. Thus, the high sensitivity (and low detection limit) of MALDI-TOF MS 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] In addition, microbes typically exist as communities comprising different species and strains; thus, any microbial identification technique must be able to identify individual species from a mixed population. To this end, various studies have demonstrated, under somewhat idealized conditions, the utility of MALDI-TOF MS in discriminating microbes from populations comprising multiple species and strains.[35, 36]

Sample preparation is crucial 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 in

a mass spectrometer (for laser induced ionization).[1, 23,24] In addition, a protein extraction protocol can be included for enhancing identification accuracy; specifically, higher quality mass spectra are obtained by treating cells with hydrolytic enzymes such as trypsin or lysozyme prior to MALDI-TOF MS analysis.[1, 15, 19, 23, 24] Moreover, immunomagnetic and affinity-based separation of cell extracts also help improve mass spectra quality and analytical sensitivity.[15] MALDI-TOF MS also allows multiple samples to be analyzed simultaneously; thus, increasing sample throughput while reducing analysis time. And, most importantly, unlike methods based on polymerase chain reaction (PCR) which requires 16S rRNA gene information for generating requisite forward and reverse PCR primers, prior knowledge of the microbe's identity is not required in MALDI-TOF MS microbe identification.[1]

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.399v2 | CC-BY 4.0 Open Access | rec: 5 Jun 2016, publ: 5 Jun 2016

Sample preparation and culture conditions are known to affect MALDI-TOF MS's ability at differentiating closely-related bacteria species and strains,[39] but the issue does not impact on the screening of microbes at the genus level. Moreover, delineating 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 preliminary species identity.[39] Such tiered and complementary use of standard and case specific protocols for pinpointing microbe provenance would help improve the resolution (and confidence) of subspecies level identification.

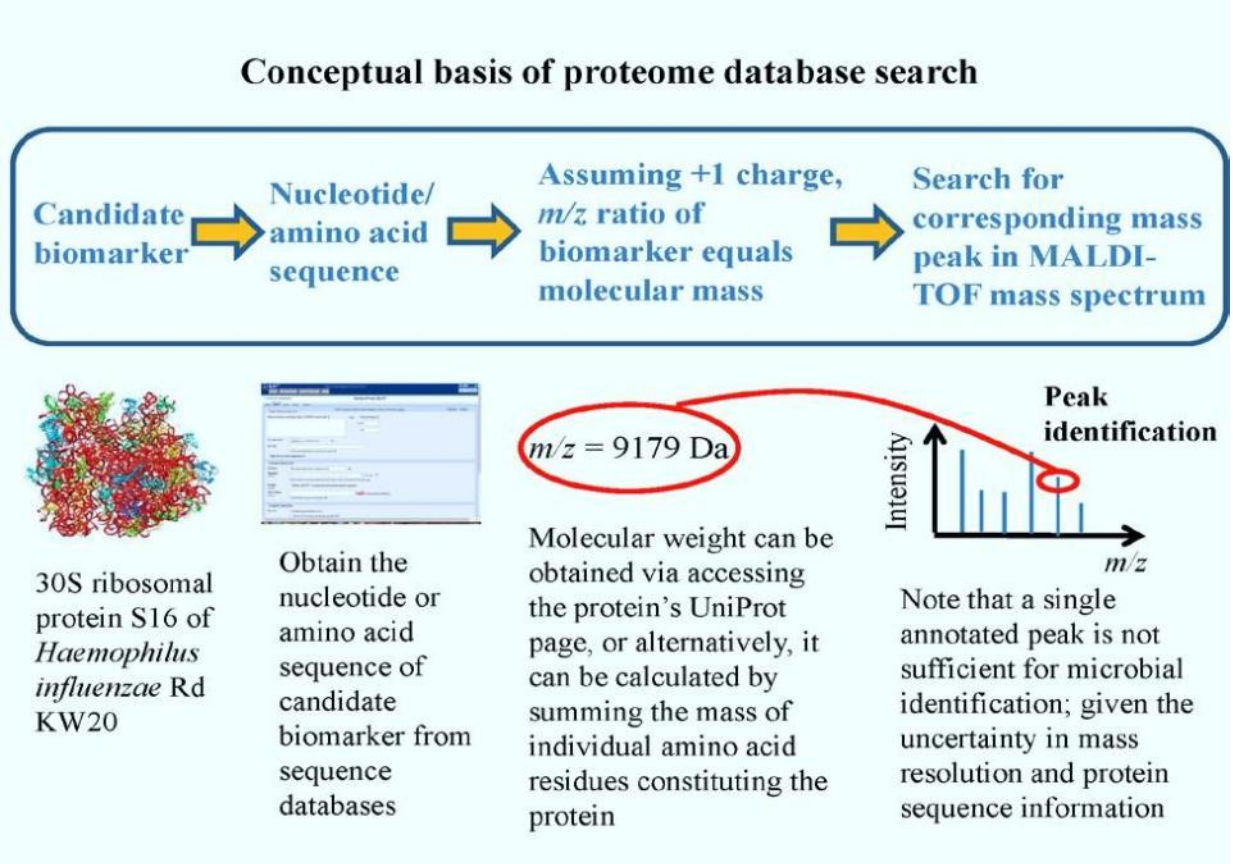
MALDI-TOF MS determination of microbe identity depends on the existence of unique sets of biomarkers in each species, which upon mass spectrometric profiling, yields a species or strain specific mass spectrum fingerprint comprising mass peaks at specific mass/charge (*m/z*) ratios.[23] Such biomarkers include ribosome proteins, unique metabolites (for example, toxins), or signaling
molecules secreted by specific species and strains. Besides using ribosome proteins as biomarkers for species identification, MALDI-TOF MS is also capable of discriminating different strains of Shiga toxin secreting *E. coli* by profiling the toxin 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; thus allowing
the differentiation of different strains of pathogenic *E. coli* based not on more conventional ribosome protein biomarkers but by the strain specific toxin secreted.[40] Use of ribosome proteins would not be useful in this instance since they are of high degree of similarity across the different strains.

