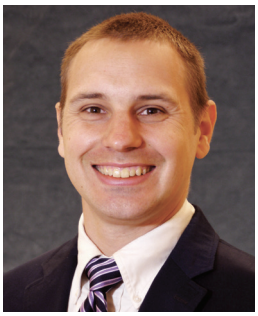

Letter from the Editor

PETER D. KAZARINOFF

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Keywords: J ATE, technician education, undergraduate research

Community and Technical College Education Community,

I'm pleased to present the Journal of Advanced Technological Education (J ATE) Volume 3, Issue 2, on behalf of the J ATE Editorial Board and Staff. This issue's theme is community college students. Many of the articles published in this issue were written by community college students based on their undergraduate research.

This year, J ATE was engaged in a professional development project called J ATE URE, the Journal of Advanced Technological Education Undergraduate Research Experience. The design of J ATE URE was to pair community college students with faculty who assisted with mentoring, research, and writing manuscripts. The J ATE URE program ran during the 2023-2024 academic year and culminated with 11 manuscript submissions. We are delighted to share that this project was a great success. All participating students and faculty involved worked hard throughout the entire process. We want to extend a huge thanks to Tanya Faltens, who led J ATE URE.

We are very proud of our growth and development within the MNT-EC family and want to thank Jared Ashcroft, our principal investigator, for his unwavering belief in the J ATE project. As such, with our success, it is time to move forward and be self-sufficient. With this in mind, this edition of J ATE will be the last issue supported by the Micro Nano Technology Education Center (MNT-EC) and the J ATE Special Project Grant. Beginning this fall, J ATE will form a business entity that generates revenue and pays its expenses independently. A key component of our ongoing sustainability plan includes offering advertising from your colleges, programs, and projects. We hope that you will be interested in participating in those efforts. For more information or opportunities, please feel free to contact me.

J ATE will continue to publish peer-reviewed articles based on technician education at community and technical colleges. We are committed to being free to submit to and publish online and will always remain open-access.

J ATE is our journal serving our community. Please support us by reading, submitting your manuscript, and serving as a valued community member by acting as a co-author or reviewer.

In Teaching and Learning,

Peter

Peter D. Kazarinoff
Editor-in-Chief

Invited Letter: Greetings from the National Science Foundation

Keywords: NSF, ATE, technician education

I am Olivia Long, and I am currently serving as a rotating program officer in the Division of Undergraduate Education at the National Science Foundation (NSF). My journey teaching in higher education has been rooted in a small, rural, regional primary undergraduate institution, where I have been deeply committed to supporting all students, particularly those transferring from local community colleges. Recognizing the unique challenges these students face, I have collaborated closely with local faculty and administrations of surrounding community colleges to make my institution more transfer-friendly. By building authentic relationships and clear pathways for students pursuing a baccalaureate degree, we have fostered a supportive and inclusive environment that encourages student success.

The theme of this issue is community college students. The articles co-authored by students highlight the potential for community college students to conduct research, analyze data, and report results with guidance from faculty mentors. Although most of my direct experience is with transfer students, I greatly respect community college faculty's vital role in shaping students' academic journeys. These educators often bring authentic experiences into their classrooms, enriching the learning environment. Similarly, helping students complete and publish their undergraduate research work has been one of the most fulfilling aspects of my career. This process encourages students to think critically, troubleshoot challenges, and gain practical experience. However, many students move on before their projects reach publication due to the short time frame before transferring or graduating. It often takes multiple students, semesters, or even years to gather enough data, making it crucial to continue these projects, publish the findings, and acknowledge the students' contributions. Publishing is essential not only for students' development and future careers, but also for showcasing the valuable research emerging from our students and faculty.

I recently attended the 2024 High Impact Technology Exchange Conference (HI-TEC) where the warmth and support of the Advanced Technological Education (ATE) community struck me. The enthusiasm shared by faculty members from various technical programs and community colleges was inspiring. Many of the sessions I attended were not only informative, but also offered research and insights that are truly worthy of publication. This experience reinforced my belief in the importance of disseminating our work through journals like J ATE, which provide a platform for the valuable contributions of community college faculty and their students.

Olivia Long
Program Officer,
Division of Undergraduate Education
The National Science Foundation

Polymeric Layer-by-Layer Microcapsules Containing Iron Oxide Magnetic Nanoparticles Exposed to Breast Cancer Cells: A Viability Study Using Tetrazolium-based (MTT) and Calcein-AM Assays (LIVE-DEAD)

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Abstract: The Layer-by-Layer capsules combined with nanoparticles are emerging as promising and secure vehicles for drug delivery. The integration of nanoparticles introduces intelligent functionalities, particularly in targeted therapy. This investigation focused on assessing the impact of drug carriers on the viability of MDA-MB-231 breast cancer cells. Specifically, polymeric capsules were synthesized using a Layer-by-Layer approach, incorporating iron oxide magnetic nanoparticles. The Layer-by-Layer method involved constructing six bilayers of PAH/PSS on a $CaCO_3$ template, with iron oxide magnetic nanoparticles (MNPs) interspersed between the layers. The core was removed by applying the chelating agenlt EDTA, resulting in the fabrication of hollow capsules. Characterization of the capsules was performed using SEM and bright-field microscopy. Successful synthesis was confirmed as capsules/MNPs responded to an external magnetic field. Various concentrations of capsules/MNPs were introduced to cells, and cell viability was assessed using the tetrazolium-based MTT assay for quantitative measurements and the calcein-AM assay (Live-dead) for real-time visualization of live and dead cells. The findings revealed that adding 20 µl of capsule suspension (containing 14,000 capsules) to 100 µl of cell culture suspension preserved 90% cell viability. This study implies a feasible concentration of capsules for delivering compounds to cancer cells without inducing toxicity to normal cells.

Keywords: Layer-by-Layer, microcapsule, magnetic nanoparticle, viability, breast cancer

Introduction

Nanomedicine encompasses a diverse array of technologies designed to diagnose, treat, monitor, and control biological systems. It spans the medical applications of nanomaterials to the development of nanoelectronic biosensors and explores potential future applications of molecular nanotechnology [1]. At the forefront of this interdisciplinary field, significant attention is devoted to research on the intelligent delivery and precise targeting of therapeutic and diagnostic agents. Current challenges in chemotherapy, such as issues related to non-specific

targeting, aqueous solubility, multidrug resistance, and limited specificity, stem from the systemic distribution of chemotherapeutic agents, leading to side effects caused by the drugs affecting both target and non-target cells [2]. Consequently, nanoparticles, designed as targeted carriers for drugs, are actively under development as non-biological alternatives to overcome the challenges associated with conventional drug delivery [3]. The benefits of encapsulating drugs in a carrier are less cytotoxicity to normal cells and prompting a triggered release only at the tumor site.

Magnetic nanoparticles (MNPs), ranging in size from 1 to 100 nm, exhibit remarkable promise in the realm of drug delivery owing to their inherent biocompatibility [4]. Their unique ability to be externally manipulated through magnetic fields enables precise control, making them a versatile tool for targeted therapies in diseases like cancer [5, 6]. MNPs offer advantages such as reduced toxicity, enhanced stability, and superior permeability compared to alternative nanoparticles [5]. MNPs have been incorporated into other drug delivery systems, such as Layer microcapsules, to develop targeted systems [6, 7].

Microcapsules created using the Layer-by-Layer (LbL) [8] technology represent microcapsules employed in drug delivery due to their straightforward fabrication process [9], biocompatibility, and impressive drug-loading capabilities [10-12]. In this approach, alternate absorption of opposite-charged polymers on a microsphere [10] followed by removing the core will result in hollow capsules with multilayered nanoshells [13]. These LbL capsules are constructed with polyelectrolyte bilayers, utilizing biodegradable materials like collagen (COL), hyaluronic acid (HA) [14], and poly-L-arginine (pARG) [7, 12, 15], as well as synthetic non-biodegradable materials such as poly(allylamine hydrochloride) (PAH) and poly(sodium 4-styrenesulfonate) (PSS) [15]. The unique properties of these capsules are that their multilayered nanoshell can incorporate nanoparticles. Past research has involved the adornment of LbL microcapsules with iron oxide magnetic nanoparticles, demonstrating a strategic approach to achieve smart [16-17] and targeted drug delivery [18-20].

While the clinical applications of microcapsules containing iron oxide magnetic nanoparticles hold promise, the comprehensive exploration of their interaction with cancer cells remains incomplete [2, 11, 21]. This study aims to fill this gap by examining the interaction between magnetic nanoparticles and LbL capsules with breast cancer cells, focusing on cell viability. The assessment involved utilizing Tetrazolium-based (MTT) [22] and Calcein-AM assays, both serving distinct functions in evaluating cell viability [23]. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay measures cellular metabolic activity by employing a yellow tetrazolium salt [22]. This salt is reduced by mitochondrial dehydrogenases in metabolically active cells, forming purple formazan crystals. The MTT assay offers a quantitative evaluation of cell viability based on the produced formazan. On the other hand, the Calcein-AM (acetoxymethyl ester) assay assesses intracellular esterase activity. Calcein-AM is initially a non-fluorescent, cell-permeant dye that, when cleaved by esterases in live cells, generates green, fluorescent calcein [23]. This dye effectively distinguishes live cells (exhibiting green fluorescence) from dead cells, as it is cleaved and retained exclusively in viable cells. The assay enables real-time imaging of live cells under a fluorescence microscope. This study synthesized LbL microcapsules consisting of $(PAH/PSS)_6$/MNPs. Breast cancer cells were incubated with these layer-by-layer capsules, including magnetic nanoparticles, and cell viability was assessed using the MTT and calcein-AM assays. The results provide insights into the biocompatibility of MNP LbL capsules against breast cancer cells.

Methods

Materials: MDA-MB-231 were purchased from the American Type Culture Collection (ATCC). Ferrofluid iron oxide nanoparticle kit were purchased from Flinn Scientific. Growth mediums and assay kits, including fetal bovine serum (FBS), high glucose DMEM, Trypsin-EDTA solution, antibiotics, MTT assay, LIVE/DEAD assay, were all purchased from Thermofisher.

Cell Culture, Passage, and Freezing of Breast Cancer Cell Line

MDA-MB-231 cells were cultured in a medium containing F-12K/DMEM (1:1), 10% FBS, and 1% antibiotics. Similarly, MCF7 cells were cultured in culture-treated flasks using a medium composed of DMEM, 10% FBS, and 1% streptomycin and penicillin antibiotics. The cells were maintained at 37°C in a humidified 5% CO_2 incubator, with media changes occurring every 48 hours. Upon reaching 80% confluence, the cells underwent detachment using trypsin/EDTA solution and were subsequently suspended in fresh media. Cell seeding involved placing 5×10^4 cells per well in cell culture-treated 96-well plates.

For cell passage, the medium was first removed and discarded, followed by washing the dish with 5 ml of PBS twice. Subsequently, 2 ml of trypsin/EDTA was added for cell detachment, with an incubation period of 8 minutes

at 37ºC. The cells were checked under a microscope to ensure detachment, and the duration of incubation was adjusted based on cell line and plate confluency. After confirming detachment, 8 ml of medium was added to the petri dish, with at least four times more medium than the applied trypsin/EDTA to neutralize its toxicity. The sample was then centrifuged.

In the process of cell freezing, cells from the MDA-MB-231 cell line were utilized [24]. This cell line, derived from excessive lung fluid in a middle-aged female patient with breast cancer, is a triple-negative breast cancer (TNBC) cell line lacking estrogen receptors, progesterone receptors, and human epidermal growth factor 2. Hence, it serves as a valuable model for studying TNBC cells. To initiate cellular freezing, cell confluency was checked, aiming for around eighty percent confluence. Following confluency verification, the medium was removed, and the dish was washed with phosphate-buffered saline (PBS) solution twice. A solution of trypsin/EDTA was added to the dish, and after incubation, the solution was centrifuged at 200g for five minutes.

Microcapsule Fabrication

$CaCO_3$ (Calcium Carbonate) is suspended in H_2O at a concentration of 10 mg/ml in a total volume of 10 ml. PAH and PSS are both dissolved in H_2O at a concentration of 4 mg/ml. To the suspended calcium carbonate, 1 ml of PAH is added and then placed on a shaker for 10 minutes. Afterward, the mixture is centrifuged, using Thermo Scientific Sorvall ST 40R centrifuge, for 10 minutes at 4,000 G. The supernatant is removed and discarded, leaving the pellet behind. The pellet is then washed two times by adding 10 ml of H_2O, centrifuging at 4,000 G for 10 minutes, following discarding the supernatant each time. Washing will remove any unbound PAH. Next, the pellet is resuspended in 9 ml of H_2O, and 1 ml of the PSS solution is added and placed on the shaker for 10 minutes. Afterward, the mixture is centrifuged for 10 minutes at 4000 G. As in the previous steps involving the PAH, the supernatant is discarded, and the pellet is washed two times with 10 ml of H_2O each time. At this point, one bilayer of PAH/PSS has been added to the calcium carbonate. The binding of the PAH and PSS are charged-based. The carbonate has a negative charge and binds ionically to the positively charged PAH. Likewise, the negatively charged PSS will bind to the positively charged PAH [25, 26]. This process is repeated 6 times to achieve $(PAH/PSS)_6$.

Synthesis of Magnetite Nanoparticles

Magnetite, Fe_3O_4, was prepared by reacting iron(III) chloride, $FeCl_3$, and iron(II) chloride, $FeCl_2$, in a 2:1 mole ratio with dilute ammonia, NH_3. A Ferrofluid Nanotechnology Demonstration kit from Flinn Lab was used as the source of these components. A 2 M solution of iron(II) chloride in hydrochloric acid, HCl, is prepared by adding $FeCl_2$•4 H_2O to 2 M HCl. Likewise, a 1 M solution of iron (III) chloride in HCl is prepared by adding $FeCl_3$•6 H_2O to 2 M HCl. A combined solution of Fe(II)/Fe(III) is prepared by adding 1 ml of 2 M $FeCl_2$ to 4 ml of 1 M $FeCl_3$. Ammonia is added dropwise to the Fe(II)/Fe(III) to form a brownish-black precipitate, which is Magnetite. This reaction is an oxidation reaction in which the ammonia provides hydroxide ions, OH^-, which will facilitate the oxidation and reduction of iron cations and produce the Fe_3O_4 oxide with the loss of water molecules. This is represented by the following equation:

$$2Fe^{3+}(aq) + Fe^{2+}(aq) + 8OH^-(aq) \longrightarrow Fe_3O_4(s) + 4H_2O(l)$$

The Magnetite particles are collected and kept in water in the refrigerator until their incorporation into the LBL capsules [24]. The MNPs solution is added to the $(PAH/PSS)_6$ capsules suspension.

MTT Assay to Determine the Viability of Cells

Cells were seeded (5×10^4 cells/well) in cell culture treated 96 wells plates. After 24 hours, magnetic nanoparticle microcapsule solution was added to each well, and plates were incubated overnight. Next day, wells were aspirated and 100 µl fresh media and 10 µl MTT assay reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) was added to each well. Cells were incubated for 3 hours in the incubator, and then 100 µl detergent reagent was added to each well, and plates were kept for 2 hours in the dark at room temperature. The absorbance was read by the plate reader (Synergy2, Biotek, USA) at 570 nm.

LIVE-DEAD Viability/Cytotoxicity Assay

The LIVE-DEAD assay was conducted using the LIVE/DEAD Viability/Cytotoxicity Kit, adhering to the manufacturer's instructions. Briefly, cells were incubated with a combination of calcein-AM and ethidium homodimer-1 for 30 minutes at room temperature. After incubation, the cells were imaged with a fluorescence microscope. Live cells exhibited green fluorescence due to calcein-AM, while dead cells showed red fluorescence

from ethidium homodimer-1. To quantify and visualize the fluorescence signals of live and dead cells, a microplate reader equipped with green and red filter cubes was used.

Scanning Electron Microscopy Imaging of Nanoparticles and Microcapsules

Scanning electron microscopy was used to determine the morphology, size, and dimension of the MNP/LbL capsules. For collecting the samples, a small portion of the solution was applied on a clean-washed silicone surface and incubated for 1 hr. The excess liquid was taken, followed by two rinses. The surface was then left to air-dry overnight in preparation for imaging. Images were collected with a scanning electron microscope FEI Quanta 200 ESEM.

Results and Discussion

LBL capsules were created through a sequential process of introducing poly(allylamine hydrochloride) (PAH) and polystyrene sulfonate (PSS) to the calcium carbonate core. Due to the negative charge of carbonate in calcium calcium carbonate, the initial layer applied is PAH, which possesses a net positive charge. Following agitation on a shaker, centrifugation, and thorough washing to eliminate excess unbound PAH, the subsequent layer of PSS, featuring a net negative charge, is added to form a single bilayer. This bilayer addition process is iterated six times to achieve capsules with a $(PAH/PSS)_6$ structure.

As depicted in Figure 1A, a solitary bilayer on the capsules exhibits a spherical form and is notably smaller in size. During real-time microscopic observation, the capsules display dynamic movement attributed to evenly distributed repelling charges on their surface. Notably, in contrast, as illustrated in Figure 1B, capsules with six completed bilayers assume irregular shapes and tend to aggregate in solution. This suggests that the introduction of PAH and PSS, coupled with the incorporation of magnetite, introduces a level of variability, resulting in outer surfaces with non-uniform charge distributions (Figure 1C). Consequently, based on our observations, there appears to be a limitation on the successful addition of bilayers to achieve a functional capsule. In our study, we opted for six bilayers.

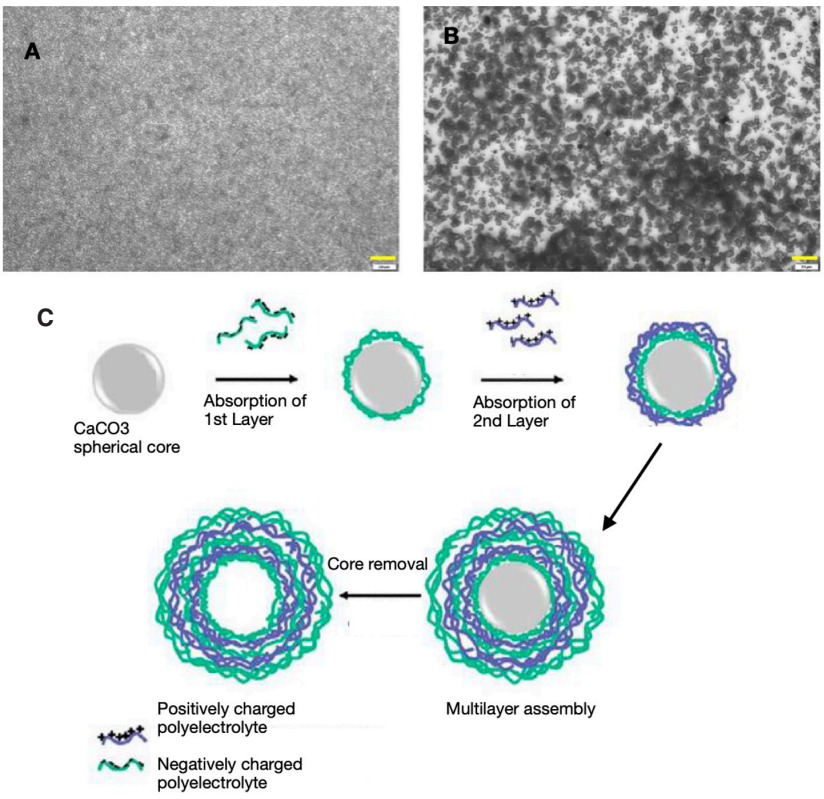

Fig. 1. (A) Capsules after a single bilayer addition of PAH/PSS to the calcium carbonate core. (B) Completed capsules of six bilayers $(PAH/PSS)_4/(PAH/MNPs)_5/(PAH/PSS)$, containing Fe_3O_4 in the fifth layer. Completed capsules appear to lose their perfectly spherical shape and clump together in high concentrations (Scale bar 20 μm). (C) Schematic image of LbL capsule assembly.

Utilizing a kit supplied by Flinn Scientific, magnetite was synthesized and stored at 4°C in an aqueous solution to prevent oxidation. In the production of capsules, magnetite replaced polystyrene sulfonate (PSS) in the fifth bilayer. A subsequent sixth bilayer, consisting of alternating PAH and PSS, was added to sandwich the magnetite between capsule layers. When observing a small droplet of the capsules on a microscope slide using an inverted microscope, with a permanent magnet positioned adjacent to the droplet, the capsules exhibited migration towards the magnet. This outcome confirmed the successful integration of magnetite nanoparticles into the capsule layers. Larger magnetite particles were discernible as black fragments, either dispersed among or situated on the periphery of the capsules.

Upon the completion of the capsules, removing the core is required for subsequent compound loading. Initially, the dissolution of the calcium carbonate core was attempted using hydrochloric acid (HCl) at an initial concentration of 0.1M, added incrementally in 100 µl portions to 1 ml of capsules in an aqueous solution. However, limited success was observed with concentrations up to 500 µl per ml of capsules in the solution. The challenge arose from the strong acidic nature of HCl, resulting in the destruction of many capsules, even at low concentrations (Figure 2). Consequently, an alternative approach was imperative to overcome this limitation.

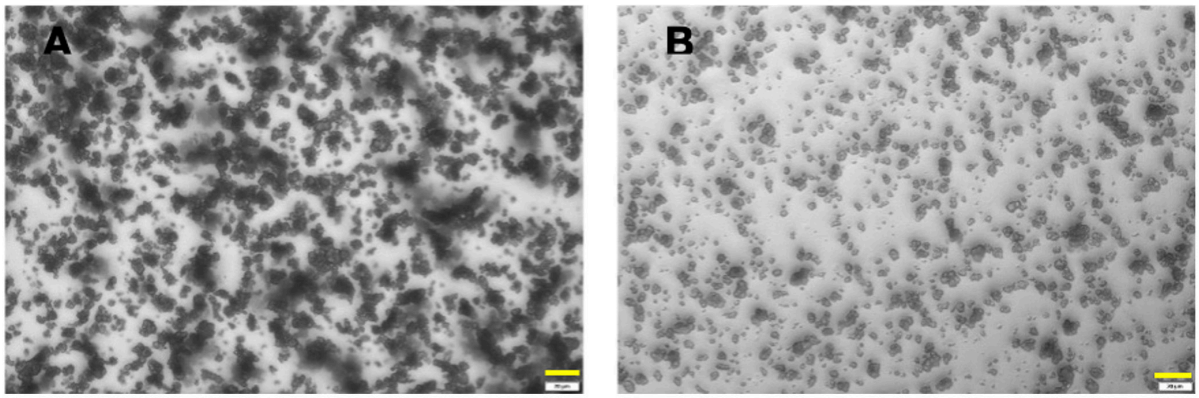

Fig. 2. Capsules with six bilayers containing MNPs in the fifth layer. (A) Capsules before the addition of HCl. (B) Capsules after the addition of 500 µl 0.1 M HCl. The image on the right shows the core has not been fully removed; however, a reduction in capsule numbers is observed (Scale bar 20 µm).

Ethylenediaminetetraacetic acid (EDTA) serves as a chelating agent with a notable affinity for calcium, and unlike HCl, it is less corrosive. To utilize EDTA for the removal of the calcium carbonate core, EDTA Disodium Salt Dihydrate from Fisher Scientific was dissolved in an aqueous solution at a concentration of 50 mg/ml. The removal process involved heating the crystals in a water bath at 50°C for one hour. Once all EDTA crystals were fully dissolved, capsules were introduced and subjected to incubation with EDTA. The results demonstrated a significantly improved reduction of the calcium carbonate core, as observed in Figure 3.

To examine the effects of EDTA treatment, capsules were imaged with an SEM (Scanning Electron Microscopy) microscope, the FEI Quanta 200 ESEM. As shown in Figure 4, calcium carbonate forms square-like crystals that are readily identifiable. In contrast, capsules treated with EDTA have irregular shapes as a result of calcium reduction.

MDA-MB-231 breast cancer cells were exposed to varying quantities of EDTA-treated capsules to evaluate the inherent toxicity of the capsules. The results of the MTT assay, presented in Figure 5, play a pivotal role in determining the optimal concentrations of capsules for delivering a potent cancer-killing compound, ensuring the capsules remain non-toxic. The depicted images in Figure 5 suggest that adding 20 µl of capsule suspension (containing 14,000 capsules) to 100 µl of cell culture suspension preserves 90% cell viability. Notably, reduced cell viability at higher concentrations could be attributed to factors such as the polymer nature (synthetic or biopolymers), competition for surface adhesion between cells and capsules, and the intracellular entry of capsules inside the cells.

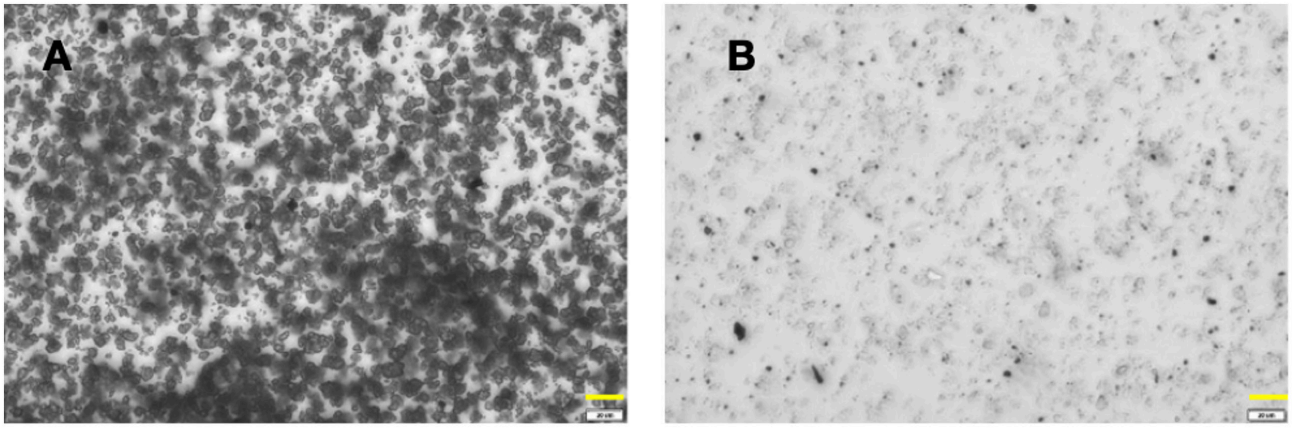

Fig. 3. (A) Capsules $(PAH/PSS)_4$/MNPs prepared on $CaCO_3$ cores A. (B) Hollow capsules $(PAH/PSS)_4$/MNPs after removing the $CaCO_3$ core with EDTA at a concentration of 50 mg/ml (Scale bar 20 µm).

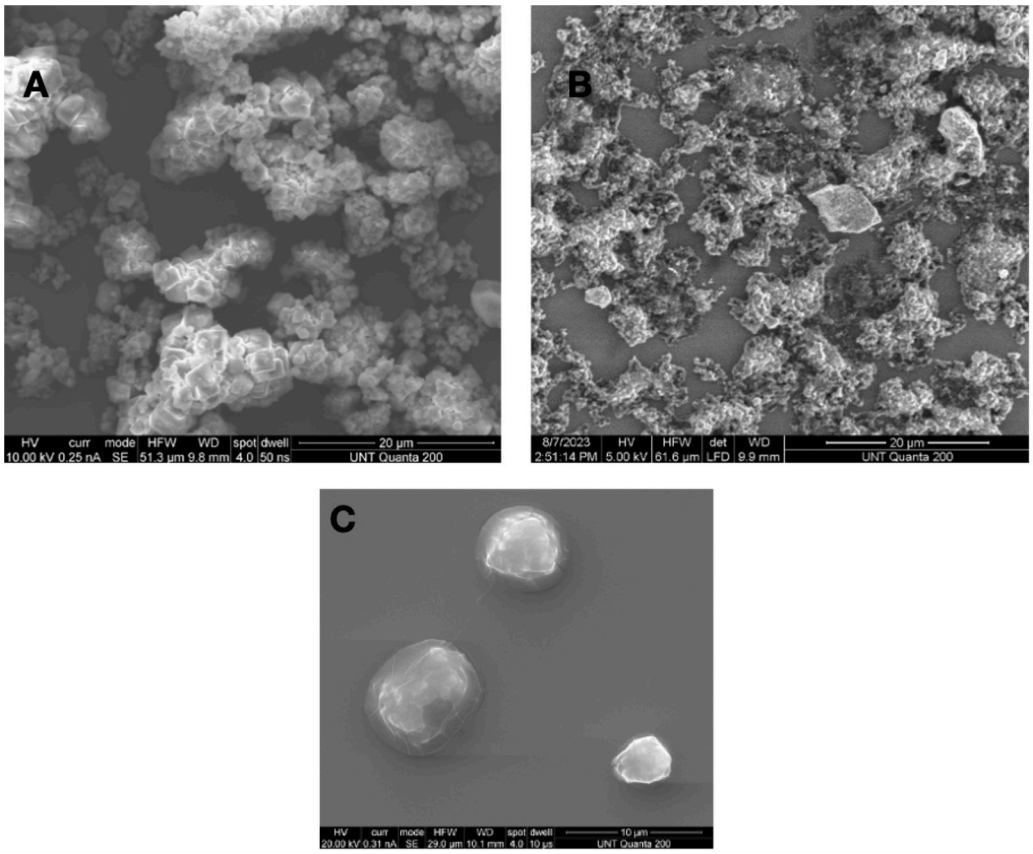

Fig. 4. SEM image of (A) Capsules $(PAH/PSS)_6$/MNPs prepared on $CaCO_3$ cores; (B and C) Hollow capsules treated with EDTA.

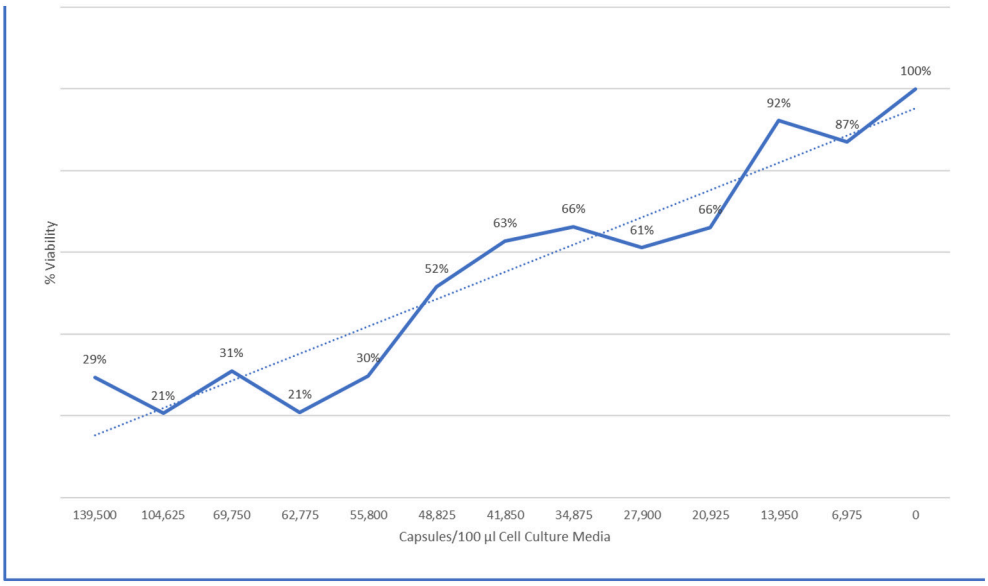

Fig. 5. MTT assay plots the viability of different amounts of capsules vs. the viability of the cells.

The LIVE-DEAD of the control cells without capsules shows that most cells are alive having green fluorescence while only few cells are displaying red fluorescence indicating being dead. The capsules at optimal concertation were exposed to cells, and Live/dead cells were analyzed. At an optimal concentration, most cells were stained green indicating being alive and few dead cells were observed (Figure 6).

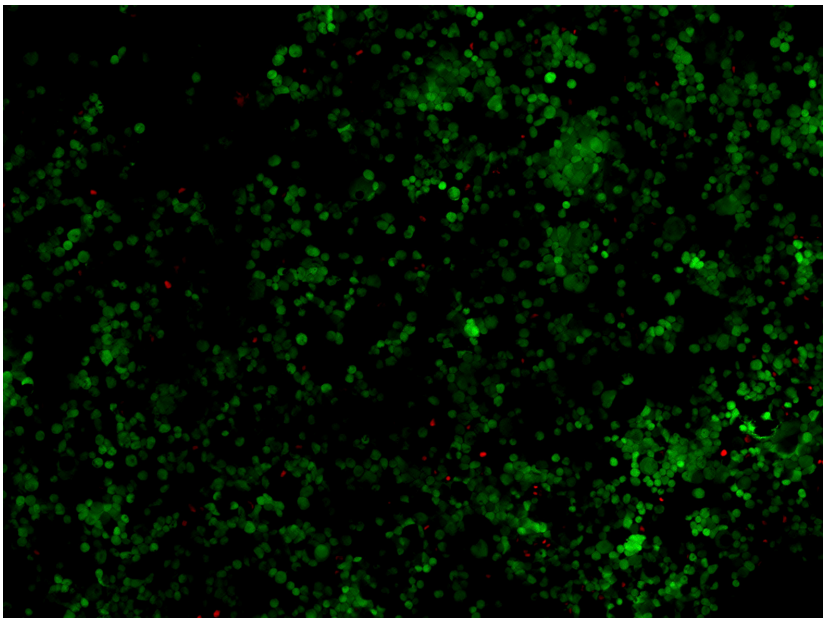

Fig. 6. Fluorescence image of LIVE-DEAD cell assay of breast cancer cells. (Live cells convert calcein AM to green, fluorescent calcein, while dead cells are stained with red fluorescent ethidium homodimer-1.)

Conclusion

In conclusion, this investigation was focused on evaluating the influence of drug carriers (PAH/PSS) MNP capsules on the viability of MDA-MB-231 breast cancer cells. Characterization of the capsules was conducted using SEM and bright-field microscopy, confirming successful synthesis as the capsules/MNPs demonstrated responsiveness to an external magnetic field. Utilizing the EDTA successfully removed the core with higher efficacy compared to HCl. Subsequent experimentation involved the introduction of various concentrations of capsules/MNPs to cells, with cell viability assessed through the tetrazolium-based MTT assay for quantitative measurements and the calcein-AM assay (Live-Dead) for real-time visualization of live and dead cells. The key finding indicated that the addition of 20 µl of capsule suspension (containing 14,000 capsules) to 100 µl of cell culture suspension preserved 90% cell viability. One of the limitations of LbL capsules is changes in pH, temperature, and mechanical forces encountered *in vivo*, which may affect the integrity and performance of the capsules, potentially leading to premature drug release, aggregation, or clearance from the body. The efficiency, yield, and size of layer-by-layer capsules are influenced by factors such as the properties of the polyelectrolytes used, pH and ionic strength, and the characteristics of the colloidal template. Optimizing these factors allows for precise control over capsule formation, leading to tailored structures with desired properties for applications in drug delivery, sensing, and controlled release systems. This outcome suggests a viable concentration of capsules for delivering compounds to cancer cells without inducing toxicity to normal cells. In summary, the study underscores the potential of the synthesized capsules as effective drug carriers for targeted cancer therapy.

Acknowledgments. We thank The Micro Nano Technology Education Center of Pasadena City College for helping initiate this collaboration and for ongoing support. We also acknowledge support from the National Science Foundation, NSF 2000281 and NSF 1720625, and National Institutes of Health grants U24 EB028887, R01 GM122424, and R16GM150848.

Disclosures. The authors declare no conflicts of interest.

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Soil is Alive: Biological, Physical, and Chemical Analysis

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Abstract: We analyzed soil samples from LA Pierce College and local farms. To assess life in the soil, 16S metabarcoding was performed. Industrial Hemp was grown traditionally at Western Fiber in Tulare CA; organic JinMa, a fiber variety, and CBD-type rootzone soil from The Rodale Institute at Camarillo CA was sampled. Noxious weeds in Marquis field at Pierce, soil from Pierce Arboretum, and a fallow proposed vineyard were also sampled. The purpose was to investigate the physical and chemical properties of soils from farms in California and to discover plant growth-promoting and antibiotic-producing bacteria from the rootzone. The hypothesis was that each environment would have a unique microbial community, and each of the fields would have similar fertility levels since they shared a prime farmland classification. We also hypothesized that the organic farm would have more diverse and abundant microbes performing ecological functions compared to the traditionally farmed soil. The main results indicated that there were significant differences in nitrogen, potassium, pH, and TDS between pairs of fields. The hypothesis was partially supported; organic matter and phosphorus levels were similar across farms. Potential plant growth-promoting bacteria and nitrogen fixers differed in proportion between sites. Greater than 80% of the *Chitinophaga* and 80% of the *Agromyces* reads identified in the study were from Camarillo, as well as more than 80% of the reads for *Pseudomonas* and greater than 60% of the *Massilia* sp. reads. The hypothesis that each environment would have a distinct community was partially supported. The hypothesis that the organic farm would have higher abundance and variety of microbes performing these functions was supported. The microbiome of hemp seemed to be more influenced by soil type and cropping method, rather than the genetic background of the cultivars. Soils need to be conditioned to retain their fertility. Periodic monitoring and monitoring before, during, and after cropping is recommended.