However, ribosome proteins are good biomarkers for tracking phylogeny across the tree of life given their high relative abundance[41] (which allows ease of detection) and high degree of conservation across species. But small differences in protein sequence exist between various ribosome proteins from different species, which is useful for differentiating different microbes,
and more importantly, allows the delineation of their evolutionary relationships. Thus, the remaining discussion will focus on ribosome protein biomarkers for MALDI-TOF MS microbe identification. Specifically, mutations in ribosome protein genes translate into changes in protein amino acid composition and sequence, and mass differences detectable by most commercial MALDI-TOF mass spectrometers, which forms the basis of a microbe specific mass spectrum. With their important physiological role in mediating protein translation and presence in almost all species across the three domains of life, ribosome proteins are ideally suited for serving as molecular time keepers of evolution, where extent of sequence difference is a good measure

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.399v2 | CC-BY 4.0 Open Access | rec: 5 Jun 2016, publ: 5 Jun 2016

of evolutionary distance and relatedness. Thus, a given microbe can be identified by comparing the similarity of its MALDI-TOF mass spectrum with those of known microorganisms catalogued in a mass spectrum reference library, in an approach known as pattern recognition (or mass spectrum fingerprinting).[42] In a bioinformatics study, 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, with mass spectra of other microbes from a reference library providing comparative biomarker peaks.[16] Collectively, the described method holds promise for differentiating various microbe species and strains present in complex mixed species consortia.

Though conceptually feasible, the pattern recognition approach runs into difficulties associated with the high cost of the requisite reference library procured from the instrument maker or developed in-house. 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.



PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.399v2 | CC-BY 4.0 Open Access | rec: 5 Jun 2016, publ: 5 Jun 2016

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 microorganism 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 since different instruments have differing mass resolution) 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 ribosome proteins, annotation of ribosome protein peaks would significantly increases the chances and accuracy of identifying a microbe. Thus, a major focus of the proteome database search approach is the positive identification of peaks belonging to ribosome proteins. Although multiple biomolecules with closely similar molecular mass could potentially be assigned to a specific mass peak, greater cellular abundance of ribosome proteins meant that the probability of other biomolecules accounting for a given peak (especially those of high molecular weight) is low since ribosome proteins are typically of high molecular mass. As mentioned, utility of ribosome 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 ribosome protein genes (known as hypervariable regions) separate distinct species and strains. However, most commercial mass spectrometers have sufficient resolving power for discriminating between ribosome 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 microbe genomes are available in public databases,[45, 46] anticipated improvement in sequencing speed and declining cost (due to 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 microbe identification.