Keywords: metabarcoding, soil science, DNA sequencing, Agroecology, Microbial Ecology

Introduction

Soil Physical Properties

The physical properties of soil are an important factor in determining which crops to plant. Soil's physical properties are properties that can be seen and felt. Physical properties do not directly refer to chemical or biological properties but are still affected by both [1]. According to the Cornell University Agronomy Fact Sheet, "Soil texture determines the rate at which water drains through a saturated soil; water moves more freely through sandy soils than it does through clayey soils" [2]. From the different experiments, we expect to find the soil to have a moisture content between 10%- 45% and an organic matter content between 5%-10% according to previous research done on soils. Moisture is thought to be one of the most important, perhaps because it is the most immediately tangible characteristic of soil.

EC, pH, and TDS

pH represents the intensity of acidity in soil; calcium-saturated organic soil typically has a pH of 7.2 to 7.8, which is mild to moderately alkaline [3]. Regarding EC values, "Apparent soil electrical conductivity (EC) has shown promise as a soil survey tool" [3]. This means monitoring over time is necessary. TDS (Total Dissolved Solids) levels are important because, "Irrigation can contribute a substantial amount of salt to a field over the season" [4].

The accumulation of soluble salts may be an issue at 500 ppm for some sensitive plants, but is usually tolerated by most plants up to 1000 ppm. In these instances, soils are typically high in chlorides or sulfates. EC gives a more accurate measurement of soluble salts because electricity would not be conducted by pure water.

pH is important because it may ultimately lead to crop failure. The accumulation of salts is known to be associated with high pH. Excessive salts can lead to fertilizer burn. The pH of the soil is the most important factor for plants in terms of their ability to incorporate soil nutrients. Even if the necessary nutrients are present in the soil, they are not available to plants if the pH is not in the optimal range for that nutrient [5]. A good example is iron which is not available at high pH. Many soil elements change form because of reactions in the soil. These reactions, controlled by pH, alter the solubility of nutrients [1].

Soil Nutrients

The primary macronutrients, sometimes called fertilizer elements, are often not available in large enough amounts for optimal growth. The three primary nutrients— nitrogen, phosphorus, and potassium—are added to the soil by fertilization with synthetic mineral salts or organic amendments such as manure [1]. Visual cues may be used to detect excess or deficits but are unreliable for proper diagnosis. Many factors can cause issues such as chlorosis, and different species may take on different appearances. The fertilization regimen can become more efficient with soil testing, reducing the effects of contamination through runoff as well as reducing production costs. Keeping track of nitrogen, phosphorous, and potassium is important beyond the health of crops; it is also important in terms of protecting the environment.

Nitrogen and phosphorus pollution can both cause serious damage to surface waters. Nitrates cause eutrophication; phosphates destroy habitat. Potassium may create an environment that is too salty. Results from field studies indicate that rational use of manure and mineral fertilizers can help reduce the pollution problems arising from livestock farming practices [6]. In such systems, defining N rates where yields are maximized, and environmental harm is minimized could benefit both food production, human health, and the environment [7]. Testing the soil is the best way to find out how much fertilizer needs to be added to grow a productive crop without adding too much, which can contribute to input costs and environmental damage [5].

Fluctuations in nutrient levels within the soil can act as catalysts for proliferation of specific bacterial strains, shaping the microbial landscape. The interplay between nutrient availability and microbial diversity offers a promising avenue for understanding the relationships driving bacterial evolution. Recognizing the intricate connections between nutrient levels and microbial diversity provides insights into sustainable agricultural practices. Unraveling the impact of soil nutrients on bacterial discovery contributes not only to scientific understanding but also to the development of eco-friendly farming approaches.

Life in the Soil

Industrial hemp (Cannabis sativa) is a fiber crop with impressive carbon sequestration potential. For example, 1 ton of hemp stalks contains about 0.445 tons of carbon absorbed as a gas or 1.63 tons of CO2 [8]. The cannabidiol varieties of hemp are also purported to produce products that have radical scavenging capabilities. Thus, it may be useful for building materials or be therapeutically relevant. The rootzone microbes of industrial hemp have been little studied in a field crop setting. Previous research showed that rhizosphere microbes in cannabis are cultivar-specific. However, it was unknown to what extent soil types or cropping systems influence these microbes.

The technique used to assess this in our study was a comparative analysis of 16S soil metabarcoding results from 6 samples from each of two sites; in Camarillo, CA at Rodale Organic Institute where JinMa and CBD (cannabidiol) types of hemp were grown, and in Tulare, CA at Western Fiber's south side field, where the YuMa fiber variety was grown and harvested. The Tulare site had a history of traditional synthetic farming methods.

Purpose and Hypotheses

The purpose of this study was to discover plant growth-promoting and antibiotic-producing bacteria from the rootzones of plants. The hypothesis was that each soil environment would have a unique microbial community associated with it. This hypothesis was developed by considering the differences in geography and the fact that the soil is sandier at the Tulare site and contains more clay at the Camarillo site. We also hypothesized that the organic farm in Camarillo would have more diverse and abundant microbes performing these essential functions, when compared to the traditionally farmed soil in Tulare. This is because organic farming is generally considered to be more biodynamic than traditional or synthetic farming methods.

Overview and Importance

The soil metagenome consists of the 16S ribosomal genes detected in a soil sample by Next-Generation Gene sequencing techniques (Massive Parallel Sequencing, such as Illumina). The metagenome is a snapshot, both qualitatively and quantitatively, of the microbial community present in the soil. Biologic activity is an important indicator of the soil's health. The great multitude of functions of the soil's microbiota is impressive. Major planetary nutrient cycles are involved and dependent on the microbiota of the soil and are of vast importance to both natural ecosystems as well as agricultural endeavors. Experiments that assess the metagenome are of great importance in informing best practices associated with organic and regenerative farming.

The soil hosts billions of bacteria, but not all species can be readily cultured [9]. The number of culturable bacterial cells in soil is generally believed to be only about 1% of the total number of living cells present. However, in environmentally stressed soils, the number of culturable bacteria may be as low as 10^4 cells per gram [10]. This emphasizes the importance of metabarcoding and other cultivation-independent techniques. Metabarcoding as it now exists is novel, particularly in its ability to detect thousands of taxa quickly in a single sample and in an economically feasible fashion - opening gateways for research to further understand plant-microbe interactions, plant physiology, microbial characteristics, and anticipating potential threats from pathogens. There is also the potential to identify new species, which may be important to agricultural technologies.

The Rodale Institute has been involved in the research and development of organic regenerative agriculture and has not applied synthetic fertilizers, pesticides, or herbicides to any of the fields that have been sampled. Furthermore, the Rodale Institute has experimented with various cropping systems and policies of leaving crop residues post-harvest, cover-cropping, green manuring, and not allowing fields to lie fallow. The recent history of Western Fiber's field is in line with the current agricultural method of utilizing synthetic herbicides, insecticides, and fertilizers, with fields and orchards typically existing as monocultures and often left fallow during non-growing seasons. Conventional tillage systems have been shown to break down soil structure and diminish the quantity of soil bacteria [1].

Methods

Soil Physical and Chemical Properties

During Fall 2021- Spring 2022, 51 soil samples from Los Angeles Pierce College farm and local farms were analyzed for soil chemical and physical properties. Industrial Hemp YuMa plots in Tulare grown at Western Fiber were sampled for soil. In Camarillo, JinMa fiber variety and CBD type rootzones were sampled for soil. Noxious weeds growing in Marquis field at Pierce College, soil from the Pierce Arboretum, and soil from fallow ground from a proposed vineyard site were also sampled.

A B

Fig. 1.The Google Earth images of the sampling locations at Rodale Organic Institute (A) and LA Pierce College (B) are pictured.

Moisture, organic matter, EC, TDS, pH, N, P, and K were determined using the methods described in the St. Clair Soil Science Lab Manual. Moisture % was determined by the oven dry method. Organic matter percentage was determined by dry combustion. A 1:5 soil-to-water ratio was used for soil pH measurements. N, P, and K were determined using Hach color-changing reagents, and %T was measured using a Genesys 20 spectrophotometer. Soil physical and chemical properties from the sites were also measured, including EC, TDS, and texture by touch. Colony forming units (CFUs) were also quantified. Comparisons between fields were carried out in the R stats package. Since there was an indication of non-normality and unequal variance, the nonparametric Kruskal-Wallis regression method was used to analyze the data.

Soil Metabarcoding

During Fall 2021, 22 soil metabarcoding samples were collected from Los Angeles Pierce College farm and local farms, DNA was extracted, and samples were sequenced on Illumina. The samples and their associated locations are referred to as follows: JinMa and CBD are from Camarillo, Ventura County, and Industrial Hemp N_side, and S_side samples are from Lemoore, Tulare County.

The data consists of 16S amplicons. DNA was extracted with the Qiagen (Hilden, Germany) Power Soil DNA kit. Quantification of the DNA extracted was achieved with spectrophotometry using the Thermo Fisher MultiSkan SkyHigh Microplate Spectrophotometer. DNAs were then sent to James Madison University for 16S amplification and library preparation, and 16S amplicon NGS was pooled on the Illumina (San Diego, CA, USA) MiniSeq platform. The V4 region of the bacterial 16S rRNA gene was amplified and barcoded for each sample using the primers developed by Kozich et al [11]. Samples were pooled. A double-sided bead cleanup was carried out. The quality and concentration of the pooled library were checked using a Bioanalyzer (Agilent, Santa Clara, CA, USA) and NEB's Library Quant Kit for Illumina. The library was sequenced on a MiniSeq using a mid-output reagent cartridge. Before loading, the library was combined with Illumina's PhiX control (30:70 16s: PhiX) to ensure a high-quality run despite the problem of relatively low diversity in the 16S library. Metabarcoding data analysis was carried out using the DNA Subway Purple Line [12] using QIIME 2 [13] and Emperor [14]. DNA Subway was used for bioinformatics; the Purple Line analysis implemented DADA2 and QIIME2 for quality control, alpha rarefaction, and the output of the ASV table for

taxonomic diversity analyses. Using MS Excel pivot tables, the relative frequency of reads from different functional categories of microbes was visualized [15].

Results and Discussion

Soil Physical and Chemical Properties

Data showed that the conditions between the fields differed significantly in values for organic matter, N, K, TDS, and pH ($p<0.05$); this offered a diverse panel of substrates for the discovery of bacteria performing beneficial functions to plants and humans. A visual representation of the correlation matrix is given in Figure 2. It is apparent that Potassium concentrations are making the highest contribution to EC and TDS from the elements measured, based on the Pearson correlation matrix. It also appears that the CFU concentrations for the 10^-5 dilution were mildly associated with higher N and P levels. As expected, EC and TDS values were 100% correlated.

According to the results of nonparametric Kruskal-Wallis regression [16]. Field was associated with OM ($p=0.02$). There was a trend toward the Arboretum and Marquis C samples exhibiting the highest OM percentages. However, the results of the pairwise Wilcoxon rank sum test indicated that there was no significant difference in OM between the groups, based on the adjusted p-values using the BH correction (BH correction= Benjamini-Hochberg false discovery rate).

According to regression results, Field was associated with N ($p=0.02$). There was a trend toward higher nitrogen levels at the proposed vineyard site, and the lowest N levels were from Rodale Institute. Most samples had low values for Nitrogen in ppm. No significant pairwise differences were detected when using the BH-corrected p-values and a cutoff of 0.10.

Field was associated with K ($p=3.24*10^{-5}$). In the pairwise Wilcoxon rank sum test, the Potassium levels of Marquis A soil samples varied from the Arboretum soils, and Western Fiber soils from Tulare differed from Rodale soil K levels, using a marginally significant p-value cutoff of 0.10. According to the results of nonparametric Kruskal-Wallis regression, Field was associated with pH ($p=0.002$). In the pairwise Wilcoxon rank sum test, the pH of Marquis A soil samples varied from the Arboretum soils, and Western Fiber soils from N. side Tulare differed from Rodale soil pH levels, using a marginally significant adjusted p-value cutoff of 0.10.

According to the results of nonparametric Kruskal-Wallis regression, Field was associated with TDS ($p=8.49*10^{-6}$). In the pairwise Wilcoxon rank sum test, the TDS of the Arboretum soils was significantly different than N. side Tulare field soils ($p\text{-adj}<0.05$). Furthermore, the Marquis C Drain soil samples varied significantly in TDS from the Marquis A soils ($p\text{-adj}=0.03$), and Marquis A was significantly different in TDS than N. Side Tulare fields. Marquis B and Rodale samples were also significantly different in TDS when compared with Tulare N. Side samples ($p\text{-adj}=0.03$). The highest TDS values were from Marquis B and the proposed vineyard site (Figure 2). The BH correction was applied.

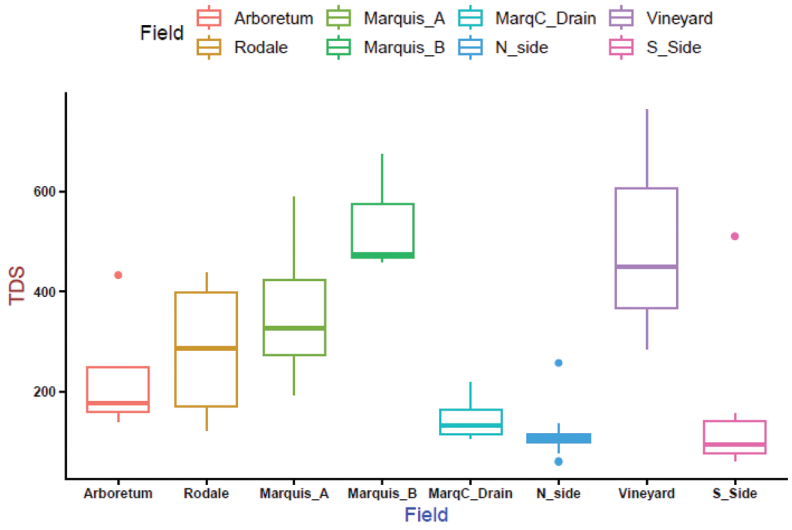

Fig. 2. A comparison between the TDS values of the fields is shown.

The tested sites, characterized by the lowest nitrogen levels among the fields, present an intriguing scenario. Nitrogen scarcity often acts as a selective pressure on microbes, encouraging the emergence of bacteria with specialized adaptations for nitrogen acquisition. Microbial communities, being highly adaptable, strategically adjust their physiology based on nutrient availability. Potassium influences microbial dynamics, and the correlation between potassium concentrations and Electrical Conductivity (EC) raises the question of whether microbial abundance may respond to varying potassium levels. Colony Forming Unit (CFU) concentrations for the 10^-5 dilution exhibit a subtle association with higher nitrogen (N) and phosphorus (P) levels. This suggests a microbial response to varying nutrient levels, hinting at a potential correlation between nutrient availability and microbial abundance.

Some bacteria can increase iron availability in conditions of high pH. *Bacillus*, which was detected in our Tulare samples, is a genus known to catabolize and exude siderophores (compounds acting as iron chelators) into the rhizosphere, which create Fe availability in soils that would otherwise have too alkaline a pH for sufficient iron mobility in the soil, thus increasing the upper limit of the pH range tolerable to any specific crop; iron availability is naturally limited at high pH [17].

Beta Diversity Analysis

In the beta diversity analysis using the unweighted UNIFRAC distance for a principal coordinates visualization of the 16S data, the Camarillo samples from both CBD and JinMa varieties appeared to be very similar based on their genetic distances (Figure 3). Meanwhile, the cluster of samples from Camarillo appeared to separate from the Tulare samples. The Tulare industrial hemp-planted and postharvest plots also appeared very similar, based on their projected distance on the PrinCoA graph.

Principal coordinates analysis is a method commonly employed in the analysis of beta diversity, which is the similarity or dissimilarity between samples or groups of samples. The UNIFRAC distance has been shown to be an effective distance metric in simulation studies [18]. The first three axes of the principal coordinates accounted for 42.54% of the variation. Industrial hemp associated soil samples from Tulare tended to be low on Axis 2 and high on Axis 3, whereas the soil samples from Camarillo were high on Axis 2 and high on Axis 1. The postharvest samples from S. Side 6 appear to be like the industrial hemp plots. These samples, six samples from Camarillo and six samples from Tulare will be the focus of the remainder of the metabarcoding analysis, due to this interesting observation and the lack of sufficient replicates for the Pierce farm samples, some of which failed sequencing.

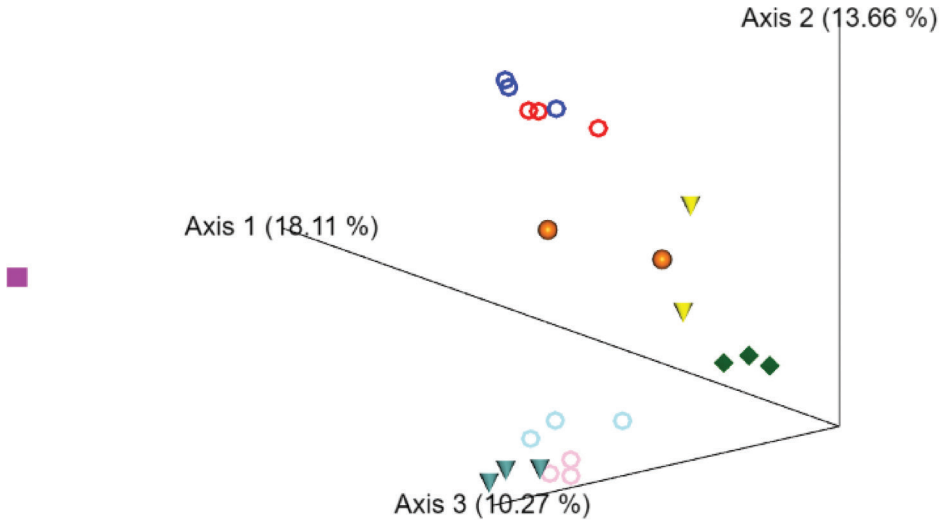

Fig. 3. The beta diversity comparison involved visualizing the first three axes of Principal Coordinates Analysis. The distance between sample groups reflects the genetic distance (between group diversity). Pink rings: Tulare S. Side Plot 3 Yu-Ma Industrial Hemp; Light Blue rings: Tulare S. Side Plot 2 Yu-Ma Industrial Hemp; Green cones: Tulare S. Side Plot 6 Postharvest; Dark Green diamonds: Tulare N. Side Plot 6 Fallow; Orange spheres: Los Angeles Marquis A fallow; Yellow cones: Los Angeles Ridge Vineyard Site Fallow; Red rings: Ventura CBD Trial; Blue rings: Ventura JinMa trial; Purple square: positive control.

Analysis of Bacterial Taxonomic Abundance

To gain further insights into which genera may be performing essential ecological functions at the sites where industrial hemp was grown, Excel pivot table analysis was performed using the methods described in the Pipeline for Undergraduate Microbiome Analysis [15]. The bacterial genera found in the different samples were compared and visualized. The functional classification was based on their putative ecological functions from the literature.

In terms of potential antibiotic producers, the genera Actinoplanes, Lysobacter, Bacillus, and Pseudomonas were well represented in the Camarillo samples. However, in Tulare samples, Actinomadura, Bacillus, and Streptomyces sp. had the highest abundance of reads. Low counts were also present for Lysinibacillus. The genus Lysinibacillus includes entomopathogenic bacteria that produce antifungal and antibacterial compounds [19].

Serratia species such as S. marcescens have been shown to promote growth in wheat seedlings and have a full suite of active nitroreductases [20]. Streptomyces are known for denitrification abilities, which is part of its suite of plant growth promotion characteristics [21]. *Vibrio* is a pathogen that uses nitrate reduction as a strategy. *Vibrio cholerae* uses nitrate reduction to determine how much it should grow its population under anaerobic and changing pH conditions [22]. Similarly, *Vibrio* sp. collected from sediments of Scottish estuaries have been shown to produce nitrite and ammonia from nitrate [23]. *Pseudomonas* such as *P. aeruginosa* are able to denitrify soil under anaerobic conditions [24]; a new nitrate-reducing species, *Pseudomonas oligotropha*, was recently discovered [25]. *Achromobacter* has denitrifying bacteria such as *A. denitrificans*, which lives only in aerobic conditions and was isolated from soil [26]. Bacilli such as *B. subtilis* are valued for their biofertilizer applications, including their ability to reduce nitrogen in agricultural soils, which reduces the loss of nitrate that typically occurs readily following a precipitation event [27]. Nitrogen reducers were represented by *Achromobacter, Bacillus, Pseudomonas, Serratia, Streptomyces*, and *Vibrio* in the Camarillo samples. In the Tulare samples, nitrogen-reducing bacteria were represented by *Bacillus* and *Streptomyces*.

In terms of potential plant growth-promoting bacteria and nitrogen fixers, 100% of the *Brevibacillus* reads identified in the study were in the Camarillo soil samples. Greater than 80% of the *Chitinophaga* and 80% of the *Agromyces* reads identified in the study were from Camarillo, as well as more than 80% of the reads for *Pseudomonas* and greater than 60% of the *Massilia* sp. reads. Furthermore, more than 70% of the reads for *Flavobacterium* sp. belonged to the Camarillo samples (Figure 4).

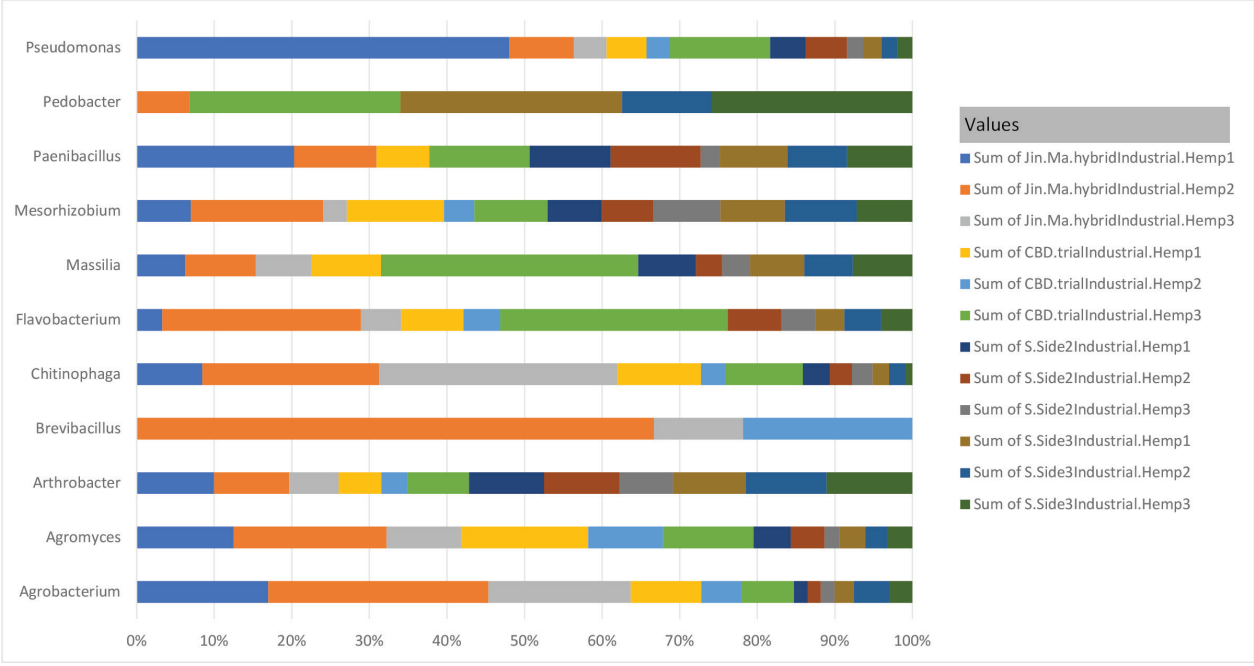

Fig. 4. The relative abundances of several potential plant growth promoting bacteria are shown for Tulare and Camarillo samples.

More than 60% of the reads from *Pedobacter* were from Tulare. It is interesting because *Pedobacter* and *Flavobacterium* are related, and known for antioxidant production but were not found to be equally abundant at both sites. Greater than 55% of *Arthrobacter* sp. reads were from Tulare. *Paenibacillus* reads were split evenly between sites; *Mesorhizobium* reads were also found in similar proportions at both soil sampling sites. Among the remaining suspected nitrogen fixing genera, reads were more prevalent in the Tulare samples for *Arthrobacter, Bradyrhizobium*, and *Devosia*. Conversely, *Sinorhizobium* sequences were only found in Camarillo soil samples, and of the sequences that were assigned to *Rhizobium*, 60% belonged to samples from Camarillo.

Potential prokaryotic contributors to essential ecological functions in the industrial hemp rootzone were identified. Although there were differences in the most prevalent species at each site, all communities had players in each of the functional roles studied here: Antibiotic production, nitrogen fixation, denitrification, plant growth promotion, and pesticide and insecticide degradation. Variations in the community may be explained by both the plants grown at the location and their associated rhizosphere, as well as the soil conditions and current cultural practices of agriculture, performed at those specific locations.

Bacteria from the plant rhizosphere alter plant growth, recruiting or repelling certain species. Soil biota from bulk soil provides the seed bank for the rhizosphere biota, creating a cycle. In this consideration, simply growing hemp or any other plant may act as a bioinoculant, which may modify edaphic soil conditions, which will, in turn, become an inoculant to future rhizospheres. This stresses the importance of not allowing a field to lay fallow for an extended period. As performed in another study [28], future work could involve the collection of bulk soil samples (edaphic soil), rhizosphere samples, and endosphere samples. A bulk soil sample would be useful to serve as a control by holding the fallow condition constant and reflecting the land use and soil factors. Future planned studies should work on the isolation of beneficial microbes that were unclassified to the species level from the organic farm in order to provide more tangible evidence of the claims made in this study. This would also lend itself to the potential discovery of novel soil bacteria. Including fungi in the next round of sequencing using an ITS (internal transcribed spacer) region [29] is also of interest.

Conclusion

The main results indicated that there were significant differences in nitrogen, potassium, pH, and TDS between pairs of fields. There was a subtle association between higher N and P levels and CFUs for the 10^-5 dilution. Therefore, the hypothesis was partially supported, particularly for organic matter and phosphorus levels, which were similar across farms. The hypothesis that each environment would have a distinct community was partially supported. The hypothesis that the organic farm would have a higher abundance and more variety of microbes performing these essential functions was supported. A surprising result was that the microbiome of industrial hemp seemed to be more influenced by soil type and cropping method rather than the genetic background of the cultivars.

This comparative analysis contributes to the literature a novel investigation of soil microbes in the industrial hemp rootzone. The limitations of the study involve the need to have a more rigorous experimental design and allow for more statistical analysis and biochemical testing. The sites with the unique combination of low nitrogen, significant potassium, and high pH have likely shaped a distinctive microbial community. This discovery emphasizes the intricate interplay between soil nutrient levels and microbial adaptations, paving the way for sustainable agricultural practices and microbial-based solutions for soil management. Soil needs to be conditioned, with cover crops and amendments over time to retain their fertility. Periodic monitoring before, during, and after cropping are necessary. In order to draw a strong conclusion about the significance and interaction between soil conditions and cultivar types, a design that tests soils from the same cultivar at multiple sites would be helpful in controlling for some of the variables.

Acknowledgments. This work was supported by training and resources from the National Science Foundation IUSE grant, NSF DUE-1821657, awarded to Bruce Nash and Ray Enke in part to support Community College faculty. This material is based upon work supported by the National Science Foundation under Grant Nos. DBI-0735191, DBI-1265383, and DBI-1743442, which supported iPlant Collaborative and DNA Subway development. Metabarcoding reads are from public project 7929 on Cyverse.org DNA Subway Purple Line.

Disclosures. The authors declare no conflicts of interest.

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The Annotation of the Complete Genome of the Mycobacterium phage Inverness

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Abstract: The gene annotation of the Mycobacterium phage Inverness was performed to establish certain genetic characteristics and qualities of the phage. Our research was designed to investigate the potential usefulness of this phage for medical purposes as part of the SEA-PHAGES project. Due to the decline in effectiveness of antibiotics when treating bacterial diseases, demand for alternative therapies and treatment has grown substantially. Phages have the potential to meet this demand. The genome of Inverness was found to be 68,264 base pairs in length, to possess a GC content of 66.5%, and to contain 99 protein-coding genes. Based on nucleotide similarities, the Mycobacterium phage Inverness was placed into cluster B and subcluster B1. The 33 genes with identifiable functions had an almost even split between rightward (49.49%) and leftward (50.51%) oriented genes. No putative function could be identified for 66 genes. No tRNAs were found to be present within the genome for this specific phage. It should also be noted that, among those genes with identified functions, two codes for lysins, which are proteins that kill bacteria cells. This suggests potential future opportunities to use this phage as a treatment for certain cases of antibiotic resistant infections.

Keywords: bacteriophage, gene annotation, bioinformatics

Introduction

The Science Education Alliance-Phage Hunters Advancing Genomics and Evolutionary Science (SEA-PHAGES) Program is a course-embedded research program where students search for new bacteriophages in soil samples, perform a variety of laboratory techniques, and complete a complex genome annotation. The goals of the project are twofold: first, to increase undergraduate student interest and retention in the biological sciences, and second, to identify phages that can be used to treat antibiotic resistant infections [1]. The SEA-PHAGES project is jointly run by the Howard Hughes Medical Institute's Science Education division and Graham Hatfull's laboratory at the University of Pittsburgh. Participation in the SEA-PHAGES project has been shown to increase student retention and to influence career choices [1]. Students at Connecticut State Community College, Northwestern campus, participated in the SEA-PHAGES program while taking a Molecular Genetics course. As part of this course, students performed the structural and functional annotation of the Mycobacterium phage Inverness to determine its potential to treat antibiotic resistant infections.

Antibiotic resistant infections occur when mutation of the infecting bacterium prevents its destruction by antibiotics. Infections with antibiotic resistant organisms can be difficult or impossible to treat. According to the World Health Organization, 1.27 million people died in 2019 due to infections with antibiotic resistant organisms [2]. Bacteriophages have proven successful as a last resort in treating antibiotic resistant infections and are being tested in clinical trials [3].

Phages, also known as bacteriophages, are viruses that infect and replicate within bacterial cells. They are the most prevalent biological agent on Earth and are found everywhere in the environment [4]. The size, shape, and genetic structure of phages exhibit remarkable diversity [4]. All phages are made up of a nucleic acid genome covered in a capsid protein shell which protects the genetic information and facilitates its transfer to host cells. Many phages have tails that are used to deliver the genome into the host cell. The ability of phages to lyse bacterial cells makes them potentially effective in treating patients with antibiotic resistant infections [5]. To understand the potential for a phage to be used to treat patients, the genome of the phage must be

annotated. Gene annotation involves the comprehensive process of detecting and characterizing genes within a genome. It starts by using computational gene prediction tools to identify potential genes based on genomic features. Structural annotation involves identifying the boundaries of the genes and functional annotation involves assigning putative functions to gene products. Gene annotation is crucial for understanding a phage's genetic traits, which must be considered when evaluating a phage for the possible treatment of antibiotic resistant infections. For a phage to be used as treatment "the phage genomes should not include any genes known or suspected to be toxic" [3].

Methods

Obtaining the Sequence of the Mycobacterium phage Inverness

The work to identify, isolate, purify, extract, and sequence DNA was not performed as part of this research project. However, a brief description of those processes is described here.

The Mycobacterium phage Inverness was collected from a bag of Miracle-Gro® potting soil obtained in Fort Collins, Colorado, USA. It was isolated, purified, and amplified by Sean Anderson of Rocky Mountain High School in Colorado, as part of the Phage Hunters Integrating Research and Education (PHIRE) program [6], using *Mycobacterium smegmatis mc*^{2} *155* as a host [7].

DNA extracted from the phage was sent to the Pittsburgh Bacteriophage Institute for sequencing. The Pittsburgh Bacteriophage Institute completed sequencing on December 20th, 2020, using Illumina Sequencing, with an approximate shotgun coverage of 511. The shotgun method of sequencing involves breaking the genome up into pieces, sequencing each piece and then reconstructing the entire genome. The shotgun coverage number, in this case, 511, describes the average number of reads that align to the reference database.

The sequence information was used to create the FASTA file. The FASTA file containing the text-based sequence of the nucleotides for the phage genome was uploaded into the PhagesDB database by the SEA-PHAGES project administrators [8].

Annotation Process

The FASTA file was obtained from the PhagesDB database and loaded into DNA Master v5.0.2, a gene exploration and annotation tool used to predict the probable genes in the sequence [9]. The Mycobacterium phage Inverness genome sequence was also run through the evidential programs contained within The Phage Evidence Collection And Annotation Network (PECAAN) version 20221109 [10]. PECAAN was utilized to compile data from several other databases for comparison [10].

Within PECAAN, gene start and stop recommendations came from the Gene Locator and Interpolated Markov ModelER (Glimmer) system v3.02b, along with the GeneMark v4.28, and Starterator v1.2 systems [11,12]. GeneMark was also utilized for determining coding capacity [11].

This information along with the Z-score, gap or overlap between genes, the final score, and coding capacity were used to select the best starting position for each gene.

The Z-score provides the standard deviation of a score when compared to the best scores from all possible start positions in the genome. The Z-score provides the standard deviation of a score when compared to the best scores from all possible start positions in the genome. DNA Master and PECAAN produce Z-scores for the various possible starting positions of each predicted gene. While the exact values of the Z-scores vary for each gene, the best Z-score is one that is closest to 2. The starting position selected for each gene is based on selecting a position that has a calculated Z-score that is closest to 2.

In addition, the Genemark map was used as an effective visual reference for the regional coding potential of prospective gene candidates, and Actinobacteriophage Phamerator, version 567, was used to compare related phages in subcluster B1 [13].

Gene functions were determined by comparing the protein sequences for each gene to previously annotated genomes. PECAAN provided protein analysis recommendations from BLASTp v2.13.0., the Protein database and Non-Redundant Protein Sequences database, and HHPred v2.08 as well as NCBI_Conserved_Domains (CD) databases [14,15]. These databases are used by the PECAAN algorithms to detect similarities between proteins. The evidence selected was based on evaluating probability ratings and e-values, which measure the significance between sequences.

The Transmembrane Helices: Hidden Markov Model, TMHMM, provided evidence on transmembrane protein predictions [16]. Transmembrane proteins play a role in controlling the lysis of bacteria after infection by a bacteriophage. Phages with transmembrane proteins have the potential to kill bacteria and, therefore, to be used to treat antibiotic resistant infections.

TRNAscan and Aragorn programs were used to look for the presence of tRNAs [17,18]. The full annotation from PECAAN was run through DNA Master to create the minimal file suitable for submission to the PhagesDB website following SEA-PHAGES protocols.

A quality control check was performed before the complete genome was submitted to GenBank by the SEA-PHAGES' administrators who check to make sure that the guiding principles for gene annotation were followed and that all the functions call are allowable functions [19].

Results and Discussion

The Mycobacterium phage Inverness has a 68,264 base pair long genome with a GC content of 66.5% with a circularly permuted genome end character. Genomes with circularly permuted genomes form circular molecules upon injection into the host. These circular molecules can be used for replication of the phage. The genome annotation identified 99 protein-coding genes, with a nearly equal distribution between the rightwards (49.49%) and leftwards (50.51%) genes.

Thirty-three genes were assigned putative functions, but no functions could be identified for the remaining 66 genes.

Proteins involved in capsid structures are clustered between genes #9 and #13. Proteins involved in the tail structure are located between genes #18 and #41. The tape measure protein was identified as being coded for by gene #28. This gene determines tail length. Long-tailed phages have a tape-measure protein gene consisting of 2,000 or more base pairs.

The tape measure protein gene in the Mycobacterium phage Inverness is 5,976 base pairs long and codes for 1,991 amino acids, indicating that it has a long tail. Figure 1 shows the size of the tape measure protein gene when compared to the genes for the tail assembly chaperone and the minor tail protein.