Compared to more conventional approaches, mass spectrometry-based microbe identification techniques in general, and MALDI-TOF MS in particular, are generally not covered in undergraduate life sciences curricula. In addition, exercises designed for teaching bacteria identification do exist, but the focus is always on testing students' ability at devising a

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.399v2 | CC-BY 4.0 Open Access | rec: 5 Jun 2016, publ: 5 Jun 2016

sequence of biochemical and culture assays for identifying an unknown microbe – through progressive elimination of candidate species and narrowing of search space, with a goal of using as few tests and in as short a time as 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 education 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 education 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] Specifically, 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 further motivate them to go the extra mile in thinking about various solution strategies.[51] Finally, casting a forward glance, the next generation of students would likely judge the quality of teaching and the education they received by yardsticks such as the extent of critical thinking and active learning incorporated in course exercises (e.g., homework, laboratory classes, field work, etc.).[51] Thus, further highlighting the importance of active learning education tools in stimulating interest in the subject matter and motivating students to learn.

Drawing from teaching experiences documented in the pedagogy 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 (using flowcharts) in textbooks but seldom employ in solving problems.[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 experienced the excitement inherent in exploring the natural world through the rarely used
education tool of field work, while being equipped with technical and thinking skills useful either in industry or graduate school. More importantly, the practical 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
education activity would also help increase students' interest in science, and hopefully translates into higher retention of students in STEM (science, technology, engineering and mathematics)

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.399v2 | CC-BY 4.0 Open Access | rec: 5 Jun 2016, publ: 5 Jun 2016

fields.[55-57] With a strong interest in science and technology seeded in university, there is a higher chance that students will continue to stay engaged in 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 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 specific needs and objectives of individual cohorts and classes; for example, determining the set of microbes present on plants' leaves[59, 60] would also achieve similar education outcomes as in determining microbe species in water
samples. Finally, tips on implementing the exercise and some background information on MALDI-TOF MS are also available in the article and associated 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 enabled microbe 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, helping them gain 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, as an open access article, URL: http://www.asmscience.org/content/journal/jmbe/10.1128/jmbe.v14i1.494.

Conflicts of interest

This synopsis describes a published paper written by the author.

References

1. Wolk, D.M. & Dunne, W.M. (2011), New Technologies in Clinical Microbiology. *Journal of Clinical Microbiology,* Vol. 49, pp. S62-S67.
2. Archer, E. & Houldcroft, C.J. (2014), Rabid about whole lyssa genomes. *Nature Reviews Microbiology,* Vol. 12, pp. 316-316.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.399v2 | CC-BY 4.0 Open Access | rec: 5 Jun 2016, publ: 5 Jun 2016