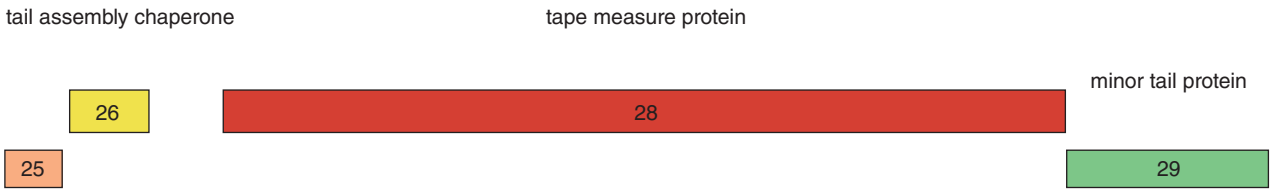

Fig. 1: This figure, taken from the Phamerator Database [13], shows the relative size of the tape measure protein gene (#28) when compared to the tail assembly chaperone genes #25 and #26 and the minor tail protein #29. The reverse gene #27 is not shown.

Genes assigned putative functions include those coding for structural proteins like RuvC-resolvase and for enzymes involved in DNA replication and packaging like DNA helicase, DNA primase, and HNH endonuclease.

DNA Helicase is the enzyme responsible for the break of the hydrogen bonds between DNA strands during the process of DNA replication. DNA primase is also used during DNA replication and is responsible for adding primers or starting sequences to the strands. This allows DNA polymerase to add new nucleotides to the growing nucleotide strands. HNH endonuclease is a small DNA-binding protein that can cleave the covalent bonds between nucleotides in a strand.

Genes that code for Lysin A and Lysin B were identified as #48 and #49, respectively. Lysins are enzymes that destroy bacteria cell walls. This leads to the death of the bacteria cells and indicates that this phage has the potential to be used to treat antibiotic resistant infections.

No tRNAs were found, indicating that the phage uses the host tRNAs in the translation process.

The complete genome annotation can be found in the National Library of Medicine's GenBank Database under accession number OR159656 [20].

The full list of genes with identified functions can be found in Table 1.

Table 1 Genes with Identified Function

Gene Number	Length in basepairs	# of Amino Acids	Product
1	567	188	Adenylate Kinase
2	1788	595	Terminase
6	555	184	RUVC-like Resolvase
8	1905	634	Portal Protein
9	2574	857	Major Capsid and Fusion Protein
10	435	144	HNH Endonuclease
12	1746	581	Major capsid Hexamer Protein
13	804	267	Major Capsid Pentamer Protein
15	309	129	Holin
18	801	266	Major Tail Protein
20	777	258	Queuine tRNA Ribosyltranferase
22	738	245	Head-to-Tail Adapter
25	423	140	Tail Assembly Chaperone
26	564	187	Tail Assembly Chaperone
28	5976	1991	Tape Measure Protein
29	1434	477	Minor Tail Protein
30	1113	370	Minor Tail Protein
31	2256	751	Minor Tail Protein
32	1347	448	Minor Tail Protein
33	1161	386	Minor Tail Protein
41	408	135	Tail Fiber
45	216	71	Helix-turn-Helix Binding Domain Protein
46	525	174	Helix-turn-Helix Binding Domain Protein
48	1329	442	Lysin A
49	1356	451	Lysin B
51	1404	476	Exonuclease
52	1704	567	DNA Helicase
57	2748	915	DNA Primase
59	1860	619	DNA Replicase
66	180	59	Ribbon Helix-turn-Helix Binding Domain Protein
68	699	232	DNA Binding Protein
82	303	100	HNH Endonuclease

Conclusion

The Mycobacterium phage Inverness was assigned to cluster B and subcluster B1 based on the nucleotide similarities to other phages in the clusters. Like other subcluster B1 phages, Mycobacterium phage Inverness infects mycobacterium and has a GC content of 66.5% and a base pair length of 68,264 [21]. Based on its gene content, it is predicted to be of the siphovirus morphotype. Phages with this morphotype have long, flexible tails that are non-contractible and have heads that are hexagonal and icosahedral.

The capsid size, head, and tail length of Mycobacterium Phage Inverness are unknown since electron microscopy was not performed. However, it was determined that gene #12 codes for a hexamer major capsid protein, confirming that the head is hexagonal. The tape measure gene (#28) was found to be 5,976 base pairs long, confirming that the tail is long.

The Mycobacterium Phage Inverness contains two genes that code for lysins. Lysin A is coded for in gene #48, and Lysin B is coded for in gene #49. Lysins disrupt the complex structures of bacteria cells, leading to rapid cell lysis [22]. This action kills the bacteria cells and offers a promising solution to combat infections caused by antibiotic-resistant strains of Mycobacterium.

Acknowledgements. We acknowledge support from the Science Education Alliance-Phage Hunters Advancing Genomics and Evolutionary Science (SEA-PHAGES), Howard Hughes Medical Institute, Chevy Chase, MD, including support from S.M.R. Gurney, Claire Reinhart, D.A. Russell, Deborah Jacobs-Sera, Vic Sivanathan, and Grahm Hatfull. The National Science Foundation (NSF) Advanced Technology Education Grant under award #1081062 provided research support for the authors.

Disclosures. The authors declare no conflicts of interest.

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Comparison of Cryopreservation Conditions on the Performance of NISTCHO Cells

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Abstract: The NISTCHO cell line is a recombinant Chinese hamster ovary cell line engineered to produce cNISTmAb, a monoclonal antibody that recognizes the fusion F glycoprotein on the surface of respiratory syncytial virus (RSV). These cells are invaluable as a standard reference material for developers of therapeutic monoclonal antibodies and serve as an educational resource in biomanufacturing training programs. This study investigates the performance of NISTCHO cells following cryopreservation at temperatures of -80°C and -150°C. Initial cell viability, maximum cell density in culture, and monoclonal antibody production were compared for cells cryopreserved for up to 30 weeks. Cells were thawed and cultured at two-week intervals to monitor their growth behavior, peak cell densities, and antibody production levels. Analysis of cell behavior in culture revealed no significant differences in cell growth or cell production between cells stored at -80°C and those stored at -150°C. These findings affirm that NISTCHO cells can be preserved at -80°C for up to 30 weeks without any adverse effects on their growth or monoclonal antibody production capabilities, an important finding for training and education programs that rely on -80° C freezers to store cell banks.

Keywords: NISTCHO, monoclonal antibody, biomanufacturing, cryopreservation

Introduction

The NISTCHO cell line, a collaborative creation by the National Institute of Standards and Technology (NIST) and Millipore Sigma, represents a significant advancement in biotechnological research tools. This open- access recombinant Chinese hamster ovary (CHO) cell line was derived from genetically engineering CHOZN™ cells [1] to express a monoclonal antibody (mAb) targeting a surface glycoprotein, the F protein of the Respiratory Syncytial Virus (RSV) [2]. The NISTCHO cell line was created using a glutamine synthetase (GS) selection system to select stable, high-producing clones by supporting their survival and growth in glutamine-free media. Monoclonal antibodies (mAbs) are the largest subset of protein biotherapeutics and represent the industry's fastest-growing segment due to their specificity and efficacy in treating a wide range of diseases. Currently, mAbs account for a significant proportion of new therapeutics, including treatments for cancer, autoimmune diseases, and infectious diseases, underscoring their critical role in modern medicine and the biotechnology industry. Monoclonal antibody therapeutics are twice as likely to succeed in clinical trials than small molecule drugs; they currently account for nearly a fifth of the FDA's annual new drug approvals, with an average of ten yearly approvals [3]. Monoclonal antibody-producing NISTCHO cells are an industry standard cell line capable of growing to high densities and producing a high mAb titer. They are an important reference cell line and provide a practical framework for studying cell growth dynamics and mAb production in a controlled, small-scale environment [4]. NISTCHO cells, like most mammalian cell lines, are cryopreserved at ultralow temperatures, such as in the vapor phase of a liquid nitrogen tank or dewar where the temperature is -150°C to -196°C. These conditions are considered optimal for storage, resulting in minimum cell damage and maximum cell performance upon resuscitation [5]. Knowing how these cells behave under different cryopreservation conditions is important since teaching laboratories often have limited access to liquid nitrogen dewars and ultralow temperature freezers. For this reason, we conducted growth studies on NISTCHO cells that were cryopreserved at two distinct temperatures, -80°C and -150°C. The cells were compared after cryopreservation for 2, 4, 6, 8, 10, 14, 26 and 30 weeks. Following resuscitation, the cells were cultured in 30ml of medium in small-scale shake flasks and monitored daily for cell viability and cell density

for nine days. The culture medium was then collected, and the mAb was purified and quantified to provide an estimated mAb titer for each culture. Comparative analysis of these metrics between the two cryopreservation conditions formed the crux of our investigation.

Methods

Cryopreservation

Working cell banks of NISTCHO cells passage 12 were established and stored at either -150°C or -80°C. Cells were cryopreserved in EX-CELL CD CHO Fusion Media (Sigma Aldrich) containing 7% DMSO. Each vial of NISTCHO p12 contained 10 million cells in a 1ml volume. Resuscitation of cells was performed using a Thawstar device (Biolife Solutions).

NISTCHO Cell Culture

NISTCHO cells were cultured in 30ml of EX-CELL CD CHO Fusion Media in 125ml disposable shake flasks. The initial seeding density was 3.33 x 10^5 cells/ml, and the cells were cultured over a period of 9 days in an environment maintained at 37°C, 5% CO_2, and agitated at 125rpm on a shaking platform. Cultures were sampled daily under aseptic conditions to monitor growth. Both the viable cell concentration and the percentage viability were assessed using a Luna fluorescence-based automated cell counter (ThermoFisher).

Cell Harvest and mAb Purification

On day 9, cell growth culture media was collected by centrifugation at 2500xg, 4°C, for 10 minutes, followed by clarification by 0.22u filtration. The cNISTmAb was isolated from 5ml of clarified medium using a 1ml Protein A Gravity Chromatography column (ThermoFisher Scientific Cat No. 21001). The binding and equilibration buffer used was a phosphate-based solution with glycerol and EDTA, adjusted to pH 8.0. The elution buffer was amine-based and adjusted to pH 2.0. Flow-through and wash fractions of 2 ml each were collected, along with 1 ml fractions of eluate. To each eluate fraction, 50 µl of 1M Tris (pH 9) was added for pH adjustment to 7.0. The concentration of mAb in each fraction was quantified using a Nanodrop spectrophotometer (ThermoFisher) at 280nm using the extinction coefficient for IgG [6].

SDS-PAGE

Samples from chromatography fractions were prepared by mixing with an equal volume of 2X sample buffer (Biorad). Samples were heated at 95°C for 2 minutes before loading onto the gel.

Electrophoresis was conducted using a Novex 4-20% Tris-Glycine gradient gel (Invitrogen). The electrophoresis was carried out at 100 volts in a 1X Tris/Glycine/SDS running buffer for 1.5h. Post-electrophoresis, the gel was stained with Coomassie Brilliant Blue stain. Kaleidoscope Precision Plus Protein standards (Biorad) were included as molecular weight markers to facilitate the identification of the heavy and light chains of the antibodies.

Results and Discussion

This study investigates NISTCHO cell growth characteristics, including cell viability and cell growth after extended cryopreservation at two different temperatures, -80°C and -150°C. The primary objective was to evaluate how cells sustain their functionality and viability post-cryopreservation. To this end, NISTCHO cells were banked in the cryopreservant DMSO in a -80°C freezer or a -150°C freezer. Vials of cells from both cell banks were resuscitated at specific intervals and grown in culture with daily monitoring of the cultures over a nine-day period. Monitoring included the determination of cell density and the percentage of viable cells using an automated cell counter. Figure 1 demonstrates sample growth curves for 30ml cultures of NISTCHO cells that were seeded with cells cryopreserved at -150°C or -80°C for eight weeks. Cell counting was performed in duplicate, and the average cell concentration was plotted against the number of days in the culture. The growth curves represent typical proliferation patterns observed for NISTCHO cells grown in CHO Fusion Media in small-scale culture. The unique phases of NISTCHO growth, lag, exponential, plateau, and decline, are clear, as is the similarity of the growth dynamics of the cultures. This indicates that cells cryopreserved at -80°C for eight weeks have equal growth capabilities after resuscitation to those stored for the same period at -150°C.

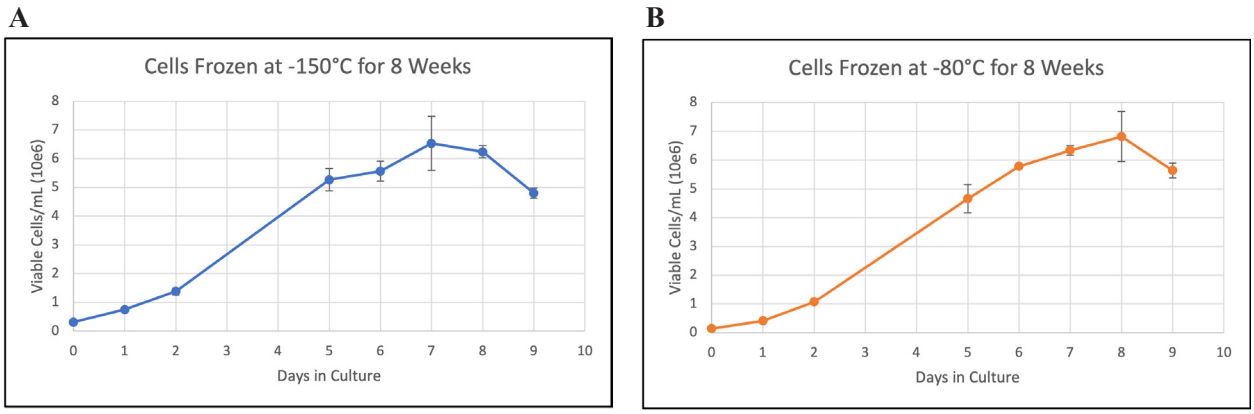

Fig. 1. Comparison of NISTCHO growth curves of cells after cryopreservation for eight weeks at -150°C (A) or -80°C (B). Cell counting was performed in duplicate, and average cell concentration and standard deviation were calculated.

The study was extended to determine if longer periods of storage at -80°C affected the properties of the cells in culture. Figure 2 demonstrates a comparison of the growth profiles and cell viability measurements of NISTCHO cells stored at either -80°C or -150°C for 14 weeks and 26 weeks.

The data demonstrates that cells preserved at either temperature maintain robust growth with no significant differences in their growth trajectories or maximum cell density (Figures 2A and B). In addition, the higher cryopreservation temperature does not negatively affect the high level of cell viability throughout the span of the culture, with both cultures maintaining cell viability above 90% into the plateau phase of the growth curves (Figures 2C and D).

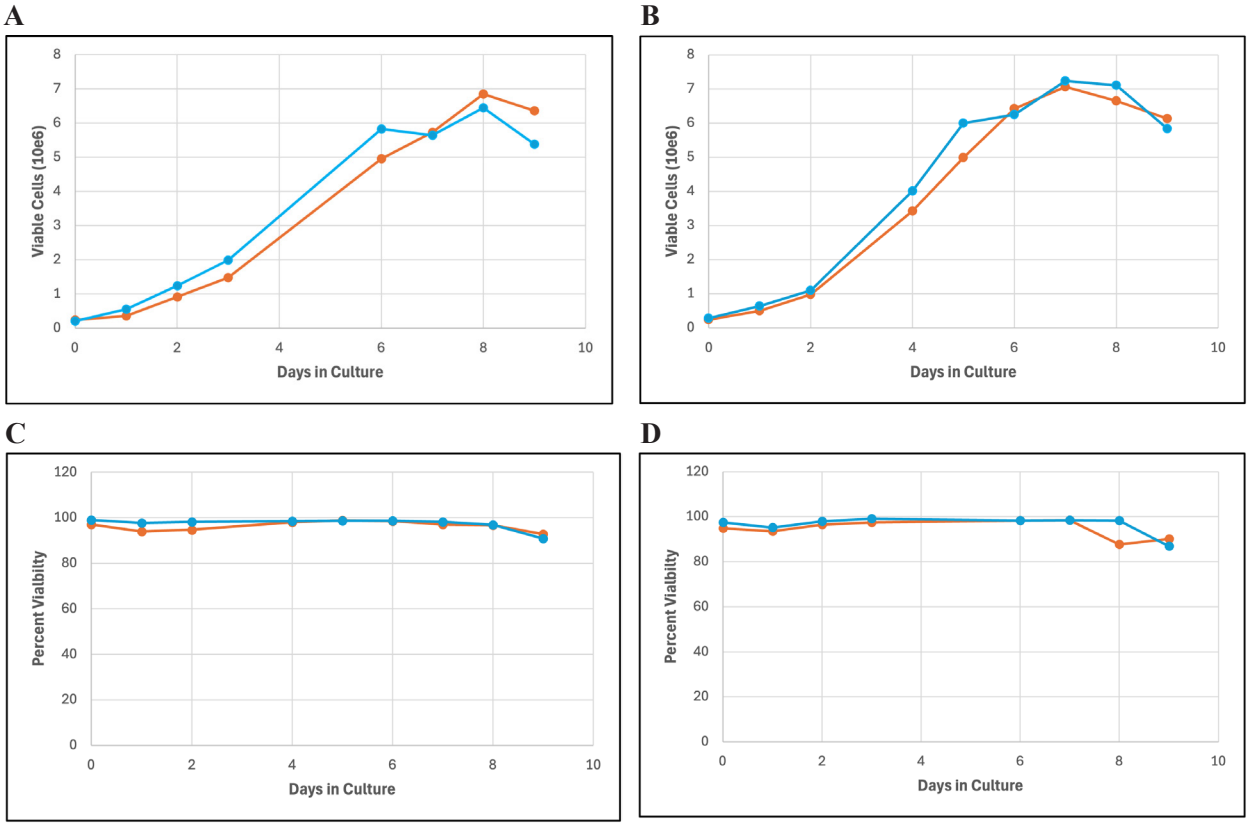

Fig. 2. Comparison of NISTCHO growth curves and cell viability for cells cryopreserved for 14 weeks (A and C) and 26 weeks (B and D). Blue lines represent the cell density and viability measurements for NISTCHO cells stored at -150°C and orange lines represent the same for NISTCHO cells stored at -80°C.

Building on this foundational data, we have compiled comprehensive growth curves that aggregate the results from growth studies from cells cryopreserved at both temperatures for 2, 4, 6, 8, 10, 14, 26, and 30 weeks. These combined growth curves provide a view of the cell behavior over the entire experimental period, enabling a broader comparison of cell behavior and performance in culture post-resuscitation. The proliferation patterns for cells frozen at the two temperatures are almost identical (Figure 3).

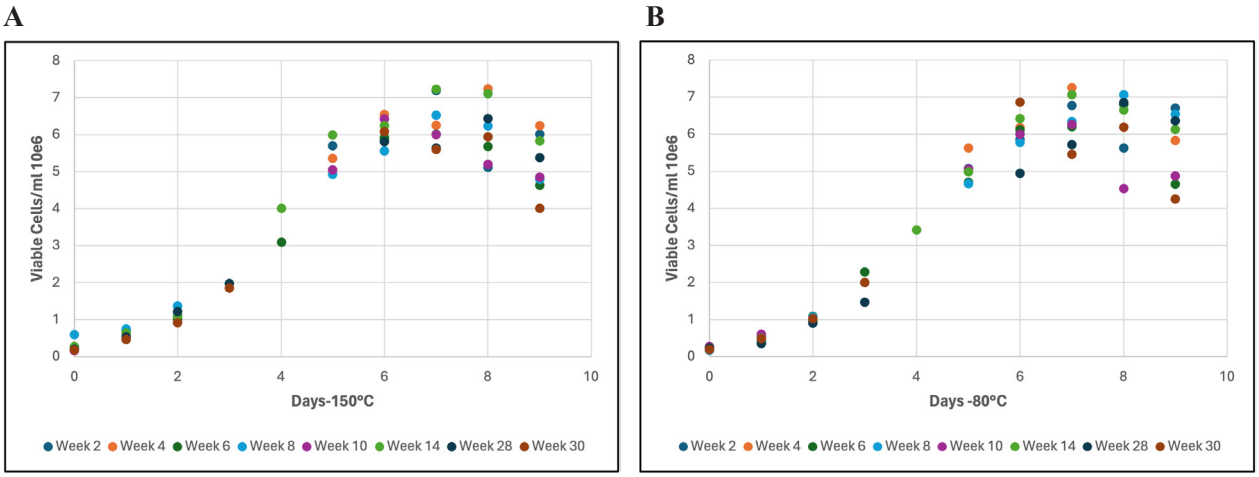

Fig. 3. Combined growth curves depicting the growth of NISTCHO cells over nine days. Cells were initially seeded at a density of 3.33 x 10^5 cells/ml and cultured in 30 ml volumes. (A) The cells used to seed the culture were cryopreserved at -150°C for the stated number of weeks. (B) The cells used to seed the cultures were cryopreserved at -80°C.

The comparative growth analysis of NISTCHO cells stored at -150°C and -80°C, demonstrated in Figures 1, 2, and 3, indicate that cells cryopreserved at -80°C, which is considered sub-optimal for most mammalian cell lines, perform as well as cells stored at the lower temperature. Notably, the initial percent cell viability immediately after resuscitation on day 0 (Figure 2) is almost identical between the cultures. Maximum cell density remains very comparable throughout the study, indicating that cryopreservation temperature did not affect cell performance. This equivalence is particularly evident in the delineation of growth phases (lag, exponential, and plateau) within the scatter plots (Figure 3), which are consistently observed in cell cultures from both conditions. The consistency across these data strongly suggests that NISTCHO cells exhibit a high degree of cryostability, which allows their metabolic and growth capacities to remain intact even after extended storage at -80°C.

The data challenge the prevailing assumption that lower storage temperatures are inherently superior for maintaining cellular integrity over long periods. Our data indicate that -80°C, a more commonly accessible and less costly cryopreservation option, does not compromise the growth characteristics of NISTCHO cells when cells are stored for up to 30 weeks.

NISTCHO cells in culture produce cNISTmAb and secrete it into the culture medium. Currently there isno commercially available functional assay, such as an ELISA, to easily quantify the concentration of cNISTmAb (i.e., titer) in the culture medium. To compare cNISTmAb titer in cell cultures from cells cryopreserved at each of the temperatures, the mAb was purified and quantified. Cell culture medium from each of the cultures was collected on culture day 9, and a representative 5ml sample was used to purify the cNISTmAb using protein A chromatography. The purified cNISTmAb was then quantified using spectrophotometry, absorbance at 280nm, and an estimated titer determined. Evaluation of Protein A Sepharose affinity chromatography purified antibodies typically indicate approximately 95% purity; hence, the vast majority of the purified material is the target protein. To assess the purity of cNISTmAb post chromatography, SDS-PAGE was performed under reducing conditions. In these conditions, the heavy and light chains of the mAb are separated and can be visualized as bands of approximately 50kDa and 25kDa.

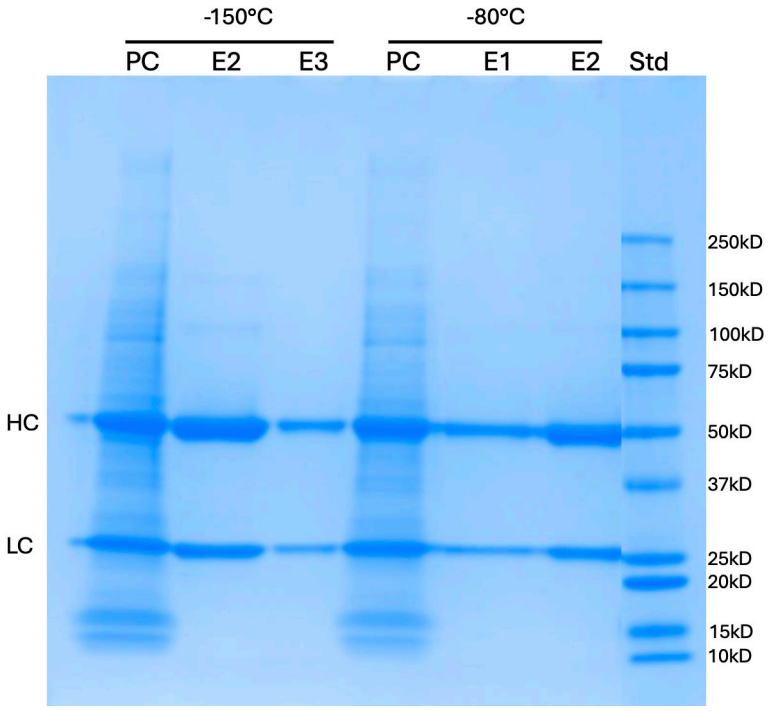

Fig. 4. SDS-PAGE Analysis of clarified culture media and cNISTmAb after purification by Protein A Chromatography for NISTCHO cultures of cells preserved at -150°C or -80°C. At -150°C storage PC: 6 ul Pre-Column clarified media, E2: 2ul of elution fraction 2 (1.650ug/ul), E3: 2 ul of elution fraction 3 (0.239 ug/ul). At -80°C storage PC: 6 ul Pre-Column clarified media, E1: 2 ul of elution fraction 1, (0.605ug/ul), E2: 2ul of elution fraction 2 (1.285ug/ul). Std: Kaleidoscope protein standards. Bands corresponding to the heavy chain (HC) and the light chain (LC) of cNISTmAb are the predominant bands in each lane.

Figure 4 shows SDS-PAGE analysis of pre-protein A column material from cultures seeded with cells cryopreserved at -150°C or -80°C for two weeks and associated eluted fractions containing cNISTmAb for each. Lanes containing fractions from the column elution E2, E3, E1, and E2 show strong representation of the heavy chain (HC) and light chain (LC) components, bands at approximately 50 kDa and 25 kDa respectively, with only very light contaminating bands visible. These minimal contaminants are likely residual host cell proteins or culture media components. This data confirms the purity of the mAb and confirms that the vast majority of the protein in these eluted fractions represents the cNISTmAb.

The concentration of mAb in each eluted fraction was quantified using a Nanodrop spectrophotometer, with a conversion factor of 1 Absorbance unit equaling 0.73 mg/ml of IgG mAb. This conversion uses the extinction coefficient for IgG when determining the concentration of the analyte from the absorbance at 280nm [6]. From these readings, the total amount of cNISTmAb in the elution fractions was calculated and divided by the volume of culture medium loaded onto the column (5ml) to produce an estimated mAb concentration, i.e., titer in ug/ml.

Table 1 shows data collected for each cell culture performed and allows a comparison of the effect of long-term storage at each of the two cryopreservation temperatures used. Overall, the titer for the cultures remains constant over time, and levels are comparable between the two cryopreservation temperatures. Cells that were stored for 26 weeks -80°C have a slightly lower titer (294ug/ml versus 332ug/ml for -150°C), and the amount of mAb produced per cell (pg/cell) for both 26 weeks and 30 weeks is slightly lower for the -80°C storage cells. This could indicate a slight downward trend in cell performance for cells stored for longer than the 30-week period of the study at -80°C. Future studies will analyze the performance of cells cryopreserved for one year at each temperature.

Table 1. Tabulated data for cell cultures cryopreserved from 2 to 30 weeks at -150°C or -80°C. Comparisons of % cell viability, maximum cell density, mAb titer (in ug/ml), and normalized mAb production (in pg/cell).

NISTCHO Cryopreservation -150°C					NISTCHO Cryopreservation -80°C				
weeks	Day 0 % Viability	Max Cell Density x 10^6 cells/ml	Titer ug/ml	mAb pg/cell	weeks	Day 0 % Viability	Max Cell Density x 10^6 cells/ml	Titer ug/ml	mAb pg/cell
2	90.20	7.19	320.00	44.51	2	97.70	6.77	312.00	46.08
4	98.80	7.24	308.30	42.51	4	96.40	7.26	292.30	40.26
6	92.90	6.00	284.80	47.47	6	99.00	6.26	305.30	48.77
8	98.30	6.53	276.90	42.41	8	98.60	7.06	253.50	35.99
10	97.00	6.43	285.40	44.39	10	96.50	6.26	276.80	44.22
14	99.10	7.24	259.00	35.70	14	97.00	7.05	278.00	39.43
26	97.40	6.44	332.00	51.50	26	94.90	6.85	294.00	42.90
30	98.70	7.11	260.10	40.40	30	100.00	7.33	259.80	35.44

Figure 5 illustrates data trends over the length of the study and clearly demonstrates the similarity of these trends for cells cryopreserved at -150°C and -80°C. Figure 5A demonstrates that initial cell viability in culture is comparable as the length of cryo storage increases, with cells from both conditions performing equally well. Figure 5B demonstrates a similar result for maximum cell density in culture. The stability of the cells in producing mAb after cryopreservation is shown in Figure 5C. mAb production is expressed as pg/cell to normalize for the variance in peak cell density between cultures. This normalization metric offers a more accurate depiction of cell productivity irrespective of the overall biomass, allowing for a direct comparison of mAb synthesis efficiency per cell under different conditions. Both cryopreservation temperatures yielded similar values for mAb production per cell over time, with a slight dip at weeks 26 and 30 for cells stored at -80°C. This suggests that the cellular machinery involved in mAb synthesis remains unaffected by the storage temperature.

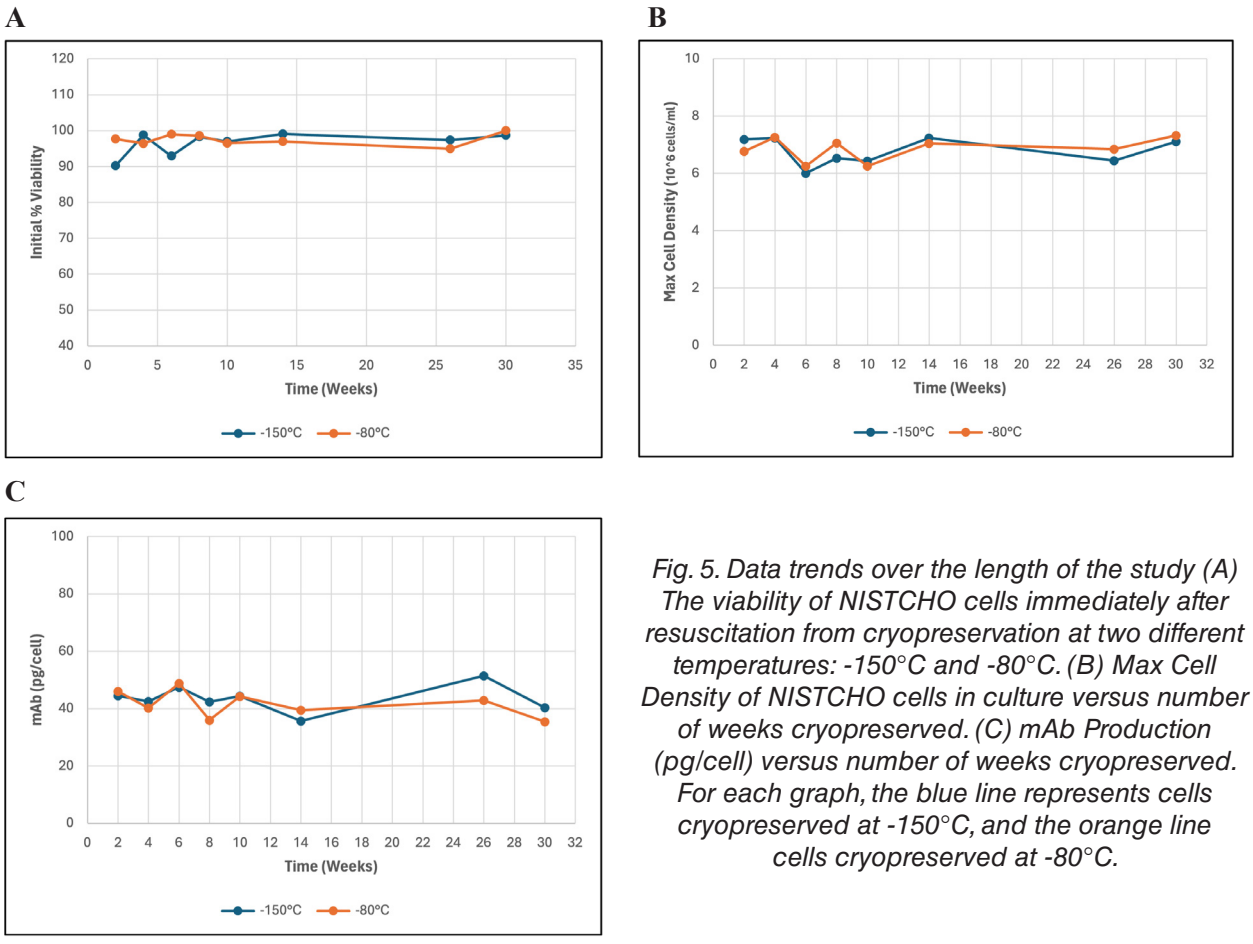

Fig. 5. Data trends over the length of the study (A) The viability of NISTCHO cells immediately after resuscitation from cryopreservation at two different temperatures: -150°C and -80°C. (B) Max Cell Density of NISTCHO cells in culture versus number of weeks cryopreserved. (C) mAb Production (pg/cell) versus number of weeks cryopreserved. For each graph, the blue line represents cells cryopreserved at -150°C, and the orange line cells cryopreserved at -80°C.

For biomanufacturing, understanding individual cell performance under various conditions is not only crucial for scaling up processes but also essential in ensuring consistent product quality and yield in large-scale productions. This study's findings underscore the potential for using NISTCHO cells in scalable biomanufacturing frameworks, given their demonstrated stability and robustness under long-term cryopreservation [4]. Moreover, NISTCHO stability at higher cryopreservation temperatures promotes their adoption and use in a wider variety of laboratory environments, especially those that rely on -80°C freezers for cell bank storage.

Conclusion

The primary objective of this investigation was to assess the impact of different and potentially suboptimal cryopreservation conditions on the viability, growth, and monoclonal antibody (mAb) production of NISTCHO cells. Specifically, we examined the effects of storing these cells at -80°C and compared the data with cells preserved at -150°C over 30 weeks. Our findings demonstrate that NISTCHO cells cryopreserved at these temperatures exhibited comparable growth patterns when revived and grown in 30ml shake flask cultures. Key metrics such as initial cell viability and peak cell density did not differ significantly between the two storage conditions. This indicates that the lower temperature of -150°C offers no distinct advantage over -80°C in preserving these parameters.

Furthermore, the consistent mAb production across both temperatures suggests that the cells' functional capacity to synthesize antibodies remains intact, irrespective of the cryopreservation temperature. This is particularly noteworthy as it highlights the robustness of NISTCHO cells under varying storage conditions, making them a reliable resource for biomanufacturing processes where cell stability and consistent output are needed.

The implications of these findings are significant for biotechnological applications, especially in settings where cost and energy efficiency are considerations [5]. Moreover, institutes that will use NISTCHO cells for education and training purposes, such as community colleges commonly store cell lines at -80°C if they do not have access to more expensive freezer options such as -150°C or liquid nitrogen dewars. The outcomes reported here will assure these laboratories that storage at -80°C for up to 30 weeks does not compromise cell performance.

The ability to maintain cell viability and functionality at -80°C without compromising the production of cNIST monoclonal antibodies (mAbs) supports the integration of these cell lines into biomanufacturing curricula. Students can gain hands-on experience with these cells, understanding the critical aspects of cryopreservation, cell culture, and mAb production [6]. Furthermore, this adaptability allows institutions with more modest facilities to engage in cutting-edge biotechnological education and research, ensuring that a lack of access to specialized equipment does not disadvantage students.

In conclusion, this study supports the use of -80°C freezers as a viable and efficient option for the long-term cryopreservation of NISTCHO cells. The data supports more economical and accessible biomanufacturing practices with this NISTCHO cell line producing cNISTmAb. Future studies extending beyond 30 weeks could provide deeper insights into the long-term viability and functional stability of cryopreserved cells, potentially expanding their utility in the biotechnology field.

Acknowledgments. We extend our deepest gratitude to members of the biotechnology department at Montgomery County Community College for their tireless support and contributions throughout the research process. We also would like to thank the National Institute for Innovation in Manufacturing Biopharmaceuticals, NIIMBL, Dr. Brad O'Dell from the National Institute of Standards and Technology, and Dr. Lori Kelman from Montgomery College for their advice and support. This work was performed under the following financial assistance award: 70NANB21H085 from the US Department of Commerce, National Institute of Standards and Technology, and NSF ATE award DUE #2055036, Principal Investigator Dr. Sandra Porter. The Corresponding Author would like to acknowledge that authors J. T. Fredericks and S. Milligan contributed equally to the study.

Disclosures. The authors declare no conflicts of interest.

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From *In Vitro* to *In Vivo*

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Abstract: The purpose of this review is to compare and contrast eukaryotic cell culture experiments with ecological microcosms and will emphasize the areas where the two approaches overlap. Cell culture experiments involve cultivating cells outside their natural setting to study cellular behavior, disease mechanisms, and drug responses. Conversely, microcosms replicate natural ecosystems in controlled environments, providing insights into ecological processes and substance fate. While cell culture focuses on cellular and molecular biology, microcosms lean towards ecological and environmental studies. However, both techniques overlap, especially in environmental toxicology, where cell culture findings are validated in microcosms to assess ecological impacts. Advancements in whole genome sequencing, metagenomics, and metabolomics have enabled the linkage of cell culture-based studies and microcosms to investigate molecular biology. Cell culture experiments contribute significantly to biomedical research, drug development, and regenerative medicine, while microcosms are valuable for understanding ecosystem dynamics and assessing environmental risks. The review discusses the historical development of cell culture and microcosms, highlighting key milestones such as the creation of the first human cell line (HeLa cells) and the emergence of stem cells and organoids. It also explores the future applications of cell culture, including cell-based screening for drug testing and the transition from 2-D to 3-D cell screening techniques for more accurate results. Addressing global health and environmental challenges through small-scale experiments using microcosms or "within the glass" (*in vitro*) cell line models is essential. Despite the time-consuming nature of these experiments, they contribute to developing theories and practical solutions for responding to climate change and emerging diseases. In summary, cell culture experiments and microcosms are indispensable in scientific research, offering unique insights into biology and ecology and hold immense potential for addressing pressing challenges in various fields.

Keywords: *in vitro*, microcosm, metagenomics, stem cells, research model systems

Introduction

Eukaryotic cell culture-based experiments and microcosms are both *in vitro* techniques used in scientific research, but they serve different purposes and are used in other contexts. Cell culture experiments involve growing cells in a controlled environment outside their natural setting, typically in a laboratory. The first report of eukaryotic cells cultured *in vitro* came from Harris and colleagues in 1906, who adapted techniques that Robert Koch used for bacterial cell culturing that he developed in the later 1800s to culture frog tissues *ex vivo* [1]. This technique allowed researchers to study cellular behavior, responses to stimuli, and disease mechanisms in a controlled and reproducible manner. However, having a stable and immortal human cell line capable of generating reproducible data would come much later and be essential in uncovering the unknown biology of human cells. Henrietta Lacks suffered from a severe form of cervical adenocarcinoma. During her diagnostic evaluation, a tissue biopsy was taken, providing extra samples for Dr. George O. Gey's tissue culture lab at Johns Hopkins in Baltimore, Maryland. Though Lacks passed away from cancer in 1951, the cancer cells, known as HeLa cells, quickly proliferated in cell culture and became the first established human cell line, still used today [2]. Cell culture experiments are often used in biomedical research, drug development, and regenerative medicine, among other fields.

While cell culture systems attempt to replicate organismal function, microcosms are miniature ecosystems contained within vessels designed to replicate natural environments on a smaller scale. Microcosms are used to study ecological processes, such as nutrient cycling, species interactions, and the effects of environmental stressors. Although microcosms have likely been in use by early scientists for centuries, if not millennia, the earliest recorded instance of microcosms in scientific literature occurred in 1851 [3]. In a meticulously balanced aquatic micro-ecosystem housed within a twelve-gallon glass aquarium, Robert Warington demonstrated the interaction between goldfish, snails, Vallisneria plants, and a multitude of attendant microorganisms. From his later work, we gain insight into the intricate roles played by each organism in the cyclical flow of materials within this aquatic community [3]. They provide a simplified yet realistic representation of natural ecosystems, allowing researchers to manipulate variables and study ecological dynamics in a controlled setting and reproducible manner.

While cell culture experiments focus on cellular and molecular biology, microcosms are more oriented toward ecological and environmental studies. However, there can be overlap between the two techniques, especially in areas such as environmental toxicology, where researchers may use cell culture experiments to study the effects of pollutants on cellular function and then validate their findings in microcosm experiments to assess their ecological impacts. With the recent improvements in metataxonomic, metagenomics, and metabolomics and increased affordability of whole genome sequencing, cell culture-based studies and microcosms can be linked to a better understanding of molecular biology at the species and community levels [4]. Tumor cells exist within an ecosystem that includes both the tumor cells themselves and their surrounding microenvironment. Emerging spatial genomic, transcriptomic, and proteomic technologies provide new methods for studying cancer evolution with detailed molecular and spatial insights [5]. Overall, both cell culture experiments and microcosms are valuable tools in scientific research, each offering unique insights into different aspects of biology and ecology.

Research conducted *in vitro* allows scientists to conduct experiments that might be impossible or unethical to conduct on whole organisms or ecosystems. Using eukaryotic cell culture to mimic models of human disease has profoundly contributed to our understanding of factors influencing everything from cancer to molecular evolution. For example, current drug candidates and toxicity screening processes depend on early-stage *in vitro* cell-based assays, which are anticipated to accurately reflect key aspects of *in vivo* (under normal conditions in a living organism) pharmacology and toxicology [6]. *In vitro* models have recently attracted attention as methods that might reduce and eventually replace the use of animals in research. Advancements in 3D bioprinting, spheroids, organoids of human tissues, and microfluidics have significantly progressed in replicating human physiology, which may one day allow for the replacement of animal models entirely [7, 8]. Practical applications relying on cell culture techniques have emerged across diverse domains, encompassing the evaluation of new drug efficacy and toxicity, the production of vaccines and biopharmaceuticals, and the advancement of assisted reproductive technologies. With recent advancements enabling the reprogramming of somatic cells, researchers worldwide are engaged in intense competition to spearhead progress in regenerative medicine. Similarly, within this field, cell culture technology is recognized as a cornerstone for continued advancement and widespread adoption.

Similarly, microcosms are *in vitro* models of natural ecosystems contained within vessels and originated to bring nature's intricacies into educational and domestic settings worldwide. Over time, these compact "worlds" have evolved into a significant research instrument. For instance, they help address complex questions like the impact of biodiversity on ecosystems. A Rutgers study on decomposer bacteria revealed that decomposition rates increased with greater bacterial species diversity compared to the higher abundance of a single species [9]. Such broad-scale questions are challenging to investigate in a controlled setting without using microcosms. Microcosms are particularly valuable because they offer a method to study the effects of various factors on entire ecosystems using simplified models, and their replicability allows for cost-effective experimental studies.

The following review provides historical context for the development and implementation of eukaryotic cell culture-based systems and microcosms and provides insight into how recent advances in the field make these approaches more powerful tools for understanding the impact of pollution at the species and community levels.

Discussion

What is Cell Culture?

Cell culture is the practice of maintaining and continuous propagation of cells outside of their natural environment. Under *in vivo* conditions, the homeostatic regulation of metabolism and gene expression in tissues would be governed via cell signaling, and the tissue's microenvironment would promote appropriate growth. Under cell culture conditions *in vitro*, cells are given the proper nutrients and growth factors to be maintained outside of the organism. Today, we use this practice to investigate the response of eukaryotic cells to various conditions. For example, if a contaminant is released into a community, and little is known about what it does to mammalian cells, cell culture-modeled experiments allow us to study its effects under controlled conditions. This process is also used to screen drugs to determine their efficacy and toxicity [3].

The Origin of Cell Culture and Common Applications

Attempts to culture cells *in vitro* began very early in history, in 1885, when Wilhelm Roux isolated a chicken embryo and sustained it outside its normal conditions [10]. *In vitro* stems from the Latin term "in glass," which refers to the act of removing a cell line and placing it into a glass container. Later, in 1910, Carrel, Burrows, and Montrose improved the technique by holding tissue samples for 2-3 months [11]. The American Type Culture Collection (ATCC) later crafted the regulatory language for cell culture to fit certain standard assessments, which was the backbone for developing the much-needed aseptic techniques to ensure samples' purity and improve cell preservation. In 1949, American virologists John F. Enders, Thomas H. Weller, and Frederick C. Robbins revolutionized virology when they successfully cultured the three poliomyelitis viruses *in vitro* using non-nervous cell cultures [12]. This groundbreaking achievement earned them the Nobel Prize in Physiology and Medicine in 1954. Their discovery quickly led to the development of effective poliomyelitis vaccines by J. E. Salk in 1953 [13]. From 1952 to 1955, Gey created the first human cell line from HeLa cells as mentioned previously [2]. Establishing a stable, immortal human cell line ushered in a new era of biomedical research. This section will discuss the utility of HeLa, CHO, and HEK 293 cells.

HeLa cells were the first human cell line able to be propagated indefinitely in culture. Henrietta Lacks died from an aggressive form of Cervical Cancer in 1951. During her prognosis and treatment, her cells were preserved without her existing family's consent. This led to many ethical debates among the scientific community and the rules and regulations regarding the patient's consent and privacy. The Department of Health also enforces its model for patient consent depending on the situation, and acting professionals can be persecuted when performing research on patients or their Biospecimens [14]. Ethical questions notwithstanding, research using HeLa cells continues to pay dividends to the scientific world. In fact, "Knowledge of almost every process that occurs in human cells has been obtained using HeLa cells" making them perhaps the most historically valuable cell line [15].

CHO cells, also known as Chinese Hamster Ovary cells, are a mammalian cell line that is easy and affordable to mass culture, which is why they are used to produce 70% of therapeutic proteins [16]. Recently, CHO cells have begun to play a more significant role in antibody production for therapeutic applications [16]. How they respond in cell-based drug assays are a good indicator of how other mammalian cells react when treated with medication, exposed to a chemical, or transfected with a pathogen [8].

HEK293, or human embryonic kidney-derived epithelial cells, are among the most frequently utilized cell lines in cell biology research. This cell line is a frequent choice for research and therapeutics because of its rapid division rate, robustness, and proficiency in post-translational modification of heterologously expressed proteins [17]. Additionally, HEK293 cells are highly amenable to transfection, making them a preferred choice for transient and stable transformation experiments, protein expression and production, and even electrophysiological studies [17].

Stem Cells and Organoids

Stem cells are nonspecific human cells that can be taken from an adult or embryo and manipulated to differentiated into various human cell types, depending on the origin of the stem cell. Though not cell lines, their *in vitro* research and clinical applications make them a powerful tool. These cells can be classified into

three categories for simplicity's sake, which will be introduced when narrowing the applications. The first classification would be Totipotent, which can create both embryonic and extra-embryonic structures, an example being a zygote. The second classification would be Pluripotent, which can form only embryonic structures such as organelles and be gathered from the Blastocyst inner cell mass four days after development. Lastly, multipotent stem cells can only generate specific cell lines, such as generating macrophages from hematopoietic stem cells. The discovery of embryonic and adult stem cells was revolutionary because of their potential in regenerative medicine. Currently, stem cell-derived organs and organoids have been successfully demonstrated in mice from mammary to prostate glands [18]. Not only can it have major beneficial effects in regrowing already existing damaged organs, but it can also theoretically create whole organs, also known as organoids, which can be risk-free from rejection, unlike organs donated from other humans, which we use in transplants today [19]. Organoids are artificially created organs from existing stem cells to perform the function of organs. Though our understanding of organogenesis is quite limited within the organoids' microenvironment, the potential for organoid structures to faithfully mimic organs may change the future as we know about transplant procedures, organ donors, and cell-to-cell interactions within organ systems [20].

Limitations of Cell Culture Models

Various cell lines are used in scientific research, yet they come with certain disadvantages and limitations, especially in drug development. One such limitation is the acquisition of additional genetic aberrations associated with increasing passage numbers. As most cell lines are transformed or derived from cancer cells, they possess mutations in cancer genes, including those associated with genomic instability which may result in genetic changes with the passage of cells [21]. Even without severe genetic changes, genotypic and phenotypic drift in continuous culture can shift cellular responses over time, which can differ significantly from patient responses to the same drugs [21]. Additionally, there are disparities between the microenvironments of original tumors and cancer cell cultures (both 2D and 3D) and cross-contamination with HeLa cell lines. Culture conditions can alter morphology, gene expression, and several cellular pathways, while mycoplasma infections can change culture properties [21]. Establishing long-term cancer cell lines for specific tumor types can be challenging, and the cell culture environment differs from the original tumor's. Furthermore, the natural heterogeneity of the tumor or tissue, the interplay of multiple cell types influencing each other, is often lost in cell culture environments which are monocultures.

The Future of Cell Culture Applications

Due to the increasing need for treatment, cell-based screening methods have become more efficient and cost-effective for drug testing than animal testing. Animal testing is a solid model for understanding the effects of drugs on mammals. However, it creates multiple problems, including the cost and ethical dilemmas of harming animals [21]. Furthermore, the drug action in animal models might differ greatly from how it acts in clinical trials, showing animal models and cell culture models will require improvements to represent human physiology better. After decades of performing anti-cancer drug screening in mice, the National Cancer Institute (NCI) significantly shifted to a novel approach: screening 10,000 diverse compounds per year against a panel of 60 human tumor cell lines *in vitro*. Alley and colleagues introduced this new "NCI-60" screening method in a landmark 1988 Cancer Research article, showcasing the technological foundation for this large-scale microculture screen [22]. The core idea was that the NCI-60 screen would accelerate the discovery of innovative cancer drugs, particularly those effective against specific solid cancers, which was not previously feasible. Currently employed in modern molecular target-based drug discovery, these panels are revolutionizing biomarker-driven precision oncology and improving the lives of cancer patients [23]. Clearly, cell-based screening creates less of an ethical dilemma and can be more accurate in determining the actual outcome of a particular drug treatment [24].

2-D vs 3-D Cell Screening

Currently, 2-D cell screening is the most popular technique; however, 3-D cell screening will allow for more accurate drug treatment effects *in vivo*. 2-D screening is a more cost-effective and convenient form of *in vitro* model for screening; in its more simplistic forms, it tends to be made of a monolayer cell culture, added with molecules to perform a biological function also known as a biological library and the use of a microplate reader or microscope. 3D cell screening is more complex and tends to be a replica of the environment in

which the cell resides; thus, it tends to be more costly but is more comparable to *in vivo*. Recently, a cancer research study showed that certain breast cancer cell lines developed dense 3D multicellular spheroids (MCSs), and these spheroids exhibited reduced sensitivity to the chemotherapeutic drugs doxorubicin (DXR) and paclitaxel (PTX) compared to cells cultured in 2D environments [25]. This demonstrates how 3D culturing of cells will produce more reliable data on the future efficacy and toxicity of cancer drugs. Researchers suggest that their downstream applications will take advantage of the 3-D model but with the manufacturing cost of the 2-D model [16].

History of Microcosms and Their Applications

Ecological microcosms are small ecosystems held in containers used as a research tool for studying the way ecosystems work and determining what happens to different substances in ecosystems. They are a simplified way to model whole ecosystems and can be replicated for experimental studies at reasonable cost [26]. Basically, microcosms are of different shapes, sizes, and compositions, but they possess some or all of the following properties: origin, isolation, size, genotypic heterogeneity, spatial heterogeneity, and temporal heterogeneity [27]. Microcosms find their origin in the natural world, and are isolated systems of reasonable size, no longer in contact with the natural world. Microcosms may be as small as a few milliliters or as large as a building. Genotypic heterogeneity refers to the fact that microcosms typically exist as mixed populations of organisms. Spatial and Temporal heterogeneity implies that the microcosms differ at places within the system and change over time. Ultimately, due to the advantages of controllability, replicability, and low cost, microcosm experiments have been used in practically every area of modern ecology, terrestrial and aquatic [26].

Modern biological science regards ecological microcosms as a relatively new research technique. However, Robert Warington introduced his work on a balanced aquarium in 1857, considered one of the first scientific papers on microcosms. He pointed out the interaction of producers, consumers, and decomposers, laying the basis for the aquarium concept, with its contained organisms functioning as a unit [27]. Microcosms vary greatly in their construction and contents but tend to follow at least a few general criteria. They typically contain multiple species to reflect the systems interactions within ecosystems, they contain artificial boundaries created by experimenters and not seen in nature, and the experimental system is at least partially isolated from the natural world [26]. Therefore, we can see the great value in using a closed system where all of the variables can be controlled or manipulated in order to gain insight into the workings of actual ecosystems throughout the world.

Since the adoption of microcosms as models for use in the laboratory, there have been great strides in their complexity and utility. Natural ecosystems, besides being challenging to define precisely, frequently exhibit considerable size and are influenced by a range of environmental factors affecting their boundaries. These factors include variations in light intensity and duration, fluctuations in temperature encompassing both average conditions and extreme events, the presence of suspended or dissolved materials in the surrounding fluid medium, and the dynamics of importing and exporting non-living materials and organisms [28]. If one is unable to control the boundaries of an experiment, determining the relationship between the independent and dependent variables becomes nearly impossible. It is also important to note that to purposefully alter an ecosystem for experimental purposes could prove quite destabilizing and result in the death of many living things, an unethical prospect. Therefore, the microcosm serves as a way not only to better control the variables in an experiment but also to avoid ethical quandaries about tampering with natural ecosystems.

The microcosm approach enables the creation of replicated ecosystems, even those that are rare, such as California vernal pools or nonexistent on Earth (Mars soil, for example), and the replicates provide better statistical veracity to the data generated from the experiments. To achieve replication, living and non-living components from a natural ecosystem are transferred into a laboratory setting and maintained under conditions closely resembling those of the original environment [28]. These replicated samples are distributed across multiple containers. Although variation due to the seeding of microcosms occurs, a greater number of replicates helps to eliminate statistical outliers and provide robust data sets.

Microcosms have been adapted to a wide variety of experimental subjects over time. Historically, theoretical ecology, biogeochemical cycles, radioactivity tracing, life support systems, and sanitary engineering have been studied using these systems [26]. One of the earliest papers on nutrient influx and aquatic ecosystem productivity used a concrete pond microcosm seeded with nitrates to determine their effect of productivity [29]. Shortly after, we see the first attempts to outline the microcosm method as a distinct approach, building on earlier work where scientists utilized microcosm experiments to demonstrate the presence of balanced aquatic environments [30]. Subsequent to the world entering the Atomic Age, microcosms were employed in radioactive tracer investigations [31] for primarily two reasons. Firstly, they offered a more manageable experimental environment compared to the complexities of controlling variables in natural ecosystems. Secondly, they helped circumvent potential pollution issues. Later, the Space Race and population explosion ushered in an era of microcosm research to address the burgeoning population and survival of humans in space. In space, the challenge revolves around sustaining human life outside of Earth's biosphere. Meanwhile, on our planet, the expanding population necessitates more effective waste management solutions to handle the growing volume of waste generated. Microcosm research began to demonstrate how closed systems could be balanced to sustain human life in space travel and how the same approach could be used to manage the production of vast amounts of human waste in the industrialized world [32, 33].

Aquatic mesocosms and microcosms are experimental systems designed to replicate natural ecosystems, such as small freshwater ponds or wetlands, in a controlled environment. They contain physical, chemical, and biological components similar to those of the natural ecosystem. These systems consist of populations and communities of organisms at multiple trophic levels, including algae, plants, invertebrates, and fish, that interact with their environment and each other in complex ways.

In ecotoxicology, microcosms are used to examine the effects of environmental stressors, including chemicals, on the ecosystem. Specifically, they have been used to study the impact of chemical pollutants on amphibian and insect metamorphosis as well as other physical and biological components of the ecosystem. These effects can include changes in nutrient cycling, primary productivity, species interactions, and community structure. Microcosms can also be used to investigate the direct and indirect effects of chemicals and other stressors on ecosystem structure and function, the recovery process from these effects, and to inform ecological risk assessment.

Using microcosms, researchers can examine how chemicals and other stressors impact ecosystems in a way that is impossible in the natural environment. For example, the exposure of plankton microcosms to Nickel (Ni) demonstrated noticeable effects on a few individual species, which allowed the researchers to determine that environmental threshold concentrations for nickel in European environmental laws are adequate [34]. They can also conduct experiments over a shorter time frame than would be required in the field, allowing them to understand the mechanisms behind the observed effects better. Overall, microcosms are a valuable tool for ecotoxicologists and other researchers interested in understanding the complex interactions between organisms and their environment [35].

Furthermore, in order to increase the accuracy and realism in the ecological risk assessment of chemicals, multispecies experiments are carried out. These experiments have certain advantages over laboratory single-species tests that enable them to provide more comprehensive and reliable data. For example, they evaluate more realistic exposure regimes, assess the effects of chemicals on populations rather than individuals, and allow the assessment of recovery of populations that have been affected by chemical exposure. Additionally, multispecies tests include food netting interactions between populations, which are essential in understanding the broader ecological impacts of chemicals.

Multispecies tests come in a wide range of experimental designs, ranging from relatively simple indoor multispecies assemblages to more complex model ecosystems and field monitoring studies. These designs are tailored to the specific needs of the experiment and the complexity of the ecosystem being studied. For instance, simple indoor multispecies assemblages are useful for preliminary screenings of chemical toxicity. Meanwhile, model ecosystems and field monitoring studies are more appropriate for assessing the long-term effects of chemicals on ecosystems.

In recent decades, significant progress has been made in the development of multispecies experimental designs. For example, a multispecies experiment demonstrated that the spatial organization of Pseudomonas putida and Acinetobacter was driven by metabolic cross-feeding, findings that would not be observed in monoculture [36]. Studies indicate that wastewater treatment systems consist of over a thousand species-level operational taxonomic units (OTUs), microbial mats contain more than 750 species, and oral biofilms harbor hundreds of different organisms, including both bacteria and eukaryotes [37]. The acid mine drainage (AMD) ecosystem, known for its low pH and high concentrations of toxic metals and sulfate, is one of the most comprehensively studied natural communities. 'Omics' approaches have significantly advanced our understanding of AMD, revealing that a few species dominate the community. However, rare taxa play crucial roles in processes like nitrogen fixation and sulfur oxidation [38]. This complexity underscores the importance of considering both the dominant and rare species to fully understand ecosystem function. These designs have become more sophisticated and have incorporated new technologies that allow for more precise and detailed data collection. As a result, multispecies experiments have become an essential tool in ecological risk assessment, enabling scientists to gain a deeper understanding of the potential impacts of chemicals on ecosystems [37].

Nonetheless, global environmental problems that affect the entire planet can be difficult to address with traditional scientific experiments; thus, conducting small-scale experiments using "model organisms" in microcosms or mesocosms can be a helpful approach to tackling these seemingly insurmountable issues. By experimenting on a smaller scale, different scenarios can be tested out. For example, how ecosystems respond to climate change or how to manage biodiversity through nature reserves. This experimental approach can also aid in the development of theories and inspire further research, ultimately leading to a better understanding of the issues and practical solutions. Although this process has been influential in the past, it can be time-consuming. Therefore, addressing global issues with significant policy implications would be a key move [39].

Additionally, when conducting microcosm studies, it is crucial to consider the experiment's duration. Typically, microcosm experiments only last for several weeks to months. However, it is essential to ensure that the experiment's duration is long enough to evaluate the effects on slow-responding organisms or processes. Like natural ecosystems, microcosm conditions can change over time, and these changes should be assessed throughout the study. As the experiment's duration increases, there is a higher likelihood of greater variability developing between replicates due to natural divergence. Time series sampling can be included in the experimental design to monitor changes. However, the impact of any repetitive sampling of components should be carefully considered. At the start of the experiment, a large number of microcosm replicates can be established to enable complete sampling of a subset of replicates at designated time intervals. In the laboratory, natural diurnal variations can be simulated to some extent by using controlled light-dark cycling of artificial lights. For shorter experiments, seasonal variability can be taken into account by repeating experiments on a seasonal basis. Additionally, ecological processes that occur over longer periods, such as succession, predator-prey cycles, and extinction, can be studied in microcosms with short real-time duration using organisms with very short generation times, such as microorganisms. This approach is known as the biological accelerator approach [40].

Conclusion

Cell 1 variables and study ecological dynamics in a reproducible manner. Both approaches serve to recreate the natural world in the laboratory from whole organisms in the case of cell culture and from an entire ecosystem in the case of microcosms.

While cell culture experiments primarily focus on cellular and molecular biology and microcosms are oriented towards ecological and environmental studies, there exists an overlap between the two techniques, especially in areas like environmental toxicology. Researchers may use cell culture experiments to study the effects of pollutants on cellular function and then validate their findings in microcosm experiments to assess ecological impacts. Moreover, recent advancements in whole genome sequencing, metagenomics, and metabolomics have facilitated linking cell culture-based studies and microcosms to investigate molecular biology at both the species and community levels [4].

In conclusion, both cell culture experiments and microcosms are indispensable tools in scientific research, offering unique insights into different aspects of biology and ecology. Their continued development and integration with emerging technologies hold immense potential for addressing pressing challenges in fields ranging from medicine to environmental science.

Acknowledgments. This work was in part supported by the National Science Foundation (NSF) under award DUE ATE #2325500.

Disclosures. The authors declare no conflicts of interest.

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Predicting Neoantigens for Cancer Using Next-Generation IEDB & CEDAR Tools

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Abstract: Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. The underlying cause of cancer relates to the cell cycle, during which DNA is replicated. Cancer cells accumulate DNA mutations that help them acquire cancerous features, such as evading cell death and indefinite growth [1]. If these DNA mutations are in coding regions, they are translated to mutated proteins. The epitopes that contain these mutations are called neoantigens. Neoantigens are highly tumor-specific and can be targeted with immunotherapies [2]. During cell division, tumor suppressor genes play a role in the case of DNA damage or replication errors. The p53 protein is a tumor suppressor gene product that prevents tumor formation by activating processes that block cell division when DNA damage has occurred [3]. Mutant p53 does not effectively bind DNA or activate the production of proteins necessary for the stop signal. This project explored a hypothesis that a set of distinct p53 protein mutations can be selected to serve as potential targets for cancer immunotherapy and vaccines by using immunoinformatics predictive analysis tools. By comparing these potential targets with experimental results, we can predict epitopes that may serve as neoantigen targets for immunotherapy. We identified candidate immunogenic epitopes using the NCI's TP53 Database (NCI DB - tp53.isb-cgc.org), Cancer Epitope Database and Analysis Resource (CEDAR - cedar.iedb.org), and a powerful new bioinformatics tool (nextgen-tools.iedb.org/) [4] hosted by Immune Epitope Database (IEDB - iedb.org) and CEDAR. Comparing predicted epitopes to highly mutable regions of p53 in tumor variants from NCI DB revealed areas of overlap that may be priority candidate epitopes for immunotherapy. Experimental data from CEDAR tested the immunogenicity of normal and mutated protein versions to help avoid harmful cross-reactions. These results help predict cancer epitope amino acid sequences relevant to understanding the immune system's role in cancer progression, prevention, and treatment. These studies also set the stage for important subsequent undergraduate research projects to further characterize predicted cancer neoantigens.

Keywords: p53, mutations, neoantigens, cancer, CEDAR, bioinformatics, immunotherapeutic, Open Access Resources

Introduction

The underlying cause of cancer relates to the process by which most human cells grow and repair, known as the cell cycle. During the cell cycle, DNA is replicated so that dividing cells contain their matching set of chromosomes. DNA can be damaged by toxins, radiation, or other sources, leading to mutations that will be passed onto offspring cells if the cell cycle is not stopped. Cancer arises from the accumulation of mutations, which results in uncontrolled cell division and growth. Genes involved in regulating the cell cycle, including

proto-oncogenes and tumor suppressor genes, are often found to be mutated in tumors [5]. Cancer therapies that target oncogenes, such as trastuzumab (Herceptin®) and imatinib (Gleevec®, STI571), both work by targeting kinases that are part of the cell signaling pathway [6].

These drugs have revolutionized cancer treatment by inhibiting oncogenic proteins and blocking accelerated growth. However, reactivating a mutated, inactive protein such as tumor suppressor genes is challenging. While p53 is the most well-characterized contributor to tumors, no targeted drugs are available. Cellular mechanisms involving tumor suppressor genes, such as TP53, have naturally evolved to stop the cell cycle in the case of DNA damage or replication error. The p53 protein prevents uncontrolled growth and tumor formation by responding to stress-induced DNA damage via entering the nucleus and binding DNA to prompt the production of another mediator protein [3]. Mutations in the p53 protein affect DNA binding, so the mediator protein is not made. As a result, the cell loses the stop signal that would prevent uncontrolled growth. In addition, p53 binds DNA as a tetramer of four molecules of functioning p53; thus, if one allele of TP53 is mutated, it will negate the function of the unmutated p53 gene product [7].

An alternate strategy for cancer treatment focuses on leveraging the adaptive immune system to identify and destroy cells displaying foreign antigens presented on their cell surface (cell-mediated branch). Neoantigens are a class of peptides carrying somatic mutations resulting in a "new" antigen that can be identified as foreign and marked for destruction [8]. Targeting these neoantigens provides a mechanism for tumor-specific immunotherapies carried out on behalf of the adaptive immune system. Since tumor- associated mutations in p53 can produce neoantigens, cancerous cells that produce them can be distinguished from normal p53 epitopes and cleared by immune effector cells.

In the case of alterations to the TP53 gene, the mutant p53 proteins are degraded into short peptides and transported into the endoplasmic reticulum. In the lumen of the endoplasmic reticulum, the peptide fragments may bind with major histocompatibility complex (MHC) Class I proteins present in all nucleated cells to mediate antigen presentation. This epitope-MHC complex is displayed on the cell's surface, where it may be found by a T-cell with a complementary receptor (TCR), forming a tight MHC-TCR complex. T-cell receptors are specific to foreign antigens and bind only epitope-MHC 'peptides in a bun' shaped complexes. The resulting immune response makes neoantigens promising immunotherapeutics, especially for highly immunogenic epitopes on the surface of tumor cells [8]. Current bioinformatics techniques such as sequence analysis, machine learning-aided binding, and immunogenicity predictions [9-10] (see Figure 1) help identify tumor-specific neoantigen epitopes that may be effective immunotherapeutic targets and cancer vaccines. Maximizing computational predictions helps minimize expensive and laborious experimental approaches.

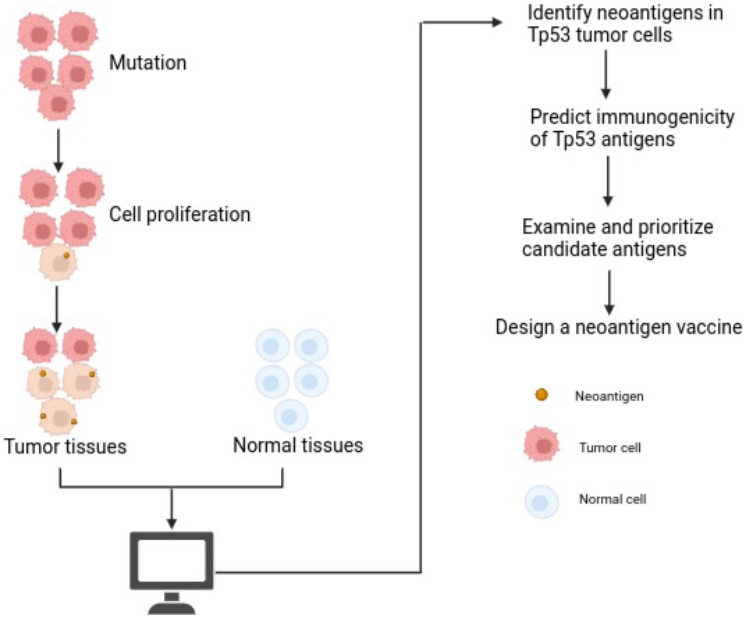

Fig. 1. [10-11] Bioinformatics tools enable prediction of candidate immunotherapy targets. (Figure adapted from "Neoantigen vaccine: an emerging tumor immunotherapy" and made with BioRender. (https://bit.ly/3TLZV4T))

The undergraduate research described here is at the forefront of cancer immunotherapy; leveraging the cancer-specific NCI TP53 database, CEDAR, and IEDB tools to predict immunogenic p53 tumor antigen peptides computationally. By utilizing a next-generation pipeline tool to predict the processing of intracellular events, the results are then compared to experimental p53 epitope data to identify epitopes most likely to elicit an immune response to a large set of tumors while minimizing cross-reactivity to normal tissue. This project combines a current understanding of p53 role in cancer with available databases and bioinformatics tools to identify and characterize priority peptide epitopes that may serve as powerful neoantigens for targeted immunotherapies.

Methods

Database and Resource Tools Used for Bioinformatics Analyses

This study used a select set of open access resources. In 2021, The Cancer Epitope and Analysis Resource (CEDAR), (https://cedar.iedb.org/) funded by the National Cancer Institute NCI, was developed as a companion to the Immune Epitope Database Analysis Resource (IEDB) (iedb.org) created by National Institute of Allergy and Infectious Diseases in 2003 with ongoing updates [12]. CEDAR serves as a repository of cancer-specific experimental peptide and epitope data as it catalogs experimental data on antibodies and T cell epitopes studied primarily in humans regarding cancer disease. CEDAR and IEDB collectively host next-generation tools that assist in predicting and analyzing epitopes (nextgen-tools.iedb.org/). For given protein sequences, the tools predict each step in the antigen processing and display process, including proteasomal cleavage, transporter associated with antigen processing (TAP), MHC Class I binding, cell-surface display, and T-Cell recognition (see Figure 2). The underlying machine learning algorithms have been trained on extensive empirical data sets to predict how each candidate epitope will behave at each step, thus avoiding costly empirical testing for large sets of new candidate epitopes. The sequence processing workflow follows the biological process by which peptides are internally processed and externally displayed for interaction with T-cell receptors. This project leveraged the newer CEDAR database to predict and analyze immunogenic p53 cancer epitopes.

The National Cancer Institute's (NCI) TP53 Database (https://dceg.cancer.gov/tools/public-data/tp53-database) has nearly 28,000 mutations of TP53 tumor variants characterized and available to the public. This extensive TP53 mutation variant dataset was used to map mutation frequency across the p53 protein and align it with computation predictions. The computational predictions from the generation tools in CEDAR were compared to experimental data in NCI TP53 DB and the CEDAR database, as described below.

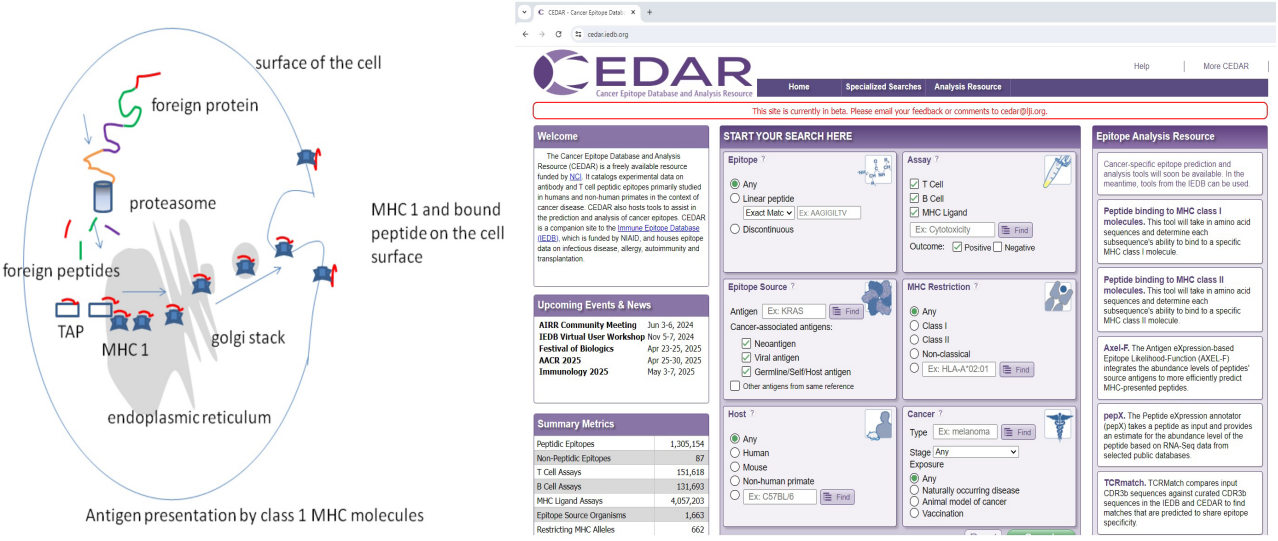

Fig. 2. [12-13] The CEDAR database and Next-Generation tools enable computational prediction of all steps in the antigen processing and presentation pathway (Figure adapted from Colm and Koşaloğlu-Yalçın et al.)