3. Bauer, K.A. et al. (2010), An Antimicrobial Stewardship Program's Impact. *Clinical Infectious Diseases,* Vol. 51, pp. 1074-1080.
4. Hatfull, G.F. (2014), Mycobacteriophages: Windows into Tuberculosis. *PLoS Pathogens,* doi:10.1371/journal.ppat.1003953.
5. Singh, A.K. et al. (2014), Laser Optical Sensor, a Label-Free On-Plate *Salmonella enterica* Colony Detection Tool. *mBio,* doi:10.1128/mBio.01019-13.
6. Kaleta, E.J. et al. (2011), Use of PCR Coupled with Electrospray Ionization Mass Spectrometry for Rapid Identification of Bacterial and Yeast Bloodstream Pathogens from Blood Culture Bottles. *Journal of Clinical Microbiology,* Vol. 49, pp. 345-353.
7. Ecker, D.J. et al. (2008), Ibis T5000: a universal biosensor approach for microbiology. *Nature Reviews Microbiology,* Vol. 6, pp. 553-558.
8. Devault, A.M. et al. (2014), Ancient pathogen DNA in archaeological samples detected with a Microbial Detection Array. *Scientific Reports,* doi:10.1038/srep04245.
9. Kloß, S. et al. (2013), Culture Independent Raman Spectroscopic Identification of Urinary Tract Infection Pathogens: A Proof of Principle Study. *Analytical Chemistry,* Vol. 85, pp. 9610-9616.
10. Safari, M., Amache, R., Esmaeilishirazifard, E. & Keshavarz, T. (2014), Microbial metabolism of quorum-sensing molecules acyl-homoserine lactones, γ-heptalactone and other lactones. *Applied Microbiology and Biotechnology,* Vol. 98, pp. 3401-3412.
11. Li, X.-R., Lv, Y., Meng, H., Gu, J.-D. & Quan, Z.-X. (2014), Analysis of microbial diversity by pyrosequencing the small-subunit ribosomal RNA without PCR amplification. *Applied Microbiology and Biotechnology,* Vol. 98, pp. 3777-3789.
12. Vital, M., Howe, A.C. & Tiedje, J.M. (2014), Revealing the Bacterial Butyrate Synthesis Pathways by Analyzing (Meta)genomic Data. *mBio,* doi:10.1128/mBio.00889-14.
13. Park, S.J. et al. (2014), Detection of microorganisms using terahertz metamaterials. *Scientific Reports,* doi:10.1038/srep04988.
14. Lleo, M.M. et al. (2014), Detecting the presence of bacterial DNA by PCR can be useful in diagnosing culture-negative cases of infection, especially in patients with suspected infection and antibiotic therapy. *FEMS Microbiology Letters,* Vol. 354, pp. 153-160.
15. Sandrin, T.R. & Demirev, P.A. (2014), Using Mass Spectrometry to Identify and Characterise Bacteria. *Microbe,* Vol. 9, pp. 23-29.
16. Mahé, P. et al. (2014), Automatic identification of mixed bacterial species fingerprints in a MALDI-TOF mass-spectrum. *Bioinformatics,* doi:10.1093/bioinformatics/btu022.
17. Seng, P. et al. (2009), Ongoing Revolution in Bacteriology: Routine Identification of Bacteria by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry. *Clinical Infectious Diseases,* Vol. 49, pp. 543-551.
18. Chen, J.H.K. et al. (2013), Direct Bacterial Identification in Positive Blood Cultures using two commercial MALDI-TOF mass spectrometry systems. *Journal of Clinical Microbiology,* doi:10.1128/jcm.03259-12.
19. Steensels , D., Verhaegen , J. & Lagrou , K. (2011), Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for the identification of bacteria and yeasts in a clinical microbiological laboratory: A review. *Acta Clinica Belgica,* Vol. 66, pp. 267-273.
20. Ng, W. (2013), Teaching Microbial Identification with Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) and

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.399v2 | CC-BY 4.0 Open Access | rec: 5 Jun 2016, publ: 5 Jun 2016

Bioinformatics Tools. *Journal of Microbiology and Biology Education,* Vol. 14, pp. 103-106.