MHC Class I Presenting Peptide Predictions Using Next-Generation Tools

The Next-Generation Epitope Prediction Tools platform (https://nextgen-tools.iedb.org/) was used to predict a set of peptide epitopes that MHC Class 1 proteins may present on the cell surface. The tool links predictions of intracellular events of antigen processing into one workflow. The computational pipeline used included the following predictions: proteasomal cleavage, predictions of selective specificity of peptides that are transported into the cytosol of the endoplasmic reticulum lumen, and MHC1 binding. Our pipeline and its parameters (https://nextgen-tools.iedb.org/pipeline) using p53 (UniProt: P04637).

p53 Tumor Variant Mutation Frequency Distribution

TP53 mutation variant data (n=27,847) from the NCI p53 database was used to identify protein regions showing high mutation frequency. The NCI codon distribution tool (https://portal.gdc.cancer.gov/analysis_page?app=ProteinPaintApp) was applied to the variant data to build a tumor variant distribution chart displaying the mutation frequency of amino acid segments along linear p53.

Aligning Predicted Peptides to Full-Length p53 Protein and Tumor Variant Data

The resulting 23 peptides were modeled on linearized p53 (Uniprot: P04637) (1-393 aa) and juxtaposed along the NCI p53 tumor variant mutation distribution chart described above to identify regions of interest for neoantigen targets.

Comparison of Predicted and Empirical Results in CEDAR to Obtain Experimental Data for NGP Peptides

To describe the immunogenicity of the 23 NGP Peptides as non-mutated (self-antigen) and mutated (neoantigen) epitopes, human T cell assays of TP53 (UniProt: P04637, E7EQX7, J3KP33) epitopes were exported from CEDAR's database and stratified. Self-antigen assays (n=25) were collected by filtering self-antigens with negative assay results. Neoantigen assays (n=76) were collected by filtering neoantigens with positive assay results. Using BLAST, sequences from CEDAR and NGP Peptides were matched, and assays for each NGP Peptide were counted.

Results and Discussion

Next-Generation Pipeline Predicted Peptides

The next-generation pipeline (NGP) feature of the CEDAR and IEDB resources was used to compute a set of candidate neoantigen targets. The NGP predicts products of intracellular steps of antigen processing to display for immune system surveillance and is a relatively new resource available to the public. (http://workshop.iedb.org/)

The results of the next-generation pipeline applied to the p53 protein sequence included a set of 23 peptides, listed in Table 1. Amino acid location on the p53 protein shows a broad distribution with a few clusters. Epitopes derived from sequences with the highest incidence of mutations must be a higher priority, as the resulting therapy will be effective against a broader set of tumors across diverse populations.

Table 1

Cluster.Sub-Cluster Number	Peptide Number	Alignment	AA Position
1.1	Consensus	FEMFRELNEALELK	338-351
2.1	Consensus	RMPEAAPPVAPAP	65-77
3.1	Consensus	EYFTLQIRGRERF	326-338
4.1	Consensus	YQGSYGFRLGFLH	103-115
5.1	Consensus	GTRVRAMAIYK	154-164
6.1	Consensus	APAPAAPTPAA	74-84
7.1	Consensus	LSQETFSDLWKL	14-25
8.1	Consensus	VEYLDDRNTFR	203-213
9.1	Consensus	NLLGRNSFEVR	263-273
10.1	Consensus	MLSPDDIEQWF	44-54
11.1	Consensus	EVRVCACPGRDRR	271-283
12.1	Consensus	DSTPPPGTRVR	148-158
13.1	Consensus	RGRERFEMFREL	333-344
14.1	Consensus	QSQHMTEVVRR	165-175
15.1	Consensus	VVVPYEPPEV	216-225
16.1	Consensus	APAPAPSWPL	84-93
17.1	Consensus	VGSDCTTIHY	225-234
18.1	Consensus	HLIRVEGNLR	193-202
19.1	Consensus	WKLLPENNVL	23-32
20.1	Consensus	RNSFEVRVCA	267-276
21.1	Consensus	RNTFRHSVVV	209-218
22.1	Consensus	RRPILTIITL	248-257
23.1	Consensus	RVEGNLRVEY	196-205

The 23 next-generation pipeline (NGP) predicted peptides and their position on the p53 protein sequence after processing through IEDB's Next-generation Pipeline tools. Each NGP Peptide is the consensus alignment of its respective clustering results.

Positioning of NGP Predicted Peptides on p53 Mutations

To determine the prevalence of each mutation and thus the real-world relevance of the predicted epitopes, the NCI's TP53 database was accessed and analyzed in the context of the linear p53 protein. As seen in Figure 3, the frequency distribution of TP53 mutation variants along full-length p53 was visualized by building a codon distribution chart, with each codon representing an amino acid. The peaks and valleys show the frequency of mutations around a specific section of the linear protein. The codon chart showed a high incidence of mutations in specific regions along p53.

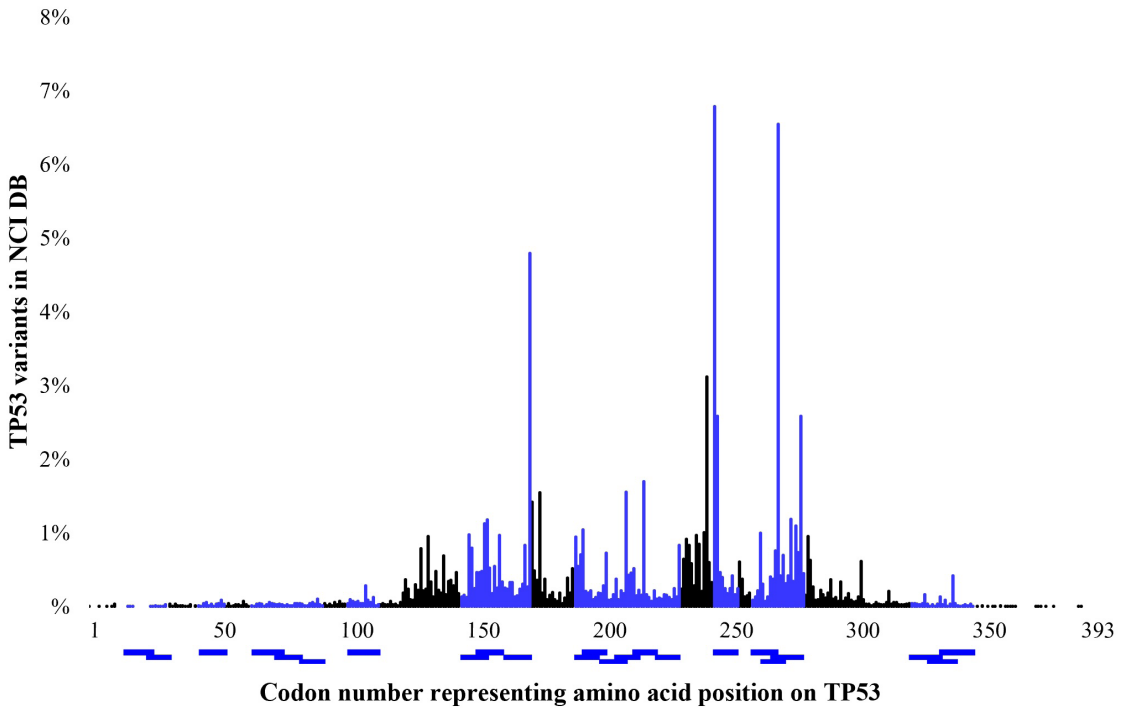

Fig. 3. The frequency distribution of known human p53 variants from the NCI's TP53 Database (n=27,847) is mapped onto the full-length p53 codons (1-393 aa), representing amino acids. In blue, the 23 NGP Peptides are both spatially aligned along TP53 and highlighted within the distribution. The association between NGP Peptides and variant data represents the mutability within each predicted peptide.

Juxtaposing the 23 NGP Peptides against the mutation distribution of computationally predicted epitopes on this frequency chart helped zone in on neoantigen epitope targets that will be effective across the broadest range of tumors and populations. Multiple spikes in prevalence are observed between the 150th and 300th codon. This area acts on the cell cycle, inhibiting the moderating actions that monitor the cell cycle, resulting in the formation of cancer cells, and it makes sense that mutations will lead to tumor development [14]. The peaks in blue show overlap between regions of high mutagenicity and predicted immunogenicity, which will be priority amino acid regions for immunotherapy.

The Summation of TP53 Point Mutation Frequencies within Each NGP Eptide

To further characterize the incidence of real-life mutations in these predicted epitope peptides, the percentage of p53 variants found in the NCI's TP53 database represented by each of the amino acids within each of the 23 peptides was combined and graphed in Figure 4.

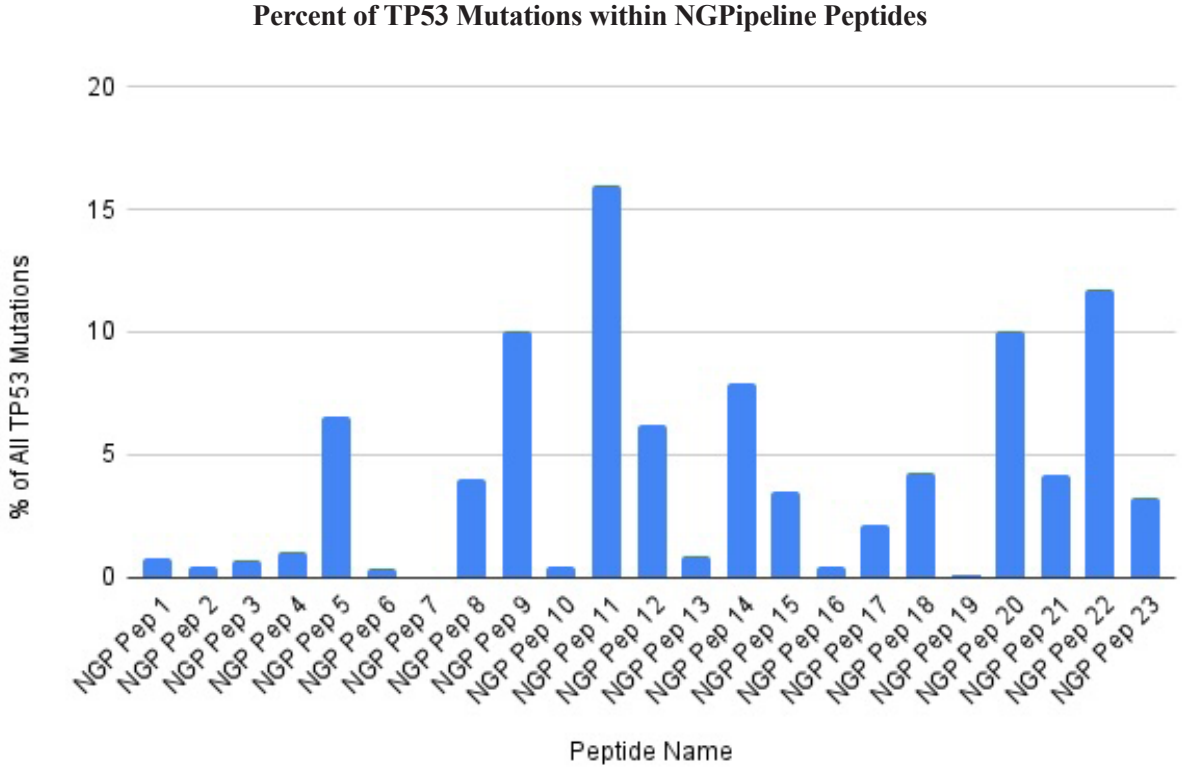

Fig. 4. This graph summarizes the frequency of tumor variants from the NCI's TP53 database, represented in each of the next-generation pipeline (NGP) predicted peptides (Peps). Calculations were performed by combining the mutational frequencies for each amino acid within the amino acid range of the NGP Peps.

Certain NGP Peptides contained higher frequencies of mutations than other peptides. Those accumulating more than 5% of known mutations were NGP Peptides 5, 9, 11, 12, 14, 20, and 22. Peptides containing 3% to 5% of the mutations were NGP Peptides 8, 15, 18, 21, and 23. Peptides with fewer than 3% of the mutations were NGP Peptides 1, 2, 3, 4, 6, 7, 10, 13, 16, 17, and 19. These data give confidence to our predictive model, where predicted epitopes can be found in the literature. The next step was to use the *in vitro* data within the CEDAR database to assess real-world immunogenicity as measured by T-cell assays.

Comparing *In Vitro* Immunogenicity of Self-Antigen and Neoantigen NGP Sequences

Non-mutated self-antigens are non-immunogenic or would otherwise be autoimmune. Depending on the mutation, variable levels of immunogenicity are possible as the neoantigen is dissimilar from the self-antigen [9]. Figure 5 shows *in vitro* human T-cell assays exported from CEDAR where the experimental epitopes matched NGP Peptides. Self-antigen assays were the accumulation of self-antigen stimulation assays that did not elicit an immunogenic response, and neoantigen assays were those from neoantigen peptides that did elicit an immunogenic response. CEDAR was vital because it collected these epitopes and their assay data to analyze post hoc. Without these open-access resources, this project would be challenging to process using currently available undergraduate research resources.

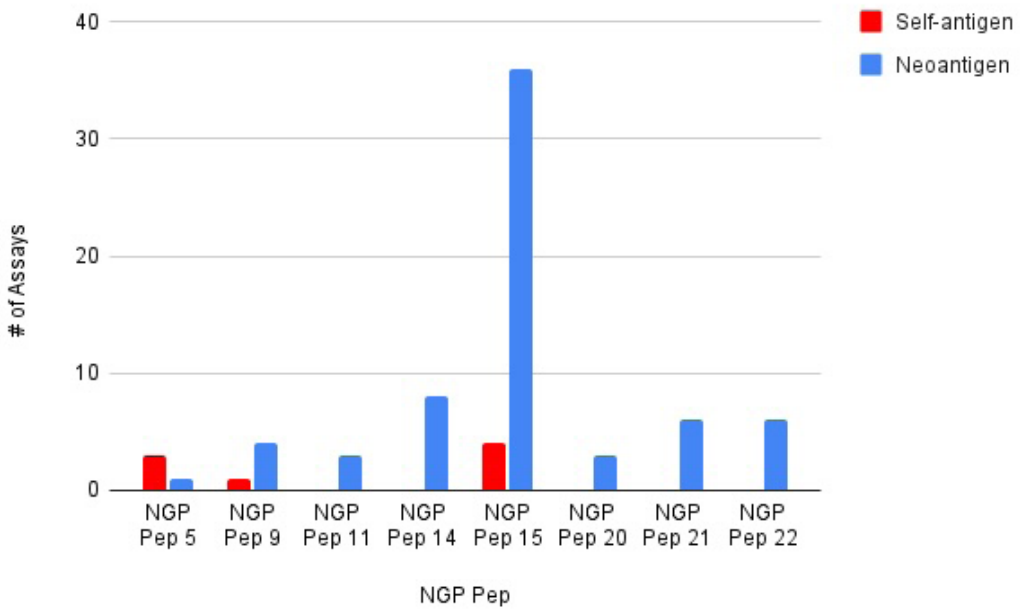

Fig. 5. The number of in vitro T cell assays exported from CEDAR with experimental epitopes matching any of the 23 NGP Peptides. Self-antigen assays were the accumulation of self-antigen stimulation assays that did not elicit an immunogenic response (n=8 of 25), and neoantigen assays were those from neoantigen peptides that did elicit an immunogenic response (n=67 of 76). NGP peptides 5, 9, and 15 are high priority as the non-mutated sequence did not produce T cell reactivity, and the mutated version did.

Conclusion

To summarize, this work identified three p53 epitope sequences representing a significant set of real-world p53 mutations found in tumors. In addition, *in vitro* data supports that the neoantigens are immunogenic, while the non-mutated sequences are not. These results suggest that the results of the CEDAR prediction tool can be used to predict real-world data. These results also help recommend further in vitro and in silico testing of epitopes to increase our confidence in whether our other NGP Peptides are suitable candidates.

These findings help support the value of computational prediction in identifying high-priority immunotherapy and vaccine targets. This is important because challenges remain in cancer immunotherapy, especially in solid tumors. p53 is an attractive target since it is a critical tumor suppressor [15]. Mutations in the p53 gene have been found in 50% of cancers, and failures in the p53 pathway contribute to almost all cancers [16]. Furthermore, prior research suggests it has a dominant negative phenotype. To aid computational predictions, an extensive set of p53 tumor antigen variants have been identified and are available in NCI TP53 DB, with supporting experimental data in CEDAR. We evaluated these resources and leveraged components that helped meet the project objective to determine whether computational predictions can successfully identify p53 immunogenic neoantigens that cover the spectrum of clinical mutations. Requirements of a successful immunotherapy target include effective antigen processing and T cell reactivity, a non-immunogenic wild-type, and tumor antigen variants that are clinically prevalent across diverse populations. Comparing predicted results with available experimental data best enables effective immunotherapy target epitope identification.

Computational prediction is critical to identifying high-priority immunotherapy targets given that the immune system sees only a tiny fraction of tumor antigens, so data alone do not give a complete picture. To predict immunogenicity, neoantigen prediction tools must cover all steps, from mutant protein production to T cell activation. The development of CEDAR and the next-generation tools platform has enabled students to

embark on medically essential and timely research to help develop broad immunotherapy targets for cancer diagnoses and vaccines. Follow-on student projects will use CEDAR and its next-generation tools to further refine the priority immunotherapy targets by assessing protein expression and the critical T-cell recognition of the candidate epitopes. As CEDAR expands, we expect future projects to utilize their growing repertoire of tools and conduct deeper analyses [12]. This research also sets the stage for future student projects that could explore features of the high-priority epitopes (overlap of computed and empirical epitopes), such as the effect of mutations on protein structure and how this may impact function and immunogenicity. Critical subsequent research will also explore how well the predicted epitopes represent diverse populations [17-18]. It will suggest ways that data can be accessed and utilized differently so that the resultant immunotherapy would benefit all populations.

In conclusion, concepts of the role of p53 in cancer were applied with the novel, open-access databases and bioinformatics tools to identify and characterize priority peptide epitopes that may serve as powerful neoantigen targets. The work sets the stage for follow-up undergraduate projects that use current bioinformatics capabilities to address and help solve immunotherapy and vaccine challenges.

Acknowledgments. The students would like to thank their faculty advisor, Sheela Vemu, Ph.D., Associate Professor of Biology at Waubonsee Community College. They would like to express their appreciation for the insight and guidance from the Cancer Epitope Database and Analysis Resource (CEDAR) Bioinformatics scientist, Dr. Zeynep Koşaloğlu-Yalçın, and Senior Project Manager Nina Blazeska, La Jolla Institute for Allergy and Immunology, La Jolla, CA. The students want to acknowledge the significant contribution of a Waubonsee Alumnus, Brady Anderson, for his research mindset that propelled the project's direction and the editing of the manuscript, and Beth Vitalis for her dedication and expertise in improving and finalizing the manuscript. The initial ideation of this project started with the Antibody Engineering Hackathons, which were supported by the National Science Foundation (NSF) under the award DUE ATE # 2055036. This work was in part supported by the National Science Foundation (NSF) under the award DUE ATE #2325500.

Disclosures. The authors declare no conflicts of interest.

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Applying Nextstrain and iCn3D to Modify and Expand an Existing Activity for Undergraduate Students Characterizing Potential Binding of Antibodies to Mutations in the Pathogens Influenza, Respiratory Syncytial Virus (RSV), or Enterovirus D68

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Abstract: Antibodies are proteins that can protect against disease using a variety of mechanisms, including binding to pathogens and targeting them for destruction. Structural modeling of antibody binding to the SARS-Cov-2 spike protein and how mutations might allow viruses to escape antibody neutralization has been previously investigated in Antibody Engineering Hackathons. The procedure for investigating immune escape can be used for students in affordable and accessible Course-Based Undergraduate Research Experiences (CUREs). In this work, we adapted and expanded the SARS-Cov-2 protocol to address new pathogens, including hookworms, Respiratory Syncytial Virus (RSV), Influenza, and Enterovirus D68. We found each presented unique challenges; however, these challenges present opportunities for student research. We describe how modifications to the SARS-Cov-2 protocol designed for SARS-CoV-2 could allow students to investigate the impact of mutations in each of these pathogens when binding to antibodies.

Keywords: iCn3D, Nextstrain, SAbDab, hookworm, enterovirus D68, RSV, Influenza, SARS-CoV-2

Introduction

Antibodies are important components of the immune system that help protect against disease. One of the functions of the antibody is neutralization, where the antibody blocks the pathogen's ability to target cellular structures. The strength of antibody binding to a particular pathogen is determined through chemical bonds and interactions that together influence the specificity of the interaction. Monoclonal antibodies are a pure collection of antibodies with identical amino acid sequences [1]. Previous hackathon teams worked to create bioinformatics activities for students involving SARS-CoV-2 and monoclonal antibodies [2-4]. In these activities, students can identify locations where antibodies bind and neutralize the SARS-CoV-2 Spike protein using 3D modeling tools (iCn3D) [2, 3, 5]. Furthermore, students can find and input new variant sequence information [6], align these variant sequences to sequences from known 3D structures, and identify mutations within the antibody binding site of the Spike protein [2, 5]. Finally, using the mutations and interactions tools within iCn3D, students can make predictions regarding the strength of antibody binding to new variants through the gain or loss of various bonds [2, 5].

The instructions and instructor guides developed through earlier hackathons [2-4] for SARS-CoV-2 were used in Bioinformatics courses at Shoreline Community College (Fall 2022), a Cellular and Molecular Biology course at Prairie State College (Spring 2023 and Fall 2023), and a Microbiology and Immunology course at Regis University (Spring 2024). The freely available databases and software employed in this project make modern research techniques available to a greater number of students and allow students to perform state-of-the-art analyses from their home or classroom computers. iCn3D [5] is used in this project because it is a free,

open-source, web-based application that does not require students to download any files. It is also the only application allowing users to share links to the annotated structure models, an important feature for evaluating student work.

Feedback from students and faculty indicated that they wanted to explore different antibody-pathogen interactions rather than continue indefinitely with SARS-Cov-2, allowing students to take ownership of the project and follow their specific interests. In this study, we broadened the approach and expanded this procedure to other pathogens and their neutralizing antibodies. To achieve this goal, we needed two important pieces of information: 1) the presence of a particular pathogen and mutation data in the Nextstrain database [6] and 2) 3D structural information for an antigenic protein in a complex with an antibody. Structures of antibody-protein complexes are deposited by researchers in the Protein Data Bank (PDB) (rcsb.org) [7] and compiled in the Structural Antibody Database (SAbDab) [8]. We describe the resources we found for four new pathogens (hookworms, Respiratory Syncytial Virus (RSV), Influenza, and Enterovirus D68), our initial results, and the challenges posed by each as they can be applied for use in student research projects.

Methods

Antibody Engineers Hackathon

The 3rd Annual Antibody Engineering Hackathon took place August 7-10th, 2023. During this event, small groups of individuals worked collaboratively and virtually to solve problems related to antibodies that could then be utilized in undergraduate classroom settings and share their results with the larger group [4]. Our group focused on exploring how we could adapt the bioinformatics approach, described previously for SARS-CoV-2 [2-4], to determine how the SARS-CoV-2 protocol may need to be adjusted to examine the impact of mutations on neutralizing antibodies, potentially resulting in escape from neutralization by antibodies. Our goal was to assist faculty who might be developing student projects or CUREs based on this procedure and their own interests.

Using SAbDab to Find PDB Antibody-Pathogen Structures

The first step of the SARS-CoV-2 protocol [2] involves locating structure files that contain antibodies that are bound to proteins from specific pathogens. SAbDab contains antibody structural data available as PDB files and is a useful source for antibody structures because the structures are standardized and annotated [8]. To adapt the SARS-CoV-2 protocol to different pathogens, we searched SAbDab for the following pathogens: influenza, hookworms (*Necator americanus, Ancylostoma ceylanicum, and Ancylostoma duodenale*), RSV, and Enterovirus D68. Then, we downloaded all available SAbDab data to a Microsoft Excel file. Available data includes the PDB file name, antibody heavy and light chain names associated with the NCBI Molecular Modeling Database (MMDB) [9], antigen names associated with the MMDB [9], organism, species where the antibody heavy and light chain was made, antigen species, references to publication(s), notes about the structure, and more [8].

Alignment of Sequence Data to Structure Data

The next step from the SARS-CoV-2 protocol [2] was to align protein sequence data from antigenic variants to the pathogen protein sequence in the structure. Using sequence data available in the Nextstrain database [6] for each of the new pathogens we analyzed, we aligned the mutation data to the structure using tools in iCn3D [5]. For influenza, sequence data was also obtained from the Influenza Virus Database. The Influenza Virus Database will be redirected to the NCBI Virus site in Fall 2024 [10]. Viral sequences can also be found through the Influenza Virus Database with a variety of filters such as nucleotide or amino acid sequence, filter for a keyword or strain name, the influenza subtype (A, B, or C), the host, the country, protein, H and N subtypes, sequence length, collection date, and release date [10].

Results and Discussion

We narrowed down a list of potential new organisms based on the availability of data and the interests of the group members. SAbDab was used in conjunction with information found in Pubmed to identify known structures for the three pathogens (RSV, Enterovirus D68, and Influenza virus) [8].

Hookworm

Parasitic hookworms may be found in contaminated soil and infect the intestines of over 400 million people globally [11]. These parasites are responsible for the loss of over 4 million disability-adjusted life years (DALY) and cause economic losses exceeding $100 billion annually [12, 13]. None of the hookworms of interest (*Necator americanus, Ancylostoma ceylanicum, and Ancylostoma duodenale*) were found in the Nextstrain database [6]. Furthermore, no structures containing hookworm proteins associated with any monoclonal antibody were detected in the SAbDab database [8]. Using the PDB database (rscb.org) [7], we identified two structures related to immune responses, *Stichodactyla helianthus* toxin (ShK)-like immunomodulatory peptide from hookworm *Ancylostoma caninum* (main host is Canids) (PDB: 2MD0) and the filarial worm *Brugia malayi* (PDB: 2MCR) [14]. Structure alignments were completed with those two peptides in iCn3D using VAST+ based on TM-align (Figure 1) [5]. Areas where the aligned sequences are highly similar (red) (Figure 1) may be good targets for antibodies in the future, as they are highly conserved regions among different hookworm species.

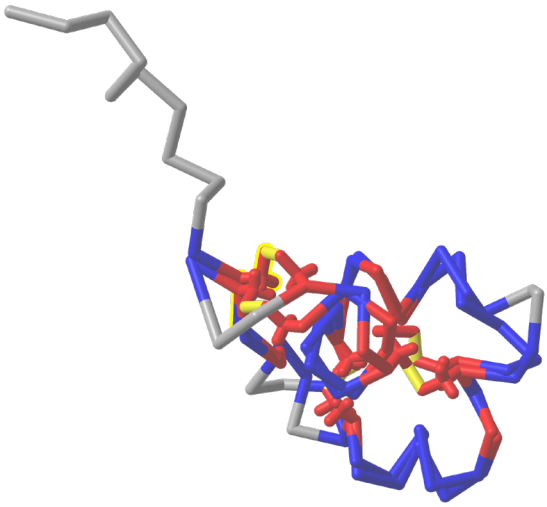

Fig. 1. ShK-like immunomodulatory peptide alignment of PDB files 2MD0 (Ancylostoma caninum) and 2MCR (Brugia malayi (filarial worm)). Red = identical amino acids, blue = different amino acids, gray = amino acids that are only found in one of the structures. Yellow = disulfide bonds https://structure.ncbi.nlm.nih.gov/icn3d/share.html?M4tnYB3gCYjoTsJa7

RSV

RSV is a non-segmented, enveloped, negative-sense RNA virus that causes cold-like symptoms that are typically mild but can be severe, particularly in the very young or immunocompromised [15]. Healthy infants under eight months of age can be given monoclonal antibodies (mAb) to prevent severe RSV symptoms [16]. Three monoclonal antibodies appear in the literature for this purpose: palivizumab, the original mAb studied for use in infants [17-19]; motavizumab, a 2nd generation derivative of palivizumab [20, 21]; and nirsevimab, a recently approved mAb, commercially known as Beyfortus [20, 22-25]. Motavizumab is not currently licensed for use due to adverse skin reactions [26, 27]. All of these antibodies bind to the Fusion Glycoprotein (F) of RSV [28, 29], which is highly conserved in RSV [30].

Using the SAbDab database [8], nine PDB structures were identified in August 2023. As of May 2024, forty-eight structures were available (Table 1). Of these structures, 64.5% contained an antibody bound to the RSV F protein F, and 14.5% contained an antibody bound to the RSV Glycoprotein (G). The remaining PDB structures included either only antibody structures or only F protein structures (Table 1). Using the Nextstrain database [6], we saw little variability in the F protein [31], so we predict that the binding region(s) will not change greatly. The G protein of RSV does mutate frequently [31], but structures of G protein-antibody complexes were not available in the SAbDab database at the time of this study (August 2023) [8].

Table 1. RSV Structures Available in SAbDab May 2024

# PDB IDs	Antigen protein	Antibody species
23	F	Human
5	F	Mouse
3	F	Llama
7	G	Human
2	F only, no antibody	Mouse
2	N/A Antibody only	Llama
2	N/A Antibody only	Cow
1	N/A Antibody only	Human
1	preF	Human
2	Other	

Structures of the RSV G protein complexed with antibodies are now available in the SAbDab database [8] and could be used for future student projects. The seven unique PDB structures in the SAbDab database [8] contain five unique neutralizing antibodies [32-34] (Table 2). Two of the structures (PDB: 5WNA (Fig. 2a) and 6BLI) include multiple copies of antibodies (3D3 and CB002.5, respectively) binding to multiple G proteins [32, 34]. One structure (PDB: 5WN9) includes the heavy chain only (antibody 2D10) bound to the G protein [32]. The other structures (PDB: 5WNB, 6UVO, 7T8W, and 6BLH (Fig. 2b)) include three different antibodies (3D3, 3G12, 3G12, and CB017.5, respectively), with each heavy and light chain in combination with a single, unique conformational state of the G protein [32-34]. The 7T8W PDB structure shows a mutated G protein bound to the 3G12 antibody [33]. To simplify the evaluation of real-time mutations for student projects, the single antibody-antigen complexes will allow students to most easily assess whether a mutation found in the Nextstrain database [6] is likely to result in loss of neutralizing activity for any of the known structures following previous protocols [35].

Table 2. RSV G Protein Containing Structures Available in SAbDab May 2024

PDB ID	Antibody name	# Antibodies	# G proteins	Reference
5WN9	2D10	H chain only	1	[32]
5WNA	3D3	2	2	[32]
5WNB	3D3	1	1	[32]
6BLH	CB017.5	1	1	[34]
6BLI	CB002.5	4	4	[34]
6UVO	3G12	1	1	[32]
7T8W	3G12	1	1, mutated	[32]

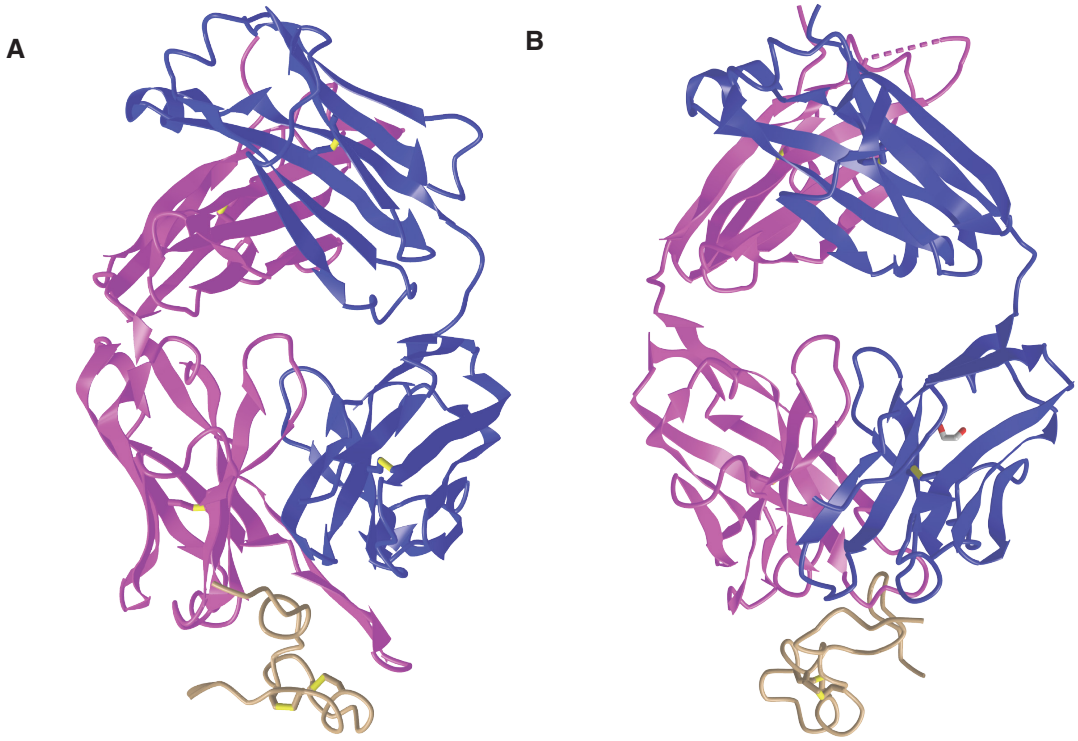

Fig. 2: Representative PDB structures showing RSV G protein and antibody complexes binding to specific portions of the G protein. (A) 5WNA: Structure of antibody 3D3 bound to the central conserved region of RSV G protein. https://structure.ncbi.nlm.nih.gov/icn3d/share.html?NDTWQ34p1pbgHPCh7 Pink = heavy chain of the antibody, Blue = light chain of the antibody, Brown = RSV G protein. (B) 6BLH: Structure of RSV G central conserved region bound to antibody Fab CB017.5 https://structure.ncbi.nlm.nih.gov/icn3d/share.html?xsQadbUuKn6CJRuz9 Pink = heavy chain of the antibody, Blue = light chain of the antibody, Brown = RSV G protein.

Influenza

Influenza A is a segmented, enveloped, negative-sense RNA virus that causes influenza. This highly contagious virus is categorized into subtypes by the Hemagglutinin (HA) and Neuraminidase (NA) glycoproteins present on the surface of the virus [36]. The mutations associated with this virus lead to frequent changes in HA/NA sequences [37] and loss of protection from previous antibodies. Thus, finding vaccines effective against the differing strains of the virus is critical due to the economic and health impacts of influenza infections [38-41].

Using the SAbDab database [8], we found many structures of antibodies associated with influenza (268 unique identifiers). We selected one PDB structure for further study, a "Broadly reactive antibody" that binds with the hemagglutinin protein (HA1) from the 2009 Pandemic strain (PDB: 4M5Z) (Fig. 3a) [42].

To see if the term “Broadly reactive antibody” was still accurate for current strains of influenza, we first looked for sequence information in the Nextstrain database [6]. Unfortunately, direct links to Genbank accession numbers were unavailable at the time of this study. To avoid manually entering all of the mutations, we searched elsewhere for sequence information on isolates. The NCBI's Influenza Virus Database provides sequence information on influenza that is linked directly to accession numbers for the gene(s) of interest [10]. As a proof of concept, we searched for sequences isolated from 2023 using the parameters of influenza type A, human host, any country, H1, N1, full length only, and collapsed identical sequences. The first sample that contained a full genome sequence and accession number in the list was from an H1N1 influenza strain (OQ615380) [43]. We aligned the HA protein sequence (WEI46805.1) from this strain to the hemagglutinin protein from the 2009 Pandemic strain (PDB: 4M5Z) in iCn3D using BLAST (Fig. 3b/c) [5].

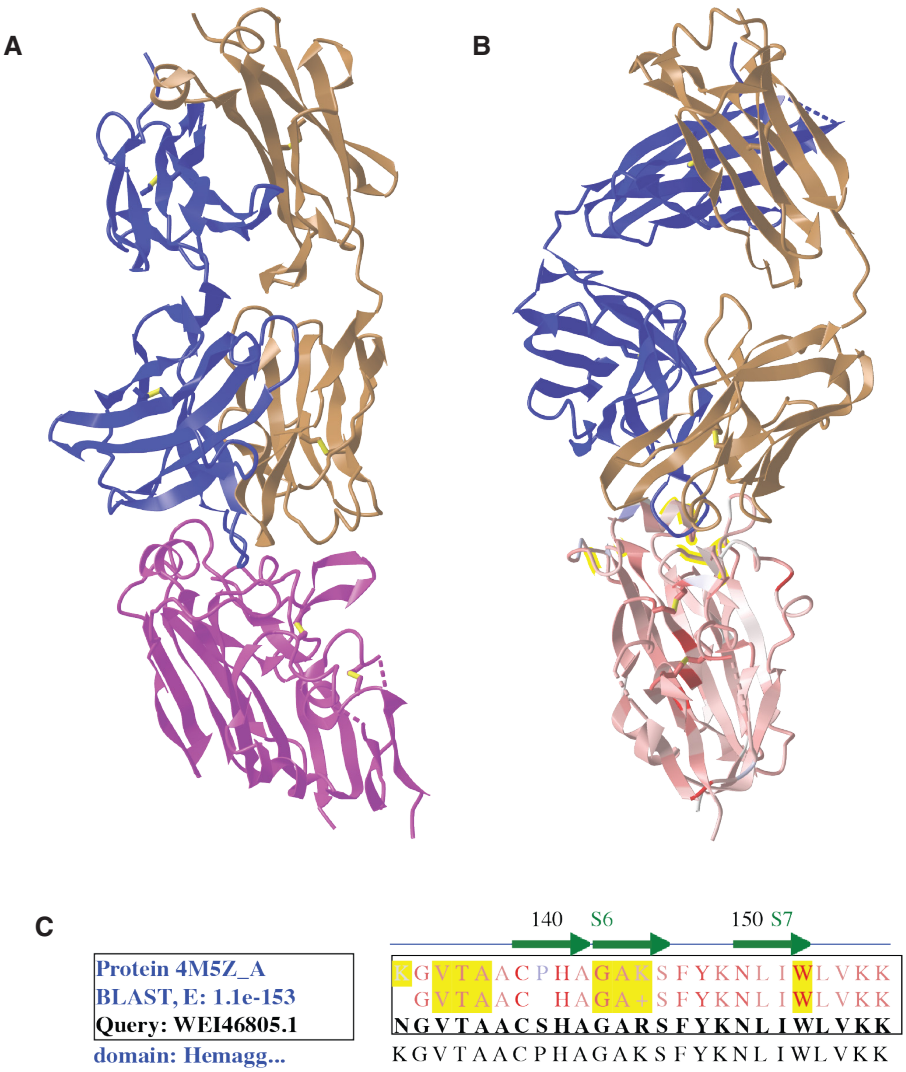

Fig. 3: Representative PDB structures showing Influenza HA1 protein and antibody complexes. (A) 4M5Z: Crystal structure of the broadly neutralizing antibody 5J8 bound to 2009 pandemic influenza hemagglutinin, HA1 subunit [5]. Pink = HA1 subunit, Blue = heavy chain of the antibody, Brown = light chain of the antibody. (B) HA protein sequence from variant (WEI46805.1) aligned to the HA structure in 4M5Z, with antibody binding domain amino acids highlighted in yellow [5]. On HA (bottom): the intensity of red represents the similarity and importance of the aligned amino acids, and Blue = different amino acids. https://structure.ncbi.nlm.nih.gov/icn3d/share.html?Ej7qWpxs48aQPGpd6 (C) Amino acid sequence alignment with amino acids in the antibody-binding domain highlighted yellow. The amino acid numbers are indicated along the top, and the green arrows indicate elements of secondary structure (S6 = sheet 6 and S7 = sheet 7). The top pink sequence is from the HA protein of 4M5Z. A blue color is used to indicate amino acids that differ (mutations) between the HA protein in 4M5Z and the amino acids in the third sequence, from the variant (WEI46805.1) (shown in black). The sequence between the 4M5Z hemagglutinin and the variant shows amino acids that are found in the same position in both HA proteins. An empty space is used when the amino acids differ and are not chemically similar. A "+" indicates that the two amino acids are different but chemically similar. The bottom sequence (black) is the consensus sequence of the hemagglutinin domain.

The HA1 sequences were very similar, as indicated by the red/pink coloration of the aligned amino acids (Fig. 3b). After verifying that both sequences came from the H1 version of hemagglutinin, we used the Select by Distance function in iCn3D [5] to identify the antibody binding region on the HA protein [2,35]. Within this binding region, most of the amino acids remained the same (Fig. 3c). We chose instances where a lysine (K) was replaced by an arginine (R) and a glutamine (Q) by a glutamic acid (E) for further investigation. The lysine (K) to arginine (R) mutation at position 145 appears to change interactions significantly (Fig. 4a). A pi-cation interaction between HA1 K145 and Y100 on the antibody heavy chain is lost, along with the following bonds from K145 to the light chain: hydrogen bonds to G29 and K31; hydrophobic interactions with T30, K31, V32, and N66; and a salt bridge ionic interaction with K51 (Fig. 4a). The glutamine (Q) to glutamic acid (E) mutation at 192 does not change interactions with the amino acids in the antibody, as indicated by the lack of different interaction lines (Fig. 4b) and only one hydrophobic interaction occurs at N32 of the heavy chain. As a consequence, this individual change is unlikely to have an effect on HA-antibody interactions. Although many amino acid interactions appear to be maintained within the predicted binding domain, and the change at 192 did not have any effect, significant changes did occur when amino acid 145 was mutated. Thus, we predict that the antibody would not be able to neutralize this variant of the virus. However, the loss of this interaction would need to be experimentally confirmed in a wet lab setting.

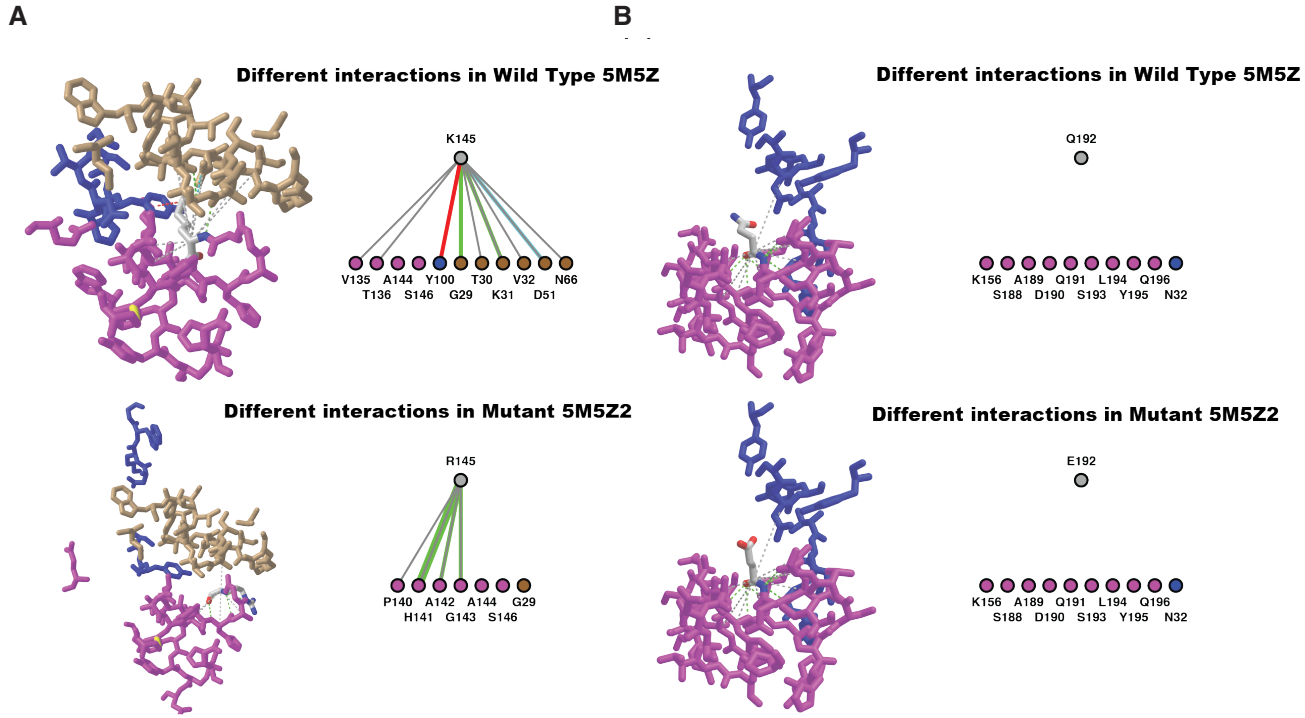

Fig. 4: Impact of mutations on predicted binding between antibody 5J8 and Influenza HA1 PDB structure 4M5Z. (A) HA1 amino acid K145 interactions with antibody 5J8. Top original K145. Bottom variant R145. Right: The color of the lines indicates the type of bond or chemical interaction. Green: H-bonds, Cyan: Salt Bridge/Ionic, Grey: Contacts, Red: π-Cation. Pink amino acids: HA1 Blue: heavy chain of the antibody. Brown: light chain of the antibody. https://structure.ncbi.nlm.nih.gov/icn3d/share.html?4RN9GsSVFZjdFZP96 (B) Neither Q192 (original) nor E192 (the variant) show different interactions with amino acids in the antibody, indicating that the binding is the same in both structures. Top original Q192. Bottom variant E192. https://structure.ncbi.nlm.nih.gov/icn3d/share.html?qFKdLueWN8ukBfD57

Enterovirus D68

Enterovirus D68 is a single, positive-stranded RNA picornavirus that typically causes cold-like symptoms, including runny nose, sneezing, coughs, and body aches [44]. Severe symptoms such as difficulty breathing or even a polio-like illness with extremity weakness, difficulty swallowing, or facial weakness can occur [44]. Children and infants are particularly at risk for disease, which can be spread by coughs, sneezes, or touching a contaminated surface [44]. Currently, no specialized treatments for this infection exist [44], so understanding the binding between potential monoclonal antibody therapeutics and Enterovirus proteins would benefit both basic research endeavors and have potential clinical implications.

The capsid of Enterovirus D68 contains four proteins: Viral Protein (VP) 1, VP2, VP3, and VP4. VP1 is the most variable protein, followed by VP2, VP3, and then VP4 [6,45]. Eight PDB structures were identified through the SAbDab database [8] in August 2023. The same eight structures remained the only available structures in May 2024 in the SAbDab database [8]. Three of the eight structures included different conformational states of the complex when the pathogen was bound with the 2H12 antibody. These three structures included all four of the viral capsid proteins (PDB: 7EBZ (Fig. 5a), 7ECY, and 7EBR) [46]. Three of the eight structures contained both antibodies and multiple capsid proteins (PDB: 6WDS, 6WDT [47], and 7EC5 [46]). The two structures where the antibody contacts only one of the capsid proteins (PDB: 6AJ9 and 6AJ7 (Fig. 5b) [48]) were the easiest to assess whether a mutation found in the Nextstrain data would be likely to result in loss of binding [35].

Table 3. Enterovirus D68 Capsid Protein Containing Structures Available in SAbDab May 2024

PDB ID	Antibody name	Capsid proteins	Reference
6WDT	EV68-228	VP1, 3	[47]
6WDS	EV68-159	VP1-3	[47]
7EBZ	2H12	VP1-4	[46]
7ECY	2H12	VP1-4	[46]
7EBR	2H12	VP1-4	[46]
7EC5	8F12	VP1, 3 genome polyprotein	[46]
6AJ7	15C5	VP3	[48]
6AJ9	11G1	VP1	[48]

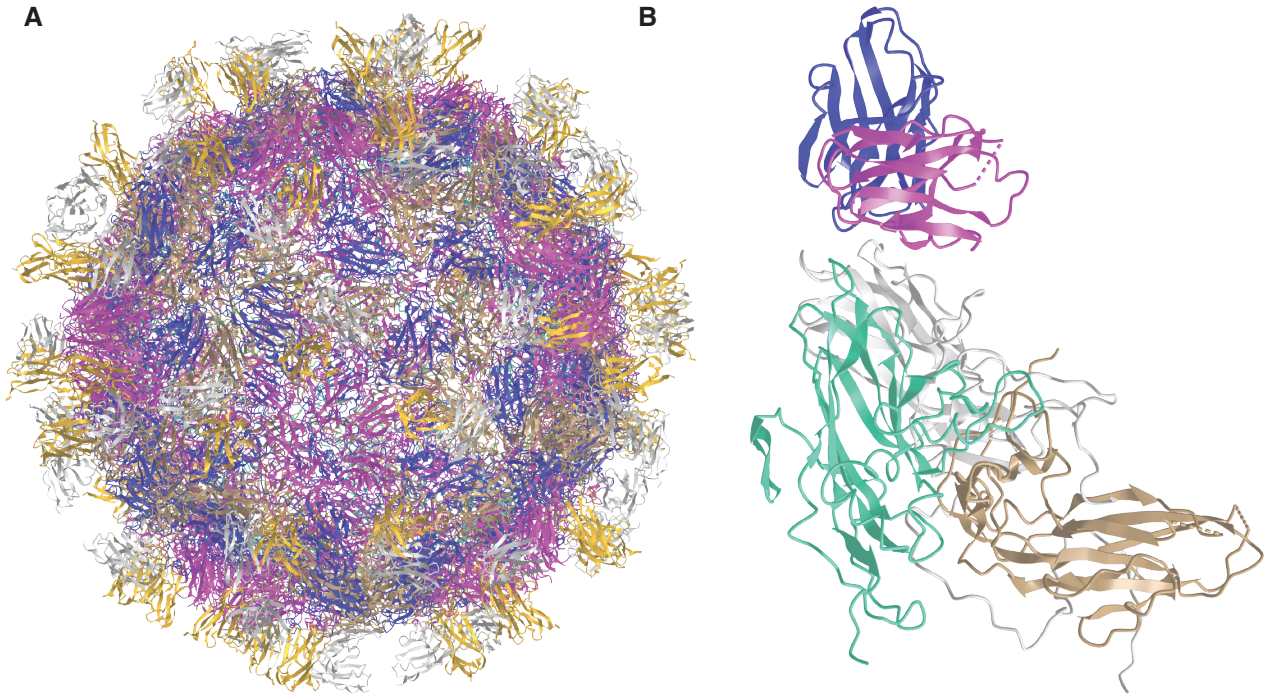

Fig. 5: Representative PDB structures showing Enterovirus D68 capsid proteins and antibody complexes. (A) 7EBZ: EV-D68 complete capsid structure in complex with 2H12 Fab. Pink: Capsid protein VP1, Blue: VP3, Brown: VP2, Green: VP4, Grey: Antibody heavy chain, Gold: Antibody light chain. https://structure.ncbi.nlm.nih.gov/icn3d/share.html?EJ3g1tq5QNXEvTKN6 (B) 6AJ7: Three EV-D68 capsid proteins in complex with antibody (Fab 15C5). Pink: Light chain of the antibody, Blue: Heavy chain of the antibody, Grey: VP3, Green: VP2, Brown: VP1. https://structure.ncbi.nlm.nih.gov/icn3d/share.html?1JMtcnqnRkzw62NQ8

Conclusion

Detailed instructions and an instructor guide are available for the original and updated protocols for analyzing the effect of mutations on antibody binding with SARS-Cov-2 [2,35]. In this work, we identified a number of specific modifications to that protocol that were needed in order to predict whether mutations in different pathogens beyond SARS-Cov-2 will be neutralized by an antibody. First, the sequence of the reference strain of the pathogen is needed. Furthermore, that pathogen should mutate rapidly and have a large number of variants within the proteins being studied, which are most likely to be antigens found on the surface of the pathogen that can interact with neutralizing antibodies. The variant protein sequence information is needed in NCBI accession number format, or a FASTA sequence, for alignment purposes. Some pathogen variant sequence information can be found on the website Nextstrain.org, but other pathogens may need to be found at the NCBI or other databases.

Additionally, PDB files containing pathogen protein/antibody complexes are needed in order to analyze the structures using iCn3D [5]. PDB files for many pathogen proteins can be found using SAbDab [8] or other databases. These structures are needed in order to align variant sequences, identify if mutations are happening within the antibody binding region on the pathogen protein, and identify any disruptions to the antibody-antigen bonds and interactions. Furthermore, we found that it can be very difficult to analyze the antibody-antigen interactions if the structure is composed of a complex of proteins. Thus, single pathogen proteins/antibody structures are preferred whenever possible. Finally, we also saw that the way the structures may be annotated with colors and chain lettering in the NCBI Molecular Modeling Database (MMDB) [9] might result in confusion, as in Figure 5a versus 5b, that can be addressed by changing color annotations in iCn3D [5].

To use this project with students, we recommend that instructors advise students to focus on structures that contain a single antibody-antigen interaction. Additionally, students need to be able to correctly identify and distinguish between the heavy and light chains of the antibody and the pathogen target. Finally, we highly recommend that instructors require students to submit the url of the structure alignments. The ability to look at the same structure as the student greatly helps with troubleshooting and identifying the source of errors. Our results demonstrate that the SARS-CoV-2 protocols can be applied to other pathogens, provided that the following conditions are met: the sequence information for the pathogen is available, the pathogen proteins of interest have a high level of mutability, and neutralizing antibody structures (PDB files) are available. A "quick start" guide is located under supplemental materials to give instructors additional information on the websites and protocols used.

Acknowledgments. This work was supported by the National Science Foundation (NSF) under the award DUE 2055036.

Disclosures. The authors declare no conflicts of interest.

Supplemental Materials. Please see https://micronanoeducation.org/wp-content/uploads/2024/09/2340461-supplemental-information-quickstart-guide.pdf

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Submerged Fermentation of *Ganoderma tsugae* for the Optimized Production of Exopolysaccharides

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Abstract: *Ganoderma tsugae*, commonly known as the hemlock reishi, has historically played a significant role in Eastern traditional medicine. Notably, *Ganoderma* species are known to have medicinal properties for potential commercial use, including cholesterol reduction, lowering blood pressure, antivirals, and antitumor therapies. To capitalize fully on these medicinal benefits for commercial use, the *Ganoderma* mycelium and its potential therapeutic metabolites, including biologically active polysaccharides, must be produced at scale. One promising approach is to employ stirred-tank bioreactors for the submerged fermentation of mycelium. The goal of this project is to maximize exopolysaccharide (EPS) production from the submerged fermentation of *G. tsugae* mycelium by locating which experimental variables are optimal. Experimental variables included media formulations and enrichments, temperature, pH, and agitation speed. Mycelium cultivation involved growing *G. tsugae* on Potato Dextrose Agar (PDA) plates, which were then used to inoculate liquid cultures in baffled shake flasks. Samples were taken every two to four days and assayed for biomass, reducing sugars, and exopolysaccharides (EPSs). The highest biomass and exopolysaccharide production was observed in a lactose-based media with a constant temperature of 28°C, pH of 5.5, and an agitation of 120 rpm. These optimized parameters resulted in a peak biomass yield of 11.4g/L and a peak exopolysaccharide yield of 1.68g/L.

Keywords: polysaccharides, *Ganoderma tsugae*, *Ganoderma lucidum*, submerged fermentation, mycelium, bioprocessing, biomanufacturing

Introduction

Over the last three decades, fungi and their chemical compounds have gained recognition from pharmaceutical industries for their effects in medicinal and dietary nutritional settings. One such fungus, *Ganoderma tsugae*, has been studied along with its close relative, *Ganoderma lucidum*, for its exopolysaccharide production. Both *G. tsugae* and *G. lucidum* excrete large amounts of these sugars as the most abundant of the bioactive compounds within them [1]. Polysaccharides from these species are known to possess anti-tumor effects but may depend on specific aspects such as molecular weight, galactose, and bound protein [2], [3]. Other *in vitro* effects of *G. tsugae* include antioxidant, anti-inflammatory, and cytotoxic properties against cancer cell lines [4-7].

Submerged fermentation of mycelium is an effective way to produce polysaccharides in large quantities compared to harvesting fruiting bodies due to the ease of transforming mycelium as a powder, as well as other factors such as maintaining a uniform distribution of the substrate, reducing contamination, and diminishing the time of growth [8]. Previous research included meta-analyses on submerged mycelium production, media formulation, and *G. lucidum* optimization in the year 2022. While other studies have identified a range of culture conditions for mycelium fermentation, additional studies are required in order to identify conditions for optimizing the production of specific bioactive compounds. The goal of the project was to optimize conditions for exopolysaccharide production. Media formulations were maximized, and media variations were tested in different batches through a sterile step-by-step plug-plate-flask-bioreactor procedure. *G. tsugae* was eventually considered over *G. lucidum* as *G. tsugae* was found to produce larger quantities of polysaccharides in lactose. This research into optimizing exopolysaccharide production of *G. tsugae* has potential commercial applications such as a nutritional supplement or food filler and an economical solution for downstream waste products.

Methods

Cultures of *G. tsugae* were obtained from Empire Medicinals, 125 Tech Park Drive, Rochester, New York, 14623, USA, and grown on Potato Dextrose Agar (PDA plates) assembled in the laboratory from PDA powder bought from VWR. To maintain cultures for use, plate-to-plate transfer of *G. tsugae* was performed by sterilely transferring a plug from a confluent culture to the center of a new PDA plate. New PDA plates were incubated at room temperature until confluency and then stored in a refrigerator.

Plugs from PDA plates were taken from the edge of the growth zone and were used to inoculate baffled flasks of liquid media. Five plugs were used to inoculate 250mL baffled shake flasks containing 100mL of media. Ten plugs were used to inoculate 500mL baffled shake flasks containing 200mL of media. Plugs were created using sterilized inoculation straws for size consistency. The shake flask media contained 20 grams per liter of lactose or glucose, 2.5 grams per liter of malt extract, 2.5 grams per liter of yeast extract, 0.5 grams per liter of KH_2PO_4, 0.5 grams per liter of Na_2HPO_4, 0.5 grams per liter of $MgSO_4$, 0.5 grams per liter of $(NH_4)_2SO_4$, and 250 micrograms per liter of ampicillin (bought from VWR). Seed cultures were incubated at 28°C shaking at 180 rpm to keep mycelium from anchoring to flask surfaces. Seed culture purity was monitored periodically with Phenol Red Broth (PR tubes), Trypticase Soy Agar (TSA plates), and microscopic examination of wet mount slides stained with Lactophenol Cotton Blue (LPCB).

After seven days of growth, flask cultures were used to inoculate either a New Brunswick BioFlo/CelliGen 115 stirred-tank bioreactor, model number BF-115 3-liter (2-liter working volume) or a Sartorius, model number 88438125-liter (4-liter working volume) stirred-tank bioreactor containing liquid media that is of the same composition as of the shake flask media and two drops per liter of Pluronic 60 to limit wall growth and foam. Samples were taken every 2-4 days and assayed for biomass (dry weight), reducing sugars (DNS assay), and exopolysaccharides (EPSs) using a phenol sulfuric acid method [9]. Batch runs testing different temperatures (25, 28, and 32°C), pH (4.5, 5.5, and 6.5), and agitation speeds (80, 100, 120, and 150 rpm) were performed. A secondary formulation of media was also tested using the conditions 28°C, 120 rpm, and 5.5pH.

The standard liquid media for the 3-liter and 5-liter stirred-tank bioreactors was formulated with the following ingredients: 2.5g/L yeast extract, 0.5g/L potassium phosphate monobasic (KH_2PO_4), 0.5g/L sodium phosphate dibasic (Na_2HPO_4), 0.5g/L magnesium sulfate ($MgSO_4$), and 2.5g/L malt extract. Each ingredient was fully dissolved using dH_2O and adjusted to 5.5pH via 1N NaOH and 1N H_2SO_4. Media to be used in a bioreactor was also given pluronic 60 to reduce foaming (2 drops per liter). The secondary media used only for the 2x experiment contained 2 times the amount of yeast and malt (5.0g/L). All media was then sterilized prior to scaling up to stirred-tank bioreactors.

To assess biomass, 10mL of homogenous samples were taken and centrifuged at 3,500 rpm for 15 minutes and the supernatant was retained for DNS and EPS assays. The remaining biomass was placed in a pre-weighed aluminum boat and then heated in an oven at 72ºC to evaporate all the liquid before being weighed.

To assess the amount of reducing sugars, lactose, or glucose as a food source, the DNS assay was utilized. From the supernatant retained in the biomass assay, a homogenized sample was diluted 1:40 with dH_2O to ensure the sample concentration was in range of the glucose/lactose standards of known concentration. The diluted samples along with the standards were then measured for reducing sugar concentration using the DNS method [9].

To assess EPS production, 2mL of supernatant retained in the biomass assay was drawn out and placed in a falcon tube. 8mL of chilled pure ethanol was added for a 1:4 dilution and sequestered in a freezer for at least 24 hours to precipitate out the exopolysaccharides. Once precipitated, the samples were centrifuged at 3,500 rpm for 15 minutes. The ethanol supernatant was decanted out, and the remaining pellet was resubmerged in 2mL of hot dH_2O and vortexed to a homogenized state. In order to ensure the EPS concentration of the sample was within the range of the **β**-glucan standards of known concentration, the samples were diluted 1:10 with dH_2O. The diluted samples, along with the standards, were then measured for EPS concentration using the phenol-sulfuric acid method [9].

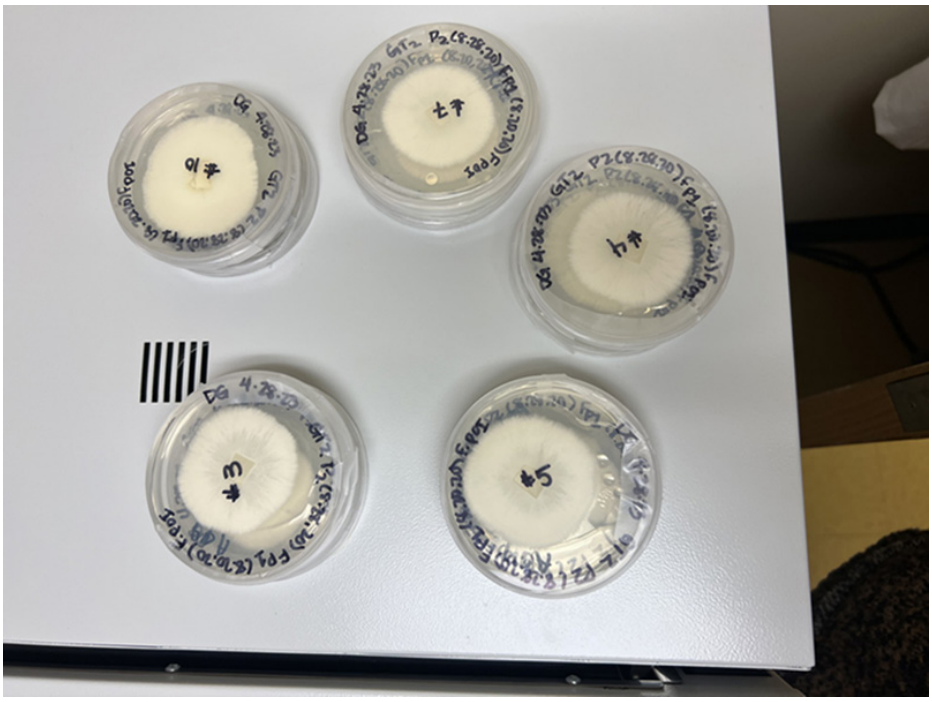

Fig. 1. 5 PDA stock plates of G. tsugae after 7 days of growth.

Fig. 2. 7-day seed flasks of G. lucidum (left) and G. tsugae (right). The elongated pellet morphology of G. lucidum is distinctly different from the circular, spider-like morphology of G. tsugae.

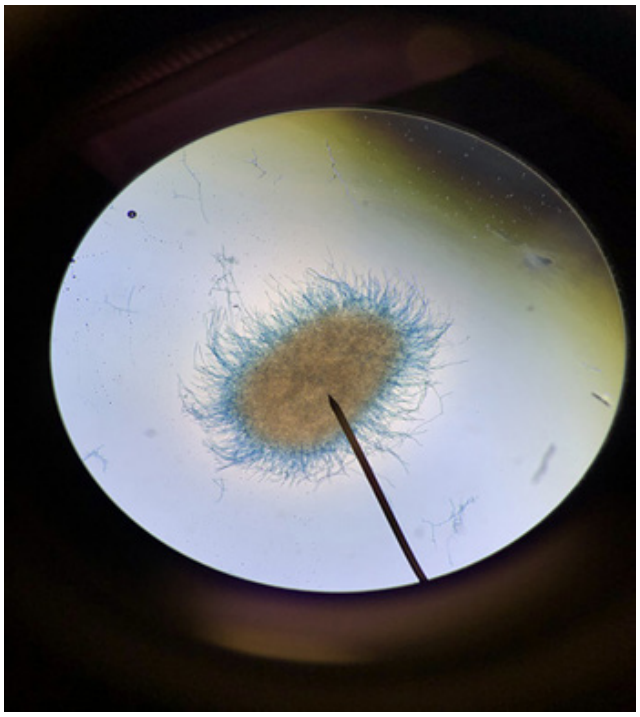

Fig. 3. G. tsugae stained with lactophenol cotton blue viewed under a microscope at 400x magnification. Lactophenol cotton blue stains chitin present in fungal cell walls.

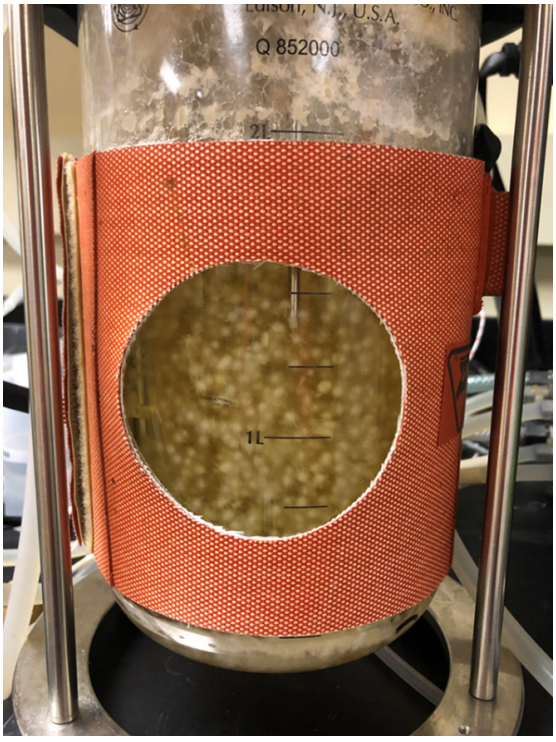

Fig. 4. G. tsugae fermented in a bioreactor after 7 days of fermentation.

Results and Discussion

The utilization of different reducing sugars as a food source, glucose, and lactose, were tested for *G. tsugae* in batches 2 and 14, respectively. Conditions included a constant temperature of 28°C, pH of 5.5, and agitation of 100 rpm over a 14-day bioreactor run. *G. tsugae* in glucose was found to have a peak EPS yield of 0.79 g/L, while *G. tsugae* in lactose was found to have a peak EPS yield of 1.21 g/L. Furthermore, both EPS and biomass production were consistently higher throughout the test run utilizing lactose rather than glucose.

The optimized food source was determined for G. *tsugae*, and other parameters were then fine-tuned for an optimized EPS yield. Optimized parameters included a 7-day shake flask incubation of *G. tsugae* in a lactose-based media at 180 rpm in a shaker, then inoculated and controlled at a constant temperature of 28°C, pH of 5.5, and agitation of 120 rpm over a 7-day run in a bioreactor (Fig. 4). These parameters resulted in a peak EPS yield of 1.68g/L and peak biomass of 11.4g/L on day 3 as seen in batch 35.

Table 1. Comparison of EPS Production (g/L) by Batch over Time (Days where samples weren't taken are denoted by "-")

Batch Number	Day 0	Day 3	Day 4	Day 6	Day 7
Batch 35 (28°C, 120 rpm, pH 5.5	0	1.68	-	1.02	0.92
Batch 34 (32°C, 120 rpm, pH 5.5)	0	-	0.45	0.39	0.34
Batch 36 (25°C, 120 rpm, pH 5.5)	0	-	0.51	0.08	0.1
Batch 37 (28°C, 80 rpm, pH 5.5)	0	0.36	-	0.72	0.44
Batch 40 (28°C, 150 rpm, pH 5.5)	0	0.81	-	0.49	0.24
Batch 44 (28°C, 120 rpm, pH 4.5)	0	0.12	-	0.14	0.15
Batch 45 (28°C, 120 rpm, pH 6.5)	0	0.38	0.34	0.30	0.32
Batch 58 (28°C, 120 rpm, pH 5.5, 2X media)	0	-	0.84	0.45	0.97

Table 2. Comparison of Biomass Production (g/L) by Batch over Time (Days where samples weren't taken are denoted by "-")

Batch number	Day 0	Day 3	Day 4	Day 6	Day 7
Batch 35 (28°C, 120 rpm, pH 5.5	0	11.4	-	10.85	8.55
Batch 34 (32°C, 120 rpm, pH 5.5)	0	-	5.85	6.30	3.90
Batch 36 (25°C, 120 rpm, pH 5.5)	0	-	0.44	0.57	0.52
Batch 37 (28°C, 80 rpm, pH 5.5)	0	2.20	-	3.10	3.20
Batch 40 (28°C, 150 rpm, pH 5.5)	0	4.70	-	4.85	3.95
Batch 44 (28°C, 120 rpm, pH 4.5)	0	1.45	-	0.90	1.10
Batch 45 (28°C, 120 rpm, pH 6.5)	0	6.30	5.75	1.35	3.00
Batch 58 (28°C, 120 rpm, pH 5.5, 2X media)	0	-	6.85	5.10	6.50

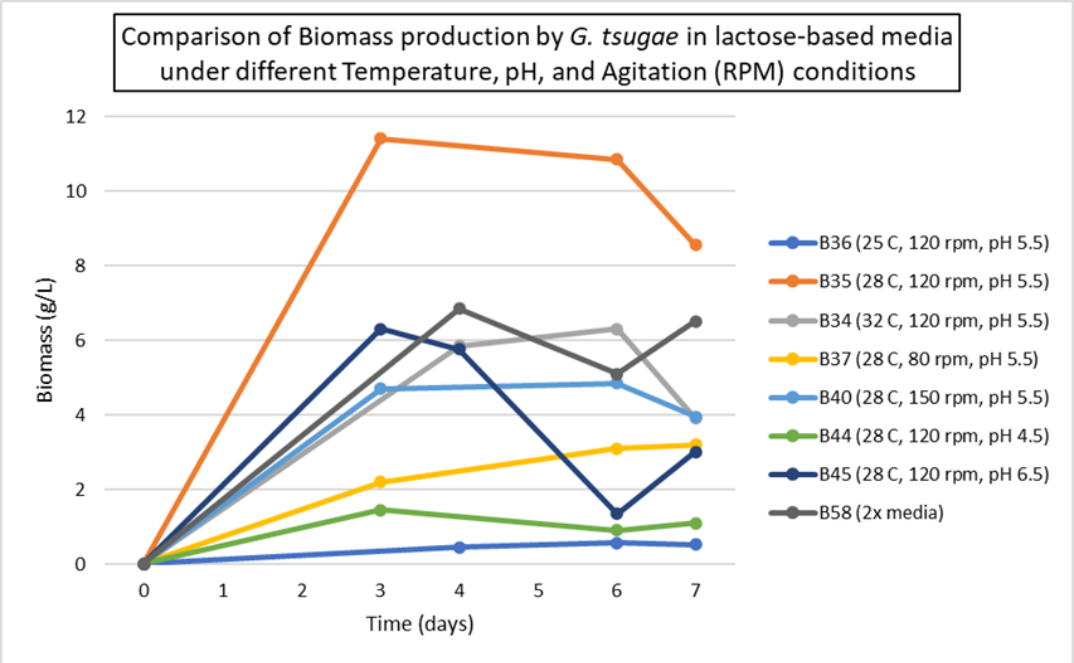

Fig. 5. The measured dried biomass for each batch over a 7-day run is plotted. Changes to the amount of dried biomass over time indicates growth or death depending on the direction of the change. Most batches peak at day 3 or 4, then decrease. This indicates that the fungi are in a growth or exponential phase until day 3 or 4, then enters a death phase. Two batches, B45 and B58, show a secondary exponential phase starting on day 6. B45 was a high pH test and B58 had twice the amount of nitrogen sources in the media. Further research is required to confirm those variables are the cause, but inducing a secondary exponential phase could have industrial applications.