21. Marko, D.C. et al. (2012), Evaluation of the Bruker Biotyper and Vitek MS Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry Systems for Identification of Nonfermenting Gram-Negative Bacilli Isolated from Cultures from Cystic Fibrosis Patients. *Journal of Clinical Microbiology,* Vol. 50, pp. 2034-2039.
22. Anhalt, J.P. & Fenselau, C. (1975), Identification of bacteria using mass spectrometry. *Analytical Chemistry,* Vol. 47, pp. 219-225.
23. Barbuddhe, S.B. et al. (2008), Rapid Identification and Typing of *Listeria* Species by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry. *Applied and Environmental Microbiology,* Vol. 74, pp. 5402-5407.
24. Murray, P.R. (2012), What Is New in Clinical Microbiology—Microbial Identification by MALDI-TOF Mass Spectrometry: A Paper from the 2011 William Beaumont Hospital Symposium on Molecular Pathology. *The Journal of molecular diagnostics: JMD,* Vol. 14, pp. 419-423.
25. van Veen, S.Q., Claas, E.C.J. & Kuijper, E.J. (2010), High-Throughput Identification of Bacteria and Yeast by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry in Conventional Medical Microbiology Laboratories. *Journal of Clinical Microbiology,* Vol. 48, pp. 900-907.
26. Usbeck, J., Wilde, C., Bertrand, D., Behr, J. & Vogel, R. (2014), Wine yeast typing by MALDI-TOF MS. *Applied Microbiology and Biotechnology,* Vol. 98, pp. 3737-3752.
27. Mellmann, A. et al. (2008), Evaluation of Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry in Comparison to 16S rRNA Gene Sequencing for Species Identification of Nonfermenting Bacteria. *Journal of Clinical Microbiology,* Vol. 46, pp. 1946-1954.
28. Eddabra, R., Prévost, G. & Scheftel, J.-M. (2012), Rapid discrimination of environmental *Vibrio* by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Microbiological Research,* Vol. 167, pp. 226-230.
29. Horká, M. et al. (2013), CIEF separation of probiotic bacteria from cow's milk in tapered fused silica capillary with off-line MALDI-TOF MS identification. *Analytica Chimica Acta,* Vol. 788, pp. 193-199.
30. Šedo, O., Vávrová, A., Vad'urová, M., Tvrzová, L. & Zdráhal, Z. (2013), The influence of growth conditions on strain differentiation within the *Lactobacillus acidophilus* group using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry profiling. *Rapid Communications in Mass Spectrometry,* Vol. 27, pp. 2729-2736.
31. Abdelhamid, H.N., Khan, M.S. & Wu, H.-F. (2014), Design, characterization and applications of new ionic liquid matrices for multifunctional analysis of biomolecules: A novel strategy for pathogenic bacteria biosensing. *Analytica Chimica Acta,* Vol., pp.
32. Dybwad, M., van der Laaken, A.L., Blatny, J.M. & Paauw, A. (2013), Rapid Identification of *Bacillus anthracis* Spores in Suspicious Powder Samples by Using Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS). *Applied and Environmental Microbiology,* Vol. 79, pp. 5372-5383.
33. El-Bouri, K. et al. (2012), Comparison of bacterial identification by MALDI-TOF mass spectrometry and conventional diagnostic microbiology methods: agreement, speed and cost implications. *British Journal of Biomedical Science,* Vol. 69, pp. 47-55.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.399v2 | CC-BY 4.0 Open Access | rec: 5 Jun 2016, publ: 5 Jun 2016

34. Biswas, S. & Rolain, J.-M. (2013), Use of MALDI-TOF mass spectrometry for identification of bacteria that are difficult to culture. *Journal of Microbiological Methods,* Vol. 92, pp. 14-24.
35. Ziegler, D. et al. (2012), *In Situ* Identification of Plant-Invasive Bacteria with MALDI-TOF Mass Spectrometry. *PLoS ONE,* doi:10.1371/journal.pone.0037189.
36. Wahl, K.L. et al. (2002), Analysis of Microbial Mixtures by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. *Analytical Chemistry,* Vol. 74, pp. 6191-6199.
37. Edwards-Jones, V. et al. (2000), Rapid discrimination between methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* by intact cell mass spectrometry. *Journal of Medical Microbiology,* Vol. 49, pp. 295-300.
38. Z, D., R, Y., Z, G., Y, S. & J, W. (2002), Identification of *Staphylococcus aureus* and determination of its methicillin resistance by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Analytical Chemistry,* Vol. 74, pp. 5487-5491.
39. Balážová, T. et al. (2014), The influence of culture conditions on the identification of *Mycobacterium* species by MALDI-TOF MS profiling. *FEMS Microbiology Letters,* Vol. 353, pp. 77-84.
40. Fagerquist, C.K. et al. (2014), Top-Down Proteomic Identification of Shiga Toxin 2 Subtypes from Shiga Toxin-Producing *Escherichia coli* by Matrix-Assisted Laser Desorption Ionization–Tandem Time of Flight Mass Spectrometry. *Applied and Environmental Microbiology,* Vol. 80, pp. 2928-2940.
41. Chubukov, V., Gerosa, L., Kochanowski, K. & Sauer, U. (2014), Coordination of microbial metabolism. *Nature Reviews Microbiology,* Vol. 12, pp. 327-340.
42. Jarman, K.H. et al. (2000), An Algorithm for Automated Bacterial Identification Using Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. *Analytical Chemistry,* Vol. 72, pp. 1217-1223.
43. Wynne, C., Fenselau, C., Demirev, P.A. & Edwards, N. (2009), Top-Down Identification of Protein Biomarkers in Bacteria with Unsequenced Genomes. *Analytical Chemistry,* Vol. 81, pp. 9633-9642.
44. Demirev, P.A., Ho, Y.-P., Ryzhov, V. & Fenselau, C. (1999), Microorganism Identification by Mass Spectrometry and Protein Database Searches. *Analytical Chemistry,* Vol. 71, pp. 2732-2738.
45. Nie, Y. et al. (2014), Diverse alkane hydroxylase genes in microorganisms and environments. *Scientific Reports,* doi:10.1038/srep04968.
46. Bohlin, J., Sekse, C., Skjerve, E. & Brynildsrud, O. (2014), Positive correlations between genomic %AT and genome size within strains of bacterial species. *Environmental Microbiology Reports,* Vol. 6, pp. 278-286.
47. Bryant, T.N. (1994), A bacterial identification teaching exercise revisited. *Computer applications in the biosciences : CABIOS,* Vol. 10, pp. 329-334.
48. Vanchieri, C. (2013), Partners for the environment. *HHMI Bulletin,* Vol. Fall 2013, pp. 34-35.
49. Drissner, J.R., Haase, H.-M., Wittig, S. & Hille, K. (2013), Short-term environmental education: long-term effectiveness? *Journal of Biological Education,* Vol. 48, pp. 9-15.
50. Dopico, E., Linde, A.R. & Garcia-Vazquez, E. (2013), Learning gains in lab practices: teach science doing science. *Journal of Biological Education,* Vol. 48, pp. 46-52.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.399v2 | CC-BY 4.0 Open Access | rec: 5 Jun 2016, publ: 5 Jun 2016