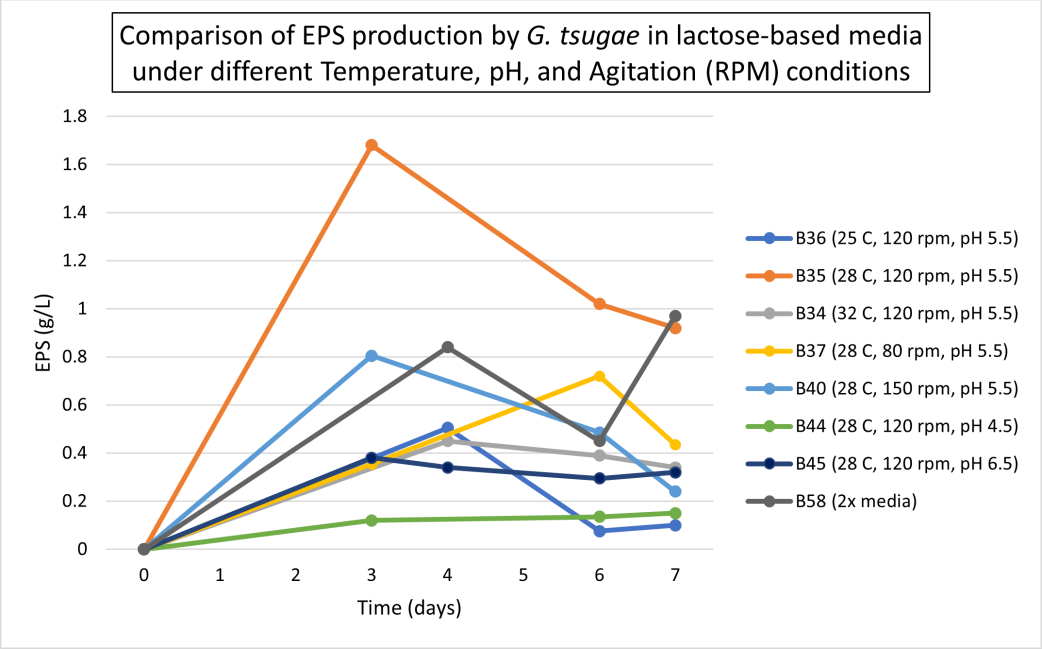

Fig. 6. The measured EPSs for each batch over a 7-day run is plotted. Like biomass, most batches have an EPS peak at day 3 or 4, then a decrease. This correlation between biomass and EPS production indicates that EPSs are created during the growth phase of the fungus life cycle. One possible explanation for the decrease is that when the fungi are in the death phase the fungi consume the EPSs as a food source. Like the biomass results (Fig. 5), B58 with double the nitrogen sources show a secondary phase of growth for EPSs. Further research is required to confirm and explain this result.

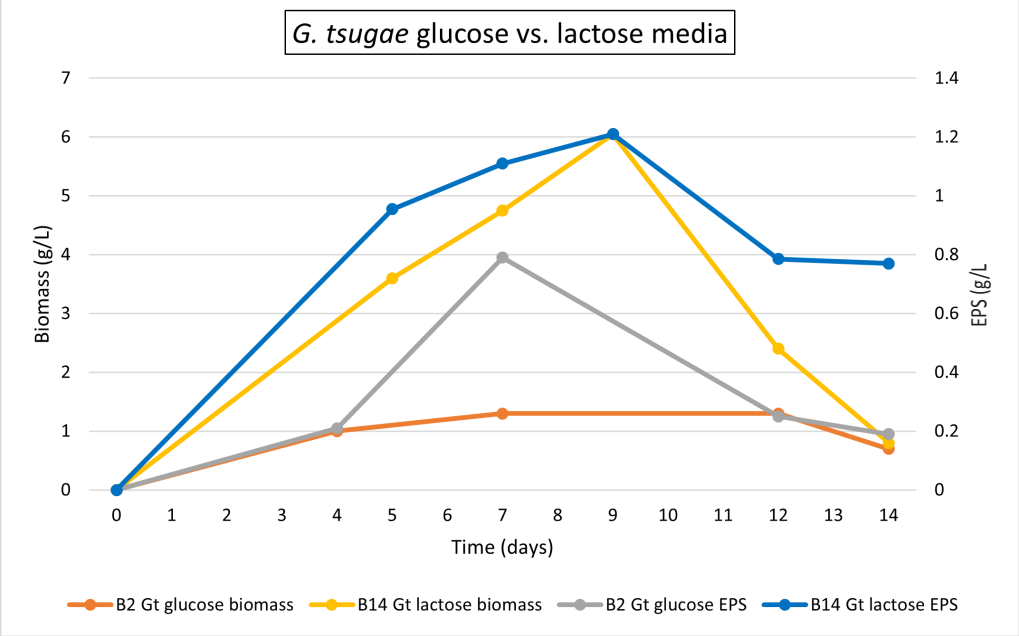

Fig. 7. G. tsugae was grown in two different media formulations containing different sugar sources, glucose and lactose. When grown in lactose, G. tsugae produces more biomass and EPS than when grown in glucose. These batches utilized standard media formulation at 28°C, 100 rpm and 5.5 pH.

It was found that when yeast and malt were doubled (2x) in media formulation (Fig. 9), although EPS production was ultimately sub-optimal, EPS production underwent a secondary exponential phase on days 6-7 with an increase of 0.52 g/L while utilizing the following conditions: 28°C, 120 rpm, 5.5 pH. This is thought to have occurred due to a death phase on days 4-6. Biomass was analogous on those days with an increase of 1.4 g/L. This second exponential phase was also observed in days 6-7 on batches that contained a constant pH different from 5.5 (6.5 and 4.5).

A 7-day two-phase pH strategy was deployed in a 5L bioreactor. The pH started at 5.5 and was increased to 6.5 on day 3 by adjusting the pH set value on the computer connected to the corresponding control tower containing acid and caustic for pH control. Optimal conditions of 28°C and an agitation of 120 rpm remained constant otherwise. While total EPS results were lower than the optimal parameters, EPS data continuing to rise is noteworthy.

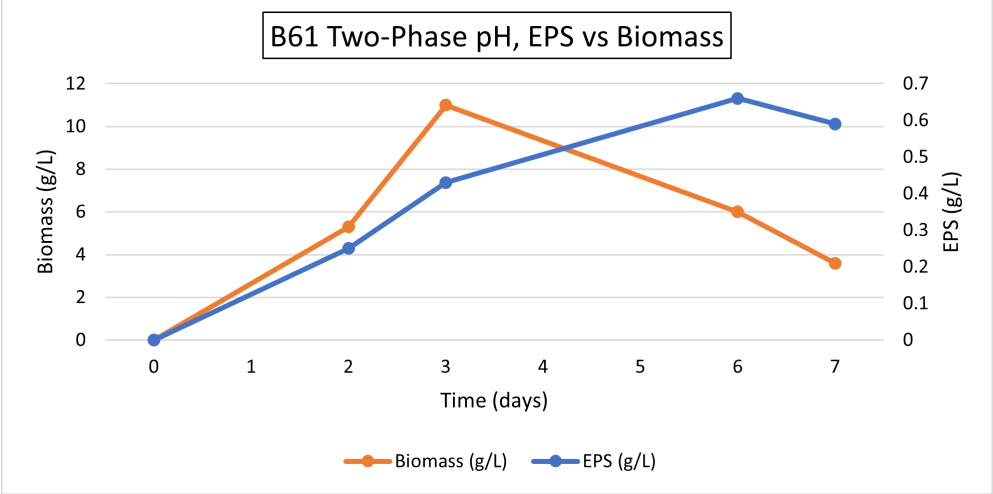

Fig. 8. G. tsugae was grown in a lactose-based media with a temperature of 28°C and 120 rpm. The pH control was done in two phases: starting at 5.5 pH, and then on day 3 the pH was changed to 6.5. This was done because batch 45, using a pH of 6.5, had a secondary exponential growth phase on day 6 (see Fig. 5). By starting with the current optimized pH of 5.5, and then switching over to 6.5 it was hypothesized that a secondary growth phase could be induced to further maximize EPS production. While biomass did not see a secondary growth phase, EPS production continued to rise.

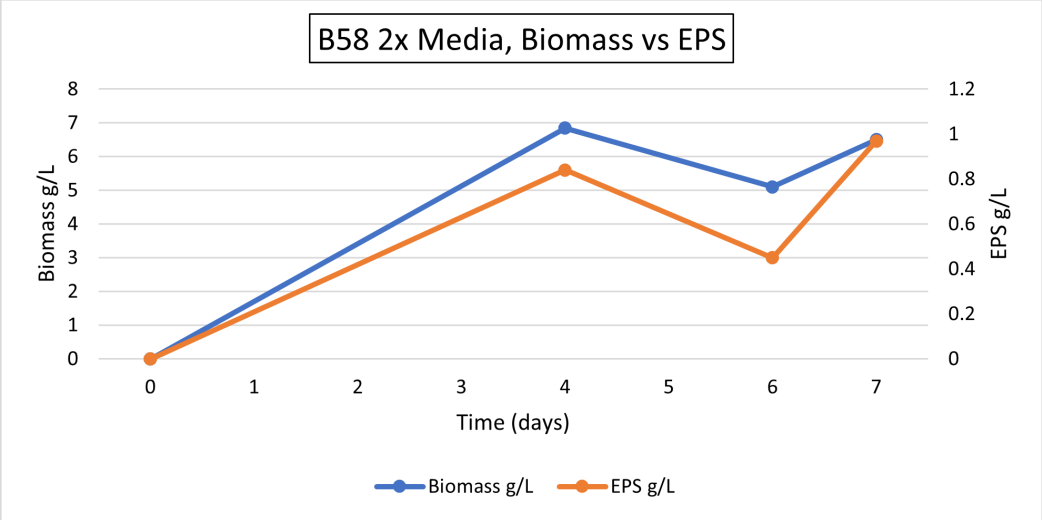

Fig. 9. G. tsugae was grown in a lactose-based media containing twice the amount of nitrogen sources with a temperature of 28°C, 5.5 pH, and 120rpm. Of note, on day 6 the batch underwent a second exponential growth phase producing more EPS.

The optimal conditions for *Ganoderma tsugae* biomass were found to be sensitive to rpm. This is theorized to be because the pellet shearing within the reactor influences the surface area to volume ratio within the submerged mycelium cells and affects the intake of nutrition. In lower rpm, *G. tsugae* was found to have a longer exponential phase, but media consumption was likely hampered due to large pellets lowering the surface area to volume ratio. As rpm increased, resulting in more breakage of the pellets, the surface area to volume ratio for the pellet cells increased, likely allowing for a better media consumption rate. Any rpm past 120 resulted in shearing the pellets, slowing growth and yields.

The optimal pH was shown to be 5.5, though days 6-7 did present signs of a potential second exponential phase. Also, low temperature and low rpm were considered lag phases, where the optimal peak was never reached until around day 6. A change in target pH rather than constant was considered as it was found to be beneficial with other submerged fungi [10] and led to time-critical experimentation in a two-pH strategy. The shake flask used for batch inoculation only progressed to day four and was believed to have affected the EPS growth rate. Contrarily to the EPSs, the Biomass was found to be comparable to optimal conditions from day 1-3 as expected. Beyond day 3, the EPSs were found to have risen, leading to the hypothesis that the age of the inoculated shake flask must be considered when describing optimal conditions for EPS. Additionally, a two-phase, 2x media strategy could also be employed because the 2x media experimentation resulted in a boost in both biomass and EPS production for days 6-7.

In the initial stages of experimentation, a media formulation was designed based on a comprehensive meta-analysis of relevant literature. To enhance the growth conditions and optimize nutrient availability, iterative adjustments by doubling (2x) yeast and malt from the original formulation were performed. This refined composition aimed to augment fungal growth and overall total polysaccharide production by increasing nitrogen sources.

While biomass is a necessary foundation for producing polysaccharides, the kinetics showed that EPSs and biomass can vary in their connectivity between strains. Other studies have focused on *G. lucidum* optimization; however, this study found that polysaccharide production was greater in G. *tsugae*. Batch 35 conditions were found to be potentially favorable in the large-scale commercialization of *G. tsugae* due to significantly higher biomass and EPS production than other batches.

Optimal EPS production by *G. tsugae* was measured in a lactose-based media with a peak concentration of 1.2g/L under initial conditions and a peak concentration of 1.68g/L under optimized parameters (Table 1). Previous research has shown that biomass production between the two species in focus was not proportional to EPS production. Peak biomass production of *G. lucidum* (28°C, 100 rpm, 5.5pH) was measured at 7.6g/L in a glucose-based media, while *G. tsugae* (28°C, 100 rpm, 5.5pH) biomass production was optimized in a lactose-based media with a peak biomass of 6.4g/L. However, EPS production was higher in said batches for *G. tsugae* (1.2g/L) compared to *G. lucidum* (0.8g/L) despite the lower biomass. Because of this data, *G. tsugae* was chosen as the successor to the project due to its ability to generate a larger quantity of polysaccharides. Batches were tested in which one variable was changed at a time from temperature, rpm, and pH. After this, it was found that the optimal conditions resulted in an even higher polysaccharide production at 1.68g/L, a 112.5% increase from the *G. lucidum* initial conditions (Fig. 10). *G. tsugae* has been found to succeed in lactose, a common waste product in food production and manufacturing. This was not the case with *G. lucidum*, as previous batches were found to grow less in lactose compared to glucose.

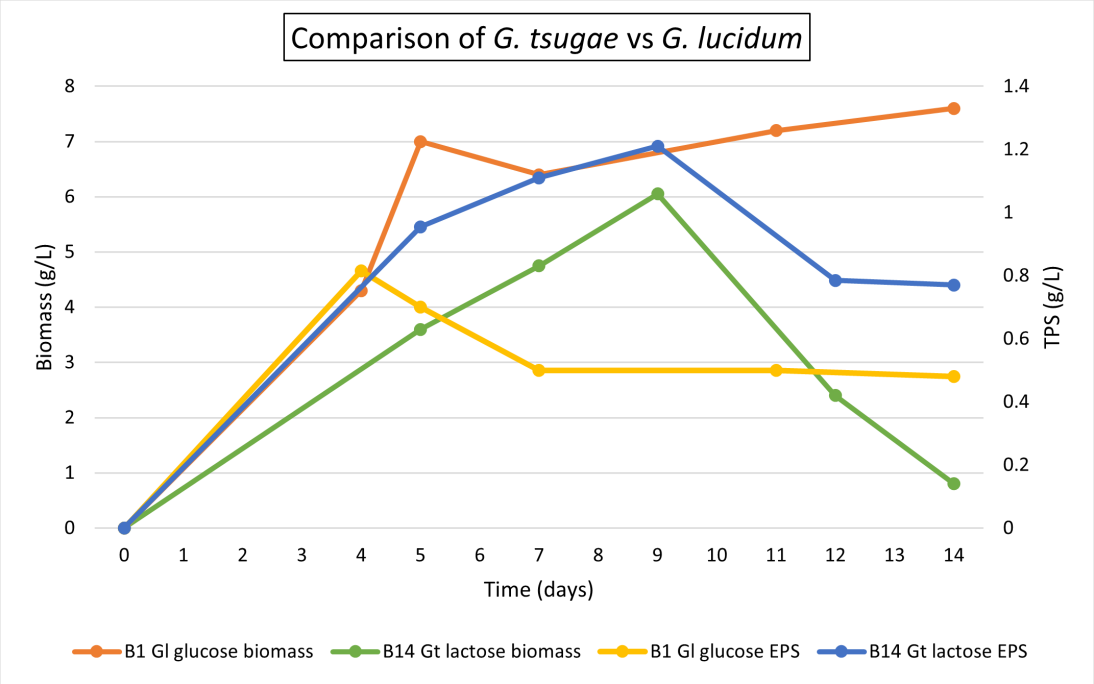

Fig 10. G. tsugae trends in its optimal media and G. lucidum trends in its optimal media against time. Though biomass was sampled higher for G. lucidum on day 5, EPS was not proportional. EPS was found to be highest in G. tsugae when using a lactose-based media.

Pluronic 60 was used as an anti-foaming agent to prevent masses of wall growth in the bioreactor. This was also believed to have lent itself to additional receivable exopolysaccharides in sample taking. This is because the mycelium excretes an extracellular matrix composed, in part, of polysaccharides for pellet cohesion. In the presence of Pluronic 60, it is hypothesized that the mycelium produces more of the extracellular matrix, and thus polysaccharides, to achieve the same level of pellet cohesion. It is important to note, however, that ultrafiltration does not work with Pluronic 60 due to its high molecular weight along with exo-polysaccharides. Therefore, crystallization may have to be used instead of ultrafiltration for downstream processing of target polysaccharides.

Conclusion

The highest biomass and polysaccharide production was observed in a lactose-based media with a constant temperature of 28°C, pH of 5.5, and an agitation of 120 rpm. These optimized parameters resulted in a peak biomass yield of 11.4g/L and a peak polysaccharide yield of 1.68g/L. Experimentation in this research concluded that lactose was a usable medium for *G. tsugae*. The implications of this research have presented a worthy competitor to *G. Lucidum* and will allow for optimum upscale processing of submerged *G. tsugae* fermentation. Lactose, a common waste product, can be utilized by *G. tsugae* to achieve EPSs and biomass, which can be leveraged in a variety of ways, such as in culinary and medicinal industries. Further research will require fine-tuning the optimal pH for each biomass and EPS production, respectively.

Acknowledgements. This work was in part supported by the National Science Foundation (NSF) under award DUE ATE #2325500.

Disclosures. The authors declare no conflicts of interest.

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Assessing the Stability of NISTCHO Cells in Long-Term Culture

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Abstract: NISTCHO is a recombinant Chinese hamster ovary cell line that has been genetically engineered to produce the monoclonal antibody cNISTmAb. This study investigates the stability of the NISTCHO cell line in long-term culture. Low passage number NISTCHO cells from a working cell bank were used to initiate a shake flask culture that was passaged over many weeks, accounting for approximately 129 cell doublings. Cells taken at two-week intervals during this period were used to inoculate fresh cultures, which were monitored over nine days for viable cell concentration, percent viability, and monoclonal antibody production. Results demonstrate consistency among growth curves over time with comparable peak cell densities and cell viabilities. Importantly, cNISTmAb production remained high, with culture titers remaining stable over the culture period and a high number of cell doublings. These findings demonstrate that the NISTCHO cell line has high stability and a sustained capability of producing cNISTmAb over extended culture periods.

Keywords: NISTCHO, Chinese Hamster Ovary, monoclonal antibody, stability, long-term culture

Introduction

The NISTCHO cell line, developed by scientists at the National Institute of Standards and Technology, NIST, in collaboration with Millipore Sigma [1] is a Chinese hamster ovary cell line derived from genetically modified CHOZN™ cells [2] by introducing genes encoding a monoclonal antibody against surface glycoprotein F of the Respiratory Syncytial Virus (RSV) [3]. This monoclonal antibody, cNISTmAb, is a fully humanized IgG1κ antibody.

The development of the NISTCHO cell line and the production of cNISTmAb provides an important reference material for the biopharmaceutical industry and a standardized platform for research and development [4]. It also provides a much-needed resource for biomanufacturing training and education programs as a modern, industry grade cell line that can be used to develop small scale processes and relevant analytical assays for teaching.

Chinese hamster ovary cells are used extensively in the production of monoclonal antibody biopharmaceuticals [5]. In the commercial production process, these cells are grown in culture for as long as six weeks, in increasing volume seed bioreactors and finally a production bioreactor. In a traditional stainless-steel bioreactor process, the culture is expanded to a volume of 10,000L or larger [5]. During this culture expansion, the cells may undergo as many as sixty cell doublings. For this reason, biomanufacturers conduct stability studies on recombinant CHO cell lines to assess the genetic stability of the cells and the ability of the cell line to sustain high production levels of the monoclonal antibody.

In this study, the stability of the NISTCHO cell line has been assessed to ensure the consistency and reliability of cNISTmAb production over a number of cell doublings that exceed a typical commercial upstream processing strategy. Analysis of the growth performance of NISTCHO that have been grown in long-term culture was conducted and the capability of the cells to continue to produce cNISTmAb was assessed.

The experiments conducted in the stability studies involved growing the NISTCHO cells in specific medium formulations. The cells were cultivated using EX-CELL Advanced CHO Production media, providing the necessary nutrients and support for cell growth and mAb production. The NISTCHO cells were developed using a glutamine synthetase (GS) selection system, where the GS gene is used as a selection marker, and the cells are grown in the absence of glutamine [6].

The stability studies conducted on the NISTCHO cell line involved growing the cells continuously in culture with passaging every 3 or 4 days; this allowed the culture to be maintained in the optimal cell density range for the cell line. For suspension cell lines such as NISTCHO passaging involves adjusting the cell concentration to a value in the low end of the optimal range for cell growth. Cells can be removed, and fresh media added, or a portion of the culture can be used to seed a new flask containing fresh medium. This allows the cells to continue to grow and double in the fresh medium without any detrimental effects of cell crowding. NISTCHO cell doubling time is between 24 and 26 hours, with three to four cell doublings between passages. In every two passages, a portion of the culture was cryopreserved. In the cryopreservation process, the cells are collected by centrifugation and resuspended in a freezing medium containing dimethyl sulfoxide. This process removes all medium from the original culture that contains cNISTmAb. The frozen cells from a particular passage number were then resuscitated and used to inoculate a fresh culture, which was monitored over an eight-day period. The culture medium was then used to purify and quantify the mAb to determine titer.

Methods

NISTCHO Cell Culture

NISTCHO cells were grown in 125ml disposable shake flasks in Production Media (EX-CELL Advanced Fed-Batch Medium, Sigma Aldrich). They were grown at 37ºC, 5 % CO_2, with shaking at 125rpm. The cultures were sampled, and growth was monitored over nine days. The viable cell concentration and percent viability were determined using a Luna fluorescence-based automated cell counter (Logos Biosystems).

Cryopreservation

NISTCHO cells were cryopreserved as 1ml aliquots at a cell density of 1.3*10^7 cells/mL in an EX-CELL® CD CHO Fusion medium containing 7% DMSO. Vials were cryopreserved at -150ºC.

Cell Harvest

On day 8 of the cell culture, the cell suspension was transferred to a conical tube and centrifuged at 2500x g for 5 minutes at 4ºC in a pre-chilled Eppendorf 5464R centrifuge. The supernatant was filtered using a 0.2 um PES membrane filter unit. An appropriate volume of Halt protease inhibitor cocktail (100X) (ThermoFisher) was added to a final concentration of 1X. The clarified cell culture medium was stored at 2-8 ºC for up to 4 days.

Purification of mAb

cNISTmAb was purified from clarified cell culture media using a 1ml protein A gravity chromatography column. 5 ml of clarified cell culture medium was loaded onto a pre-equilibrated column. Buffers and columns were provided by Protein A IgG Purification Kit (Pierce, Cat No.44667). 2mL flow-through and wash fractions were collected, and 1ml elution fractions were collected. A neutralization buffer (50ul of 1M Tris at pH 9) was added to each elution fraction to adjust the pH to pH 7. Protein concentration was determined using the Nanodrop spectrophotometer (ThermoFisher). For elution fractions, the mAb concentration was determined using the IgG extinction coefficient, i.e., conversion of 1 Abs unit = 0.73mg/ml of IgG mAb. The data collected from the Nanodrop were used to determine the mAb concentration per 1mL, i.e., titer of each culture.

SDS-PAGE

Chromatography fractions were analyzed on a Novex 4-20% gradient gel with Tris/ Glycine/ SDS Buffer (Invitrogen). Samples were combined with an equal volume of 2X sample buffer (Bio-Rad) and then heated to 95ºC for 2 minutes before loading. Precision Protein plus standards (Bio-Rad) were used. Gels were stained with Coomassie stain.

Results and Discussion

A stability study was conducted on NISTCHO cells to evaluate the production consistency of cNISTmAb across multiple passages, from p:10 to p:46, encompassing 129 cell doublings. The experiment was designed so that NISTCHO cells were cultured continuously, and cryopreservation was performed every two passages. NISTCHO cells with increasing passage numbers were resuscitated, and a growth curve and titer determination were performed for each passage number. Vials of cells, with a concentration of 1.3 x10^7 cells/mL, were thawed and cultured in 30 mL of production medium for eight days. This cultivation aimed to evaluate both the growth profile of the NISTCHO cells and the production of the mAb.

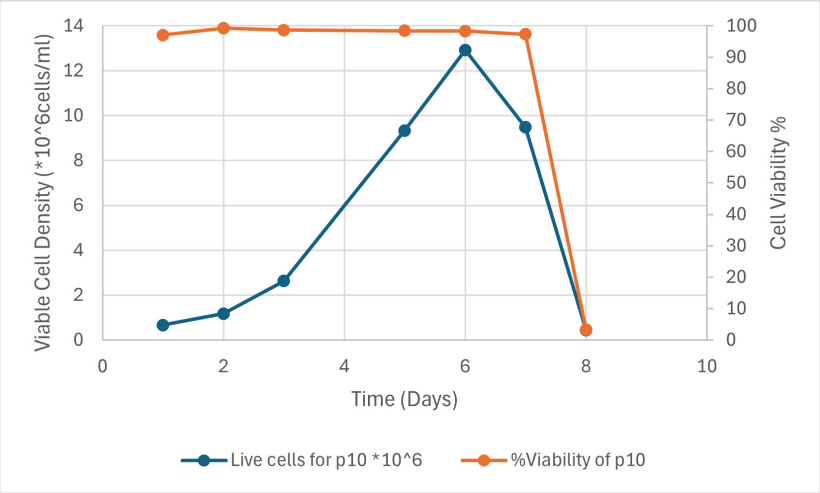

Fig. 1. Growth curve of NISTCHO cells passage 10 with viable cell density on the left y-axis (blue line) and % viability (orange line) on the right y-axis. The x-axis represents the number of days in culture. Cell count was not performed on days 0 and 4.

The growth curve shown in Figure 1 depicts the proliferation of NISTCHO cells over the course of 8 days, with an initial lag phase and then an exponential phase peaking on day six at 12.92 x10^6 cells/ml before the culture goes into a decline phase due to nutrient depletion. The percentage of viability cells remains high, greater than 90% up to day 6. This is the typical pattern for NISTCHO cells in a batch culture with no additional feed or nutrients added.

To look at cell performance for NISTCHO cells in long-term culture, cells from passages p18, p22, p32, p34, p36, p38, p42, and p46 were cultured and compared to p10. A vial of cells for each passage was resuscitated and used to inoculate 30mL of production media at a seeding density of 4.3 x 10^5cells/ml. The cultures were maintained from day 0 to day 8 with daily sampling and monitoring. The peak viable cell density stayed within the range of 12 to 15 x 10^6 cells/ml throughout, indicating that cultures seeded with higher passage number cells performed as well as those seeded with low passage number cells. Figure 2 shows data for p10, p22, p32, p36, and p46 and provides valuable insight into the growth dynamics of the NISTCHO cells.

The initial viability of cells in a culture is a critical factor that significantly influences the outcome of cell culture experiments and serves as an indicator of the health and vitality of the cell population after cryopreservation. A high initial NISTCHO cell viability, such as the values observed in this study ranging from 94.4% to 99.4%, indicates that most cells are alive and capable of proliferating on day 0 of the culture. This is crucial because cultures with high viability values have cells that are likely to maintain their functionality, replicate efficiently, and contribute to robust growth and proliferation throughout the culture period.

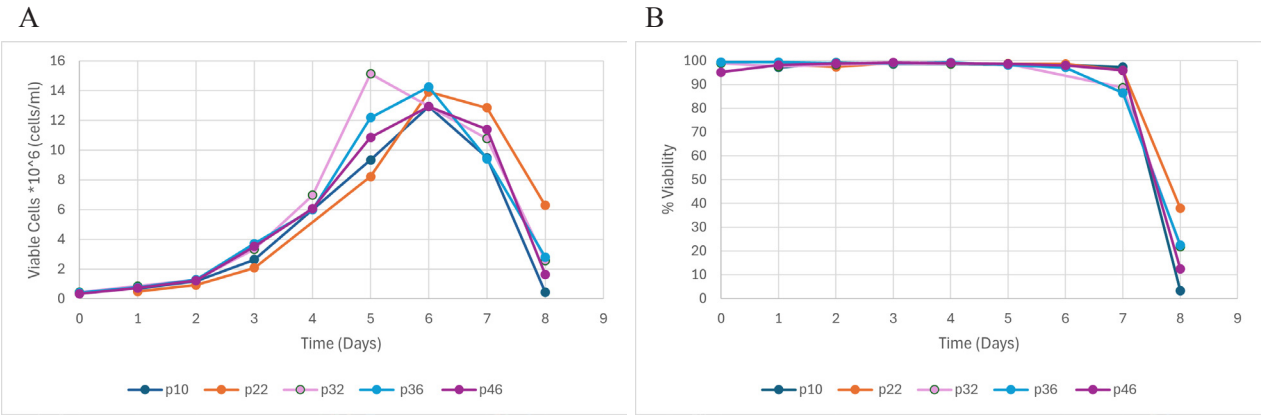

*Fig. 2. (A) Representative growth curves of viable cell concentration of cultures derived from cells with increasing passage number. All flasks were seeded with 1.3*10^7 cells by direct inoculation from cryopreserved vials of p10, p22, p32, p36, and p46 NISTCHO cells. Cell count was not performed on day 6 for p32. (B) % cell viability plotted against days in culture for cultures depicted in (A).*

The growth curves of the NISTCHO cells across passages p10, p22, p32, p36, and p46 exhibit distinct patterns over time (Figure 2A). Initially, there is a gradual increase in cell density observed in all passages, indicating an initiation of cell proliferation. This early growth phase is followed by a period of rapid exponential proliferation, particularly evident in passages p32 and p36, where cell densities increase significantly from days 2 to 5. After day six, signs of a plateau or decline in growth become apparent across all cultures. The small differences in the growth curves represent natural diversity among cultures, and overall cell performance was comparable to the base passage, p10.

Table 1 contains data from nine NISTCHO cultures inoculated with NISTCHO cells of increasing passage number with the associated number of cell doublings for each passage number, with passage 10 representing the baseline for this experiment since these cells were used to initiate the culture. It is clear that neither the peak cell density of the cultures or initial % viability are compromised in cultures seeded with high cell doubling number cells; cells that have undergone approximately 129 doublings have comparable cell density to the original passage 10. Some variance between cultures is evident as is common in cell culture experiments.

Table 1. Impact of passage number (and associated number of cell doublings) on peak cell culture density and initial cell viability in NISTCHO cell cultures inoculated with cells of increasing passage number representing the increased number of cell doublings.

Passage Numbers	Number of Cell Doublings	Peak VCD *10^6 (cells/ml)	Initial % Viability
10	0	12.92	97.1
18	31	14.56	96
22	45	13.92	98.5
32	80	15.12	98.9
34	87	15.32	96.4
36	94	14.24	99.4
38	101	13.44	94.4
42	115	13.68	95.1
46	129	12.92	95.1

Protein A Affinity Chromatography Purification and Purity Assessment of cNISTmAb by SDS-PAGE Analysis

During the cell culture process, the cNISTmAb is secreted into the cell culture media, and the concentration of mAb in the medium is known as the titer, which is typically expressed as g/L or mg/ml. In this study, the cNISTmAb was purified from the culture medium and quantified to determine atiter estimation. Protein A affinity chromatography was used to purify the mAb. In this commonly used purification technique, the protein A ligand specifically binds to the Fc region of the antibody, allowing for the isolation and purification of the desired mAb product [7]. The mAb product can then be quantified using spectrophotometry [8].

For the cultures described above, the cell culture medium containing cNISTmAb was collected by centrifugation on day eight, and a portion of it was used to purify the cNISTmAb. The chromatography fractions were collected, and the fractions representing the eluate were analyzed using SDS-PAGE under reducing conditions (Figure 3). This technique separates proteins based on their molecular weights, allowing for the detection of the target mAb and any potential contaminants.

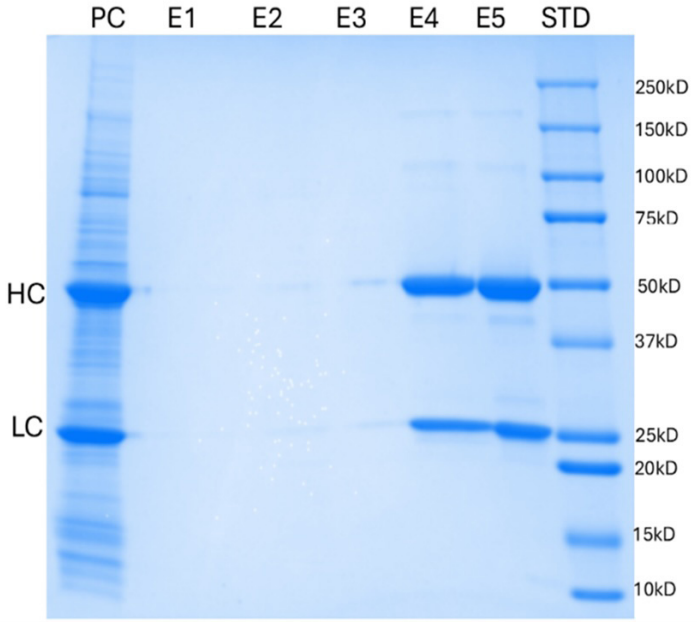

Fig. 3. SDS PAGE analysis of protein A column chromatography fractions from p10 NISTCHO cell culture medium. PC contains the pre-column clarified culture medium, 6ul. E1:Eluate1, 10ul (0.295 mg/ml). E2: Eluate 2, 10ul (0.225 mg/ml). E3: Eluate 3, 10ul (0.009 mg/ml) E4: Eluate 4, 1ul (1.771mg/ml). E5: Eluate 5,1ul (2.645 mg/ml).

In the SDS-PAGE analysis (Figure 3), the pre-column (PC) lane contains 6ul of clarified cell NISTCHO cell culture medium. Highly representative bands for the heavy and light chains of the cNISTmAb are visible with many contamination protein bands, representing CHO host cell proteins and media components. The heavy and light chains of the cNISTmAb are observed at around 50 kD and 25 kD, respectively. Lanes 2, 3, 4, 5, and 6 represent successive elution fractions from the purification process. Lane 2, Lane 3, and Lane 4 exhibit faint bands at 50 kD and 25 kD due to low concentration, and lanes 5 and 6, containing eluate fraction 4 and fraction 5 contain strong bands indicating that the cNISTmAb was eluted from the column in these fractions. To assess the purity of the mAb, high concentrations of eluate were loaded on the gel and faint bands representing contaminating proteins were visible around 170kD, 120kD, and 40kD. Despite the presence of these contaminants, the gel demonstrates a significant level of mAb purity in the region of 95% pure, post-protein A column purification. This underscores the effectiveness of the purification process in isolating the target mAb from the cellular components and other impurities.

Clarified medium (5ml) from each of the cell cultures described was used to purify the mAb byprotein A chromatography. The eluted fractions containing the mAb were analyzed using a Nanodrop spectrophotometer to measure the absorbance at 280nm. Using the extinction coefficient for IgG this value provided the concentration of the mAb in each fraction [8]. The volume of each fraction was measured, and the total amount of mAb in milligrams (mg) per fraction was calculated by multiplying the concentration (mg/mL) by the volume (mL) of the fraction. The total amount of mAb obtained was then divided by the initial 5 mL of clarified medium to determine the titer concentration.

Impact of Passage Number on Growth Performance and mAb Productivity

The evaluation of both peak cell density, titer, and monoclonal antibody (mAb) production per cell across the doubling numbers provides critical insights into the overall performance of the bioprocess. A successful bioprocess is characterized by achieving high peak cell densities while simultaneously maintaining optimal mAb production i.e. a high titer. In these experiments, the titer of each culture was estimated by purifying mAb from 5ml of clarified medium using protein A column chromatography.

Table 2 lists the estimated titer obtained for each culture and the mAb produced by each cell, taking into account the peak cell density of the culture. Culture titers remained high as the passage number increased and were comparable to the base p10 culture. mAb production per cell remained constant as the number of doublings increased, with p22 cells (45 doublings) and p42 cells (115 doublings) showing almost identical mAb production per cell, and overall cells that had undergone 129 doublings (p46) producing 35.45pg/cell compared to 40.63pg/cell for the original passage 10.

Table 2. Viable cell density observed for each culture of NISTCHO cells, with corresponding number of doublings of, culture titer, and the amount of mAb produced per cell.

Passage Numbers	Number of Cell Doublings	Peak VCD *10^6 (cells/ml)	Titer (mg/ml)	mAb pg/cell
10	0	12.92	0.525	40.63
18	31	14.56	0.516	35.44
22	45	13.92	0.527	37.86
32	80	15.12	0.533	35.25
34	87	15.32	0.51	33.29
36	94	14.24	0.47	33.01
38	101	13.44	0.477	35.49
42	115	13.68	0.508	37.13
46	129	12.92	0.458	35.45

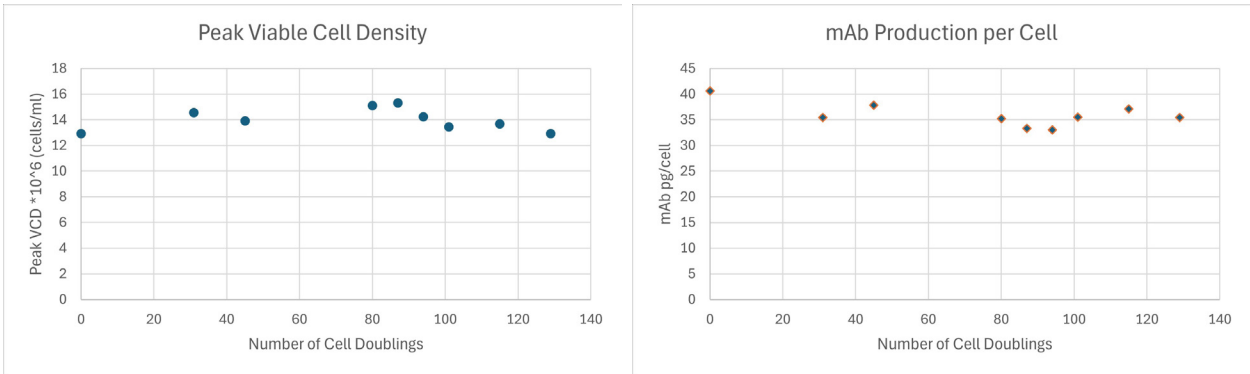

Fig. 4. (A) The chart depicts peak viable cell density plotted against number of cell doublings of NISTCHO cells. (B) The graph illustrates the amount of mAb produced per cell in cultures inoculated with cells with increasing numbers of cell doublings.

Figure 3 shows growth cell potential and mAb production over time. Data for peak viable cell density and mAb production per cell were plotted against the number of doublings of the NISTCHO cells used for culture. Despite an increase in the number of cell doublings from passage 10 to passage 46, the peak cell density for the cultures remains constant (Fig.4A), with a slight decrease starting around 100 doublings. The amount of mAb produced per cell (Fig.4B) also remains constant, with the highest level at p10 and comparable amounts of mAb produced for subsequent cultures. This data demonstrates the prolonged capability of the NISTCHO cell line to consistently produce cNISTmAb well beyond the number of doublings typically seen in upstream processing cultures.

Conclusion

Cell line stability studies are an important element of recombinant cell line characterization during biotherapeutic research and development. The ability of a cell line to consistently express the transgene at high levels over a long period in culture is critical for meeting the high liter demands of commercial biomanufacturing. The findings in this study reveal that the NISTCHO cell line is a highly stable cell line when grown long-term in culture. Cells that had undergone approximately 129 cell doublings in culture produced comparable levels of mAb to those that were taken directly from the working cell bank. High passage number cells also performed well in terms of the peak cell density they reached in culture and their ability to retain high % cell viability levels in batch cultures. These findings are of importance to biopharmaceutical companies that will use NISTCHO as a reference cell line in CHO cell development and characterization and to education and training programs that will adopt the NISTCHO in their courses.

Acknowledgments. We would like to express our sincere appreciation to everyone at Montgomery County Community College's Biotechnology Program for their support and mentoring during this study. We also would like to thank the National Institute for Innovation in Manufacturing Biopharmaceuticals, NIIMBL, Dr. Brad O'Dell from the National Institute of Standards and Technology, and Dr. Lori Kelman from Montgomery College for their advice and support. This work was performed under the following financial assistance: award 70NANB21H085 from the U.S. Department of Commerce, National Institute of Standards and Technology, and NSF ATE award # DUE 2055036, Principal Investigator Dr. Sandra Porter. The corresponding author would like to acknowledge that authors S. Nadour and K. Clement contributed equally to this study.

Disclosures. The authors declare no conflicts of interest.

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Manufacturing and Formulation Development of Lyophilized Crude *Taq* DNA Polymerase Extract

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Abstract: Finding the optimal formulation for the lyophilization of biomolecules, such as antibodies and other proteins, is an essential component in drug development to preserve the drug product and increase its shelf life. In this study, we developed a lyophilization formulation for crude *Taq* polymerase, a common DNA polymerase enzyme used to amplify DNA through the Polymerase Chain Reaction (PCR) technique. Lyophilization is a standard process to stabilize and preserve biomolecules such as antibodies, enzymes, peptides, and small molecules. Through a formulation screen, we identified optimal lyophilization (lyo) formulations that resulted in a lyo cake with good physical characteristics while maintaining polymerase activity upon reconstitution. Subsequently, we found that our lyo formulation preserved the polymerase activity even after the Taq crude extract was stored at elevated temperatures for weeks.

Keywords: lyophilization (lyo), lyo cake, crude *Taq* polymerase, *Taq* crude extract, Polymerase Chain Reaction (PCR)

Introduction

ASPIRE (Advanced Student-focused Project: Internship, Research, & Education) is a biotechnology program funded by NSF (NSF DUE 2100575) that provides internships in protein biomanufacturing and research opportunities for community college and high school students in the Greater Los Angeles Region. ASPIRE offers various collaborative research projects, including Tiny Earth [1], SEA-PHAGES [2], DNA Barcoding, and industry-relevant protein bio-manufacturing and process development projects. In collaboration with the DNA Learning Center (DNALC) of Cold Spring Harbor Laboratory, ASPIRE students have successfully optimized the production of *Taq* polymerase crude extract and the associated classroom materials (Standard Operating Procedure and Batch Record), disseminated locally and nationwide through the InnovATEBIO and the DNALC.

Taq polymerase is a DNA Polymerase I, an essential enzyme that creates copies of DNA with a miniscule error rate, which enables cells to pass genetic information from the parent to daughter cells. It was isolated from a thermophilic bacterium in the geysers of Yellowstone National Park, *T. Aquaticus*, in 1976 [3]. Since its initial discovery, *Taq* Polymerase has revolutionized the field of Molecular Biology [4]. Owing to its thermal stability, *Taq* Polymerase has become the ideal enzyme to create massive amounts of DNA copies in vitro through a process known as the Polymerase Chain Reaction (PCR), streamlining the DNA amplification process by eliminating the need to replace DNA polymerase that is destroyed in each PCR cycle [5]. PCR has become crucial to genetic engineering, forensics, and medical diagnostics, including the COVID-19 detection method [6].

Lyophilization, also colloquially known as freeze-drying, has become part of standard drug development workflows to preserve the Active Pharmaceutical Ingredient (API) by removing water through sublimation. Generally, samples are frozen and placed into a vacuum chamber to allow the water molecules to escape as gas, leaving the molecule of interest as a dry solid, commonly called the "cake." Ideal lyo cake should rehydrate easily into a homogenous mixture within a reasonable period, usually within minutes, while preserving the biological activities of the biomolecules [7]. Finding the optimal chemical combination, also

known as excipients, that protects the biomolecules during lyophilization and produces a 'good cake' can be challenging. This has spurred the growth of specialist CDMOs (Contract Development and Manufacturing Organizations), offering services to develop the lyo formulation before the drugs are manufactured at large scale for clinical trials.

ASPIRE students produce *Taq* polymerase crude extract as a part of the Biotechnology training program; products and protocols are disseminated nationally through the DNALC and InnovATEBIO, the National Biotechnology Education Center. We developed a lyo formulation for crude *Taq* extract to minimize the distribution cost. Lyophilization of a Ready-to-Use RT-PCR mix with a similar enzyme requires a combination of Trehalose, Ficoll-400, and Gelatin [8]. Trehalose comprises two glucose molecules linked together at the reducing end (glycosidic bond), making it a nonreducing end and stable against acidic conditions. Trehalose is generally considered a good lyoprotectant (protects biomolecules from denaturation during the lyophilization) and bulking agent [9], owing to its chemical arrangement that resists degradation as well as its backbone flexibility which enables it to participate in an extensive hydrogen bond network [10, 11].

Although lyophilization for the *Taq* mixture has been attempted [8], the presence of other cytosolic *E. coli* proteins in the crude *Taq* extract may render the lyo formulation ineffective. Therefore, we seek to determine the optimal formulation for the lyophilization of crude *Taq* extract. In this study, we developed a formulation screen that led to the identification of optimal lyo formulation for the *Taq* crude extract. We then performed PCR to assess the polymerization activity of the preserved crude *Taq* extract upon reconstitution. Our results suggest that the lyophilized crude *Taq* extract maintains its polymerase activity after more than 10 weeks of storage at 50ºC and two weeks at 72ºC.

Methods

Protein Expression

An open-source *Taq* polymerase (pOpen*Taq*, FreeGenes #BBF10K_003493) construct, provided by the DNALC, was transformed into the BL21 *E. coli* expression strain. Protein expression is done using the standard protocol for bacterial protein expression systems. Briefly, cells were streaked onto LB Amp plates ([amp]: 50 µg/mL) and were incubated overnight (16 – 20 hours) at a 37ºC incubator. An overnight (ON) culture was prepared by inoculating 5-10 colonies into 3 mL LB Amp broth ([amp]: 50 µg/mL), placed in the shaking incubator (250 rpm) at 37ºC for 16 hours. Two mL of the ON culture was spun down to remove secreted β-lactamase in the supernatant. The pellet was resuspended and transferred into a fresh 80 mL LB Amp in a 250 mL baffled flask. The culture was brought to OD_{600} 0.9 in a 37ºC shaking incubator before induction with Isopropyl β-D-1-thiogalactopyranoside (IPTG) (final concentration of 0.5 mM) for 16 hours. The culture was spun down, and the supernatant was removed. The pellet can be used immediately or stored at -80ºC for later use.

Crude *Taq* Extract Preparation

Crude *Taq* extract was prepared according to the previously published protocol [12] with some modifications. Briefly, the pellet was resuspended in TEN buffer (10 mM Tris-HCl pH 7.9, 1 mM EDTA, 100 mM NaCl), 10 mL per 1 g pellet. Cell lysis was done by sonication (Qsonica, 40% Amplitude, 1 sec on, 1 sec off, 1 min, repeat 3x); the sample was kept on wet ice for the entire sonication duration. The cell lysate was incubated at 75ºC for 30 minutes to denature unwanted aggregated proteins and further lyse the cells, maximizing yield without risking enzymatic activity. Lysate is then spun down (14,000x g for 20 minutes) to remove any cellular debris, and the supernatant is collected as the crude *Taq* extract. Complete SOP and Batch records are made available through InnovATEBIO.

Polymerase Chain Reaction (PCR) to Assess DNA Amplification Activity

The prepared Taq extract's amplification activity was assessed using the standard λ DNA amplification developed by the Bay Area Bioscience Education Community (BABEC). Briefly, 0.25 ng of λ DNA was amplified in a 25 µL reaction using a 0.4 µM λ primer set (PC01: 5'-GATGAGTTCGTGTCCGTACAACTGG-3', PC02: 5'-GGTTATCGAAATCAGCCACAGCGCC-3'). The thermal cycler setting is as follows: Initial denaturation (95ºC, 2 minutes), 25 cycles of amplification (95ºC for 15 sec, 37ºC for 15 sec, and 72ºC for 30 sec), final elongation (72ºC for 5 min), and storage (4ºC, infinity).

The amount of *Taq* yield was determined qualitatively by comparing the polymerase activities to the commercially available recombinant *Taq* polymerase: *Taq* DNA Polymerase (New England Biolabs, M0273S, at 5 units per 25 µL reaction). The qualitative assessment compared the relative DNA band size on the gel electrophoresis (0.8% Agarose).

0.25 T reaction uses 0.25 µL of *Taq* crude extract in a 25 µL PCR reaction.

0.5 T reaction uses 0.5 µL of *Taq* crude extract in a 25 µL PCR reaction.

1 T reaction uses 1 µL of *Taq* crude extract in a 25 µL PCR reaction.

2 T reaction uses 2 µL of *Taq* crude extract in a 25 µL PCR reaction.

10x *Taq* buffer was prepared in-house using the same recipe as the 10x Standard *Taq* Reaction buffer (NEB, Cat #B9014S).

10 mM dNTP mix: Promega, 1,000 µL (Cat #U1515)

Preparation of Stock Solutions for Lyophilization Formulation Screen

D-(+)-Trehalose (Fisher BioReagent, BP2687100), Ficoll-400 (Thermo Scientific, AAB2209518), and Gelatin (VWR, 470301-132) were used in the lyophilization process of the *Taq* crude extract. The 50% stock solution for trehalose was made by diluting 10 grams of trehalose in 20 mL of diH_2O, the 50% stock solution for Ficoll-400 was made by diluting 10 grams of Ficoll-400 in 20 mL of diH_2O, and the 30% stock solution for gelatin was made by diluting 3 grams in gelatin in 10mL diH_2O (the gelatin had to be melted to allow for the 3 grams to dissolve fully). Sterilization of the trehalose was done by sterile filtration (Pall Corporation, 0.22µm), while Ficoll-400 and gelatin stock solutions were sterilized by autoclave. All components were mixed, and the volume was brought up to 1000µL with sterile distilled water in a deep-well 96-well plate to create the lyophilization formulation matrix and stored at -80ºC before use.

Lyophilization Screen

20µL of diluted *Taq* crude extract and 20µL of each formulation mix were added to each well of a new 96-well assay plate; this mixing brings the effective *Taq* concentration to half as much compared to the original crude *Taq* extract. Upon thorough mixing, the 96-well assay plate was frozen at -80ºC. Lyophilization was done using Labconco FreeZone 2.5L Benchtop Freeze Dryers following the standard lyophilization setting—briefly, the assay plate containing frozen *Taq* lyo. Mixes were put under a vacuum (0.01 Torr) in the drying chamber as the temperature was raised slowly to room temperature. After lyophilization, Lyo cakes had an initial visual inspection. The visually good lyo cakes were reconstituted using sterile distilled water for functional assay to assess the retention of DNA amplification activity.

Thermal Stability Study of Lyophilized *Taq*

Several aliquots of lyophilized *Taq* (at 8 µL) were prepared for the stability study at six different temperatures (4ºC, 25ºC, 37ºC, 50ºC, 72ºC, 95ºC). Each temperature was strategically chosen to cover various conditions found in the lab. At 4ºC, refrigeration conditions are practiced in labs, and 25ºC represents room temperature. The physiological conditions at 37ºC mirror the conditions of the human body. Then, a moderate increase in temperature to 50ºC between 72ºC mimics the condition of PCR amplification—lastly, 95ºC, which is the denaturing temperature of the PCR reaction. One aliquot from each temperature was taken at each time point and used for comparative functional assay (λ DNA amplification) against both the non-lyophilized *Taq* mix and the crude extract that was incubated at each corresponding temperature for the same time. A sample is collected weekly from each incubation temperature for ten weeks (70 days), and its polymerase activity is assessed by PCR amplification of λ DNA, as described above.

We prepared several aliquots of 4 µL of crude *Taq* extract, mixed with 4 µL of B7 formulation, to make a total of 8 µL of B7 mix in thin-walled PCR tubes. These mixes were then lyophilized overnight. Between 5 to 15 aliquots of the B7 mix were placed at the different incubation temperatures. Given that *Taq*'s half-life at 95ºC is 40 minutes [13] and based on the preliminary temperature stability study done previously (data not shown),

we anticipated that the lyophilization would not extend its activity beyond a few days at this temperature hence, only 5 aliquots of lyophilized B7 mix were placed at 95ºC. We prepared 15 aliquots for each of the other five temperatures (F.L.-Formulation Lyophilized). Aliquots of non-lyophilized crude *Taq* extract (C.E.) were also placed at 4ºC, 25ºC, 37ºC, 50ºC, 72ºC, and 95ºC. In addition, we also asked whether the B7 mix excipients themselves, without the freeze-drying process, are sufficient to protect polymerase activity. To address this, we prepared the B7 mix without subjecting it to the freeze-drying process; in other words, we kept it as a liquid. We also stored these non-lyophilized B7 mix (F.NL.-Formulation Non-Lyophilized) at 4ºC, 25ºC, 37ºC, 50ºC, 72ºC, and 95ºC.

Samples were collected at regular intervals, and polymerase activities were compared to the aliquot that had not undergone thermal stress (i.e., Day 0). F.L. was rehydrated with sterile dH_2O to bring it to the original 8 µL volume. C.E., F.L., and F.NL. were then used to prepare a 2x master mix for the PCR assay. We used a 2T recipe (Table 1) to prepare a 2x *Taq* master mix from F.L. and F.NL. to account for the ½ dilution with the lyo stock solution, 1T *recipe* (Table 1) was used for C.E. A commercially available NEB 2x Master Mix from New England Bio-Labs (Cat. #M0270S) was used as a benchmark. The polymerase activities of these master mixes were assessed by PCR amplification of λ DNA.

Results and Discussion

Preparation of *Taq* Polymerase Crude Extract

Crude *Taq* extract was prepared using a previously developed protocol [12] with some modifications described in the method section. We observed a protein band at around 90 kDa on a denaturing protein gel, SDS PAGE, consistent with the calculated *Taq* Polymerase's size, 94 kDa (Fig. 1a). We noticed several low molecular weight bands in the crude *Taq* extract, underscoring that it is not a completely pure enzyme. This gel also shows the presence of other proteins in the extract, such as the 37 kDa and 20 kDa bands, as well as other less prominent protein bands. This underscores the crude nature of this prep (Fig. 1a). Nevertheless, we can enrich the proportion of *Taq* polymerase in the mixture through this method. We then asked whether we could see polymerase activity in the *Taq* crude extract we prepared. To do this, we performed DNA amplification using the λ DNA PCR protocol provided by BABEC. We chose this method since it is standard and has been used by many high school students for years; the PCR protocol is described briefly in the method section. Due to other protein contaminants, it is difficult to predict the enzymatic activity simply by relying on protein concentration numbers. For this reason, we performed various dilution series to determine the amount of crude *Taq* extract needed to create a 2x master mix for PCR. The degree of DNA amplification of our master mixes is then qualitatively compared to the one obtained from the commercially available NEB 2x Master Mix (NEB, Cat. #M0270S).

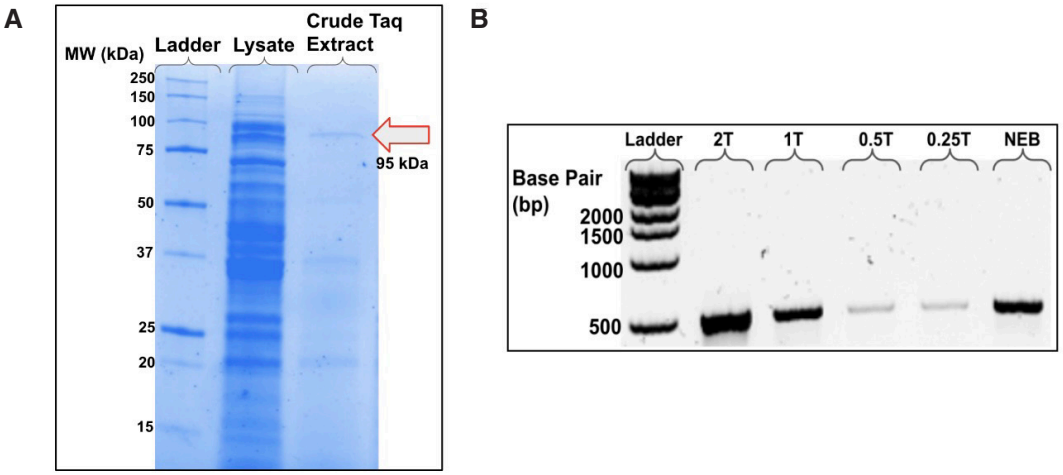

Fig. 1. (A) SDS PAGE comparing cell lysate and crude Taq extract reveals the enrichment of Taq Polymerase in the crude extract mixture. (B) Qualitative DNA amplification assay of crude Taq extract at various dilutions compared against commercially available Taq master mix.

The composition of our 2x master mix is shown in Table 1. Briefly, we prepared 50 µL of 2x master mix first. We then mixed 12.5 L of the 2x master mix with 0.25 ng of λ DNA and the λ primer mix at a final primer concentration of 0.4 µM. We assigned this 1T to describe the 2x master mix composition with 4 µL of crude *Taq* extract in 50 µL of total volume. Given that each 25 µL PCR reaction only requires 12.5 µL of the 2x master mix, only 1 µL of crude *Taq* extract is effectively used in a 25 µL PCR reaction. In addition, we ran a PCR reaction using the NEB 2x Master Mix as a comparison at the same λ DNA and λ primer mix concentration.

Table 1. *Taq* Master Mix Concentration Dilutions

Master Mix	2T (2µL *Taq*/25µL rxn)	1T (1µL *Taq*/25µL rxn)	0.5T (0.5µL *Taq*/25µL rxn)	0.25T (0.25µL *Taq*/25µL rxn)
10x *Taq* Buffer (µL)	10	10	10	10
dNTP (µL)	2	2	2	2
dH_20 (µL)	20	24	26	27
50% Glycerol (µL)	10	10	10	10
Taq Extract (µL)	8	4	2	1

Following the PCR reaction, we ran gel electrophoresis (0.8% agarose) to qualitatively compare our crude *Taq* extract at various dilutions to the NEB's *Taq* (Fig. 1b). Based on our data, we suggest that 1 µL of our crude *Taq* extract exhibits similar polymerase activity to the NEB 2x Master Mix. Given that approximately 12 mL of crude *Taq* extract was produced from an 80 mL *E. coli* culture; we produced enough *Taq* crude extract to support 12,000 PCR reactions from each manufacturing batch.

Developing Lyophilization Formulation for the Crude *Taq* Extract

Lyophilization is a common method used to preserve biomolecules. Therefore, we attempted to find the ideal lyophilization (lyo) formulation for our crude *Taq* extract. Previous attempts to lyophilize a qPCR mix have been successful [8]. Although the composition of the qPCR mix [8] is markedly different from our crude *Taq* extract, we hypothesized that the ideal lyo formulation for our crude *Taq* extract should consist of similar excipients, albeit at a different proportion. For this reason, we designed the lyo formulation screen in a 96-well format using the same excipient combination: trehalose, ficoll-400, and gelatin.

A combination of three concentrations of trehalose (10%, 20%, 30%), three concentrations of gelatin (0.2%, 2%, 6%), and seven concentrations of Ficoll-400 (2%, 6%, 9%, 12%, 15%, 18%, 20%) were chosen to create the lyo formulation screen matrix (Fig. 2a). Each stock solution was brought to a 1000 µL total volume using sterile nanopure water. For trehalose, columns 1-3 had no trehalose (0%), columns 4-6 had 10%, columns 7-9 had 20%, and columns 10-12 had 30%. For gelatin, columns 1, 4, 7, and 10 had 0.2%, columns 2, 5, 8, and 11 had 2%, and columns 3, 6, 9, and 12 had 6%. For Ficoll-400, row A had 0%, B-2%, C-6%, D-9%, E-12%, F-15%, G-18% and H-20%. Wells G11, H10, H11, and the entirety of column 12 could not be used as the percentages overshot the 1000µL limit for the stock (Fig. 2a).

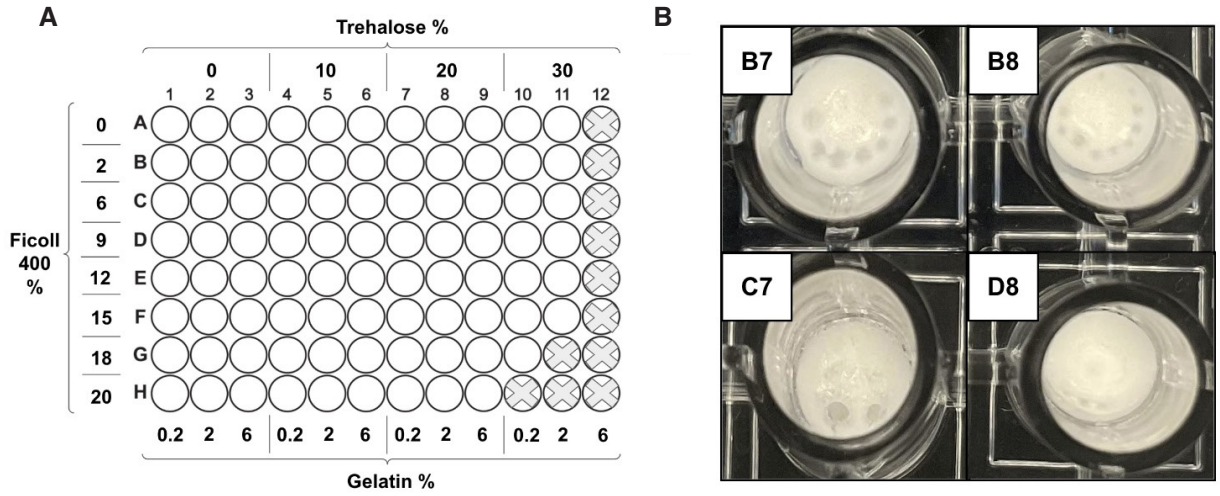

Fig. 2. (A) Lyo formulation screen design in a 96-well-plate format containing combinations of Trehalose, Ficoll-400, and Gelatin at various concentrations (B) Images of lyo formulations that yield desirable cake appearance: wells B7 mix, B8 mix, C7 mix, and D8 mix.

To start the lyophilization process, 20 µL of the crude *Taq* extract was added to each well of the new 96-well screen plate, and 20 µL of the solutions from the stock well plate was added to their corresponding wells. The screen plate was stored in a -80°C freezer while the lyophilizer was primed to reach -80°C. Once the lyo mixtures were completely frozen, the screen plate was transferred to the lyophilization vacuum chamber overnight to completely remove the water molecules through the sublimation process.

Once lyophilization was complete, a physical inspection was done to find the ideal cake, defined as white powdery solids that can be easily reconstituted within a few minutes [7]. Based on these criteria, four good cakes were identified out of the 85 formulations screened in this attempt: wells of mixtures B7 (10% Trehalose, 1% Ficoll 400, 0.1% Gelatin), B8 (10% Trehalose, 1% Ficoll 400, 1% Gelatin), C7 (10% Trehalose, 3% Ficoll 400, 0.1% Gelatin) and D8 (10% Trehalose, 3% Ficoll 400, 1% gelatin); all of them were white, dry, powdery, had minimal puffing on the surface, uniform structure throughout the cake and dissolved instantly when water was added to rehydrate them (Fig. 2b).

Lyophilized Crude *Taq* Extract Retained the DNA Amplification Capability Upon Reconstitution

To investigate the retention of polymerase activity of the lyophilized *Taq* crude extract, lyo cakes in wells B7, C7, B8, and D8 were rehydrated with 20 µL of sterile H_2O since only 20 µL of crude *Taq* extract was added to each well. We then prepared a 2x master mix using the 2T recipe (Table 1), an equivalent of 2 µL of crude *Taq* extract in a 25 µL PCR reaction, anticipating a reduction in polymerase activities, referred to as B7 mix, C7 mix, B8 mix, and D8 mix. As a comparison, we also prepared a 2x *Taq* master mix using crude *Taq* extract that has not undergone lyophilization using the 2T recipe, referred to as U (un-lyophilized). Lastly, we compared the polymerase activity of B7 mix, C7 mix, B8 mix, D8 mix, and U to the Master Mix created using NEB's *Taq* (Fig. 3).

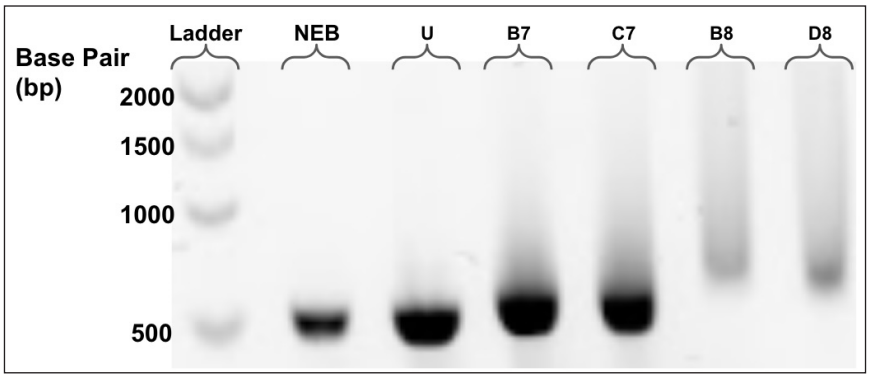

Fig. 3. PCR assay to assess the retention of polymerase activities of the lyophilized Taq crude extract.

Given that the 1T *Taq* master mix recipe exhibited comparable polymerase activity to the NEB (Fig. 1b), the non-lyophilized *Taq* master mix control at 2T recipe (U) displayed higher polymerase activity compared to the NEB 2x Master Mix control (1T recipe). Only the B7 and C7 mix showed polymerase activity comparable to the master mix prepared using non-lyophilized crude *Taq* extract (U). The B7 and C7 mixes contain a final concentration of 10% Trehalose and 0.1% Gelatin. There was no discernable difference in polymerase activity when varying the Ficoll 400 concentration between 1% (B7 mix) and 3% (C7 mix). Wells containing similar Trehalose and Gelatin concentrations (A7 mix and D7 mix), similar Trehalose and Ficoll 400 concentrations (C8 mix), and similar Gelatin and Ficoll 400 concentrations (B4 mix, C4 mix, B10 mix, and C10 mix) were not tested in this assay as they did not produce good cake (Supp. Fig. 1). While the cakes of B8 mix and D8 mix looked promising, their polymerase activities were almost non-existent compared to U, B7 mix, or C7 mix. The lyophilization process unaffected the PCR assay for the B7 mix and C7 mix as the polymerase activity is comparable to U. The B8 mix and D8 mix did not result in a high polymerase activity due to a non-optimal concentration of chemicals. We can conclude that the lyophilization process does not affect the retention of the polymerase activity.

Lyophilized Crude *Taq* Extract Retained Its Function Even After Prolonged Incubation at Higher Temperature

We performed a ten-week stability study to address whether the lyo formulation can protect the crude *Taq* extract activities against elevated temperature. The polymerase activity of lyophilized crude *Taq* extract was compared to the control of the non-lyophilized crude *Taq* extract after 10 weeks of incubation at various temperatures. Since the polymerase activity is comparable between B7 and C7 mixtures, only the B7 mix formulation was used in this stability study.

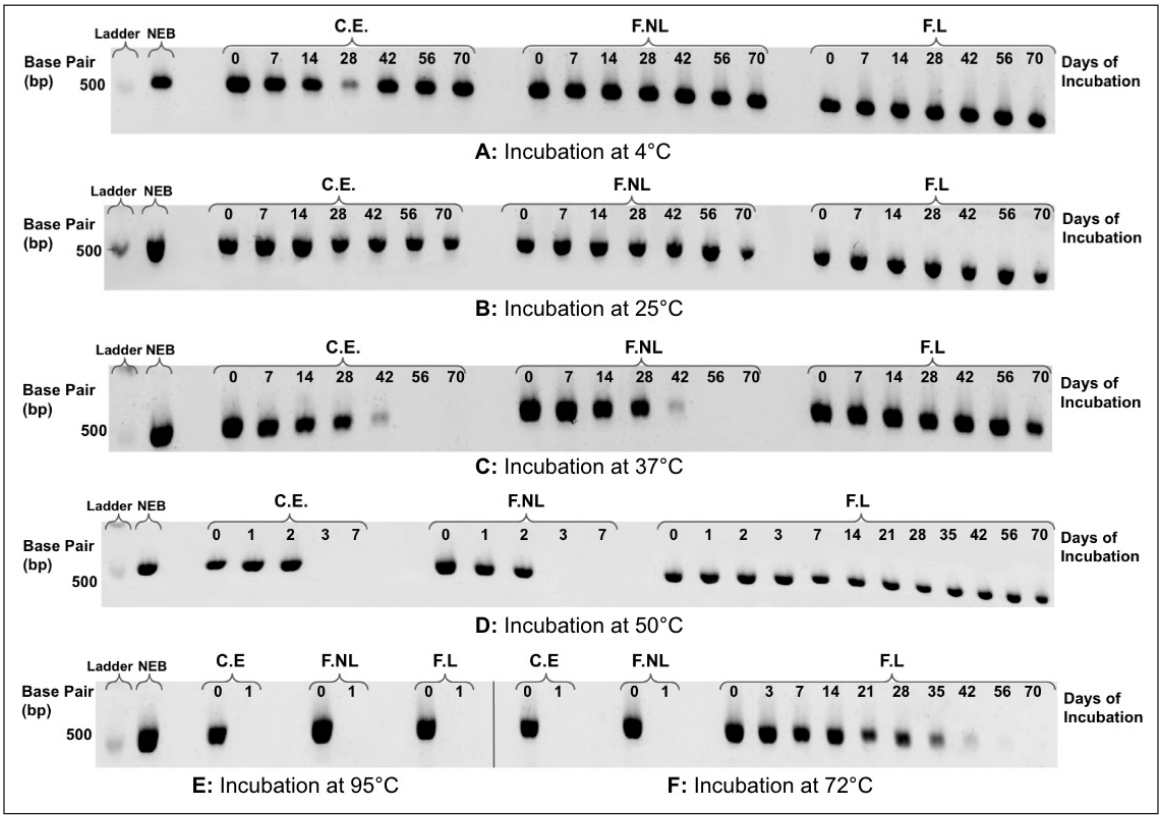

Fig. 4. DNA gel of the three different Taq polymerase solutions tested at six different temperatures.

Samples were run on 0.8% agarose gels (Fig. 4). *Taq* retained its polymerase activity after ten weeks of incubation at 4°C for C.E., F.NL., and F.L. (Fig. 4: A). This is expected since 4°C is a common storage temperature for laboratory reagents. A similar trend is also observed for the incubation at 25°C (Fig. 4: B). This is somewhat surprising since enzymes, or any biomolecules in general, are typically stored at 4°C or

frozen to avoid the loss of enzymatic activity due to degradation or aggregation. This highlights crude *Taq* extract's resiliency, making it especially suitable for introductory Bioscience classrooms.

The lyophilized crude *Taq* extract (F.L.) continued to retain its polymerase activity for 70 days (10 weeks) at 37°C, while C.E. and F.NL. displayed a loss of polymerase activity starting at day 42 (6 weeks) and beyond (Fig. 4: C). This suggests that the lyo mix alone, without the freeze-drying process, is insufficient to protect the polymerase activity of the crude *Taq* extract at this temperature. The difference in the retention of polymerase activity is even more prominent at higher incubation temperatures. Both C.E. and F.NL. were stable for only 2 days, while the F.L. was stable for at least ten weeks (Day 70) at 50°C incubation (Fig 4: D). At 72°C, F.L. continued to display polymerase activity for about two weeks (Day 14) before showing a notable decline in enzymatic activity (Fig. 4: F). This is impressive since both C.E. and F.NL. lost their polymerase activity at 24 hours of incubation at 72°C. Given the nature of our qualitative assay, however, we cannot detect the time when the polymerase activity starts to decline. This lyo formulation, however, is unable to save the crude *Taq* extract from a near-boiling temperature (95°C). All samples (C.E., F.NL., and F.L.) lost their polymerase activity within 24 hours of incubation at 95°C (Fig. 4: E). Visual inspection of the F.L. samples stored at 95°C revealed a color change from white to yellowish powder (Fig. 5). A change in the color of the cake indicates possible chemical changes that affect the stability of the crude *Taq* extract cake, consistent with the results from the functional assay (Fig. 4: E).

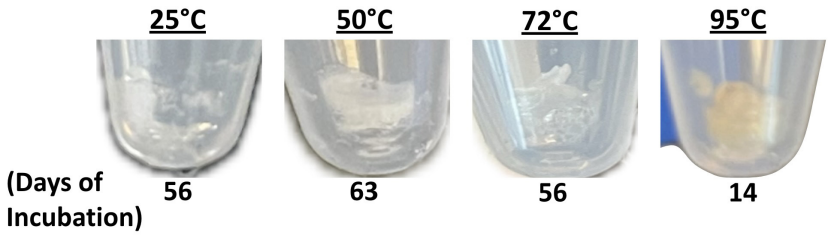

Fig. 5. Image of Lyo cakes of the crude Taq extract stored in various temperatures.

Conclusion

Overall, we developed an ideal lyo formulation that can preserve the polymerase activity of a crude *Taq* extract even after prolonged storage at elevated temperatures. In this study, we showed that non-lyophilized crude *Taq* extract maintains the polymerase activity even after being stored at room temperature for up to 10 weeks. However, polymerase activity has a qualitatively observable decline beyond 14 days of incubation (2 weeks). We also showed that our lyophilized crude *Taq* extract (F.L.) can maintain its activity even after being stored at 72ºC for about two weeks before seeing a significant decline in activity. The additional cost associated with lyophilization reagent is calculated to be roughly 30 cents per 100 PCR reactions or less than 1 cent per 1 PCR reaction. This additional cost is negligible, considering that the cost of commercial *Taq* is roughly 50 cents per 1 PCR reaction. There are ongoing efforts to develop lyo formulation using even more affordable chemical combinations. Our results will enable ASPIRE and Biotechnology programs nationwide to distribute the crude *Taq* extract to high school and community college partners without the need for expensive cold