51. Pienta, N.J. (2014), Teaching General Chemistry and Making a Difference. *Journal of Chemical Education,* Vol. 91, pp. 305-306.
52. Conway, C.J. (2014), Effects of Guided Inquiry versus Lecture Instruction on Final Grade Distribution in a One-Semester Organic and Biochemistry Course. *Journal of Chemical Education,* Vol. 91, pp. 480-483.
53. Mandler, D., Blonder, R., Yayon, M., Mamlok-Naaman, R. & Hofstein, A. (2014), Developing and Implementing Inquiry-Based, Water Quality Laboratory Experiments for High School Students To Explore Real Environmental Issues Using Analytical Chemistry. *Journal of Chemical Education,* Vol. 91, pp. 492-496.
54. Eisen, L., Marano, N. & Glazier, S. (2014), Activity-Based Approach For Teaching Aqueous Solubility, Energy, and Entropy. *Journal of Chemical Education,* Vol. 91, pp. 484-491.
55. Jordan, T.C. et al. (2014), A Broadly Implementable Research Course in Phage Discovery and Genomics for First-Year Undergraduate Students. *mBio,* doi:10.1128/mBio.01051-13.
56. Linn, M.C. et al. (2014), Computer-Guided Inquiry to Improve Science Learning. *Science,* Vol. 344, pp. 155-156.
57. Fakayode, S.O., Yakubu, M., Adeyeye, O.M., Pollard, D.A. & Mohammed, A.K. (2014), Promoting Undergraduate STEM Education at a Historically Black College and University through Research Experience. *Journal of Chemical Education,* Vol. 91, pp. 662-665.
58. Khatib, F. et al. (2011), Crystal structure of a monomeric retroviral protease solved by protein folding game players. *Nature Structural & Molecular Biology,* Vol. 18, pp. 1175-1177.
59. Müller, T. & Ruppel, S. (2014), Progress in cultivation-independent phyllosphere microbiology. *FEMS Microbiology Ecology,* Vol. 87, pp. 2-17.
60. Romero, F.M., Marina, M. & Pieckenstain, F.L. (2014), The communities of tomato (*Solanum lycopersicum* L.) leaf endophytic bacteria, analyzed by 16S-ribosomal RNA gene pyrosequencing. *FEMS Microbiology Letters,* Vol. 351, pp. 187-194.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.399v2 | CC-BY 4.0 Open Access | rec: 5 Jun 2016, publ: 5 Jun 2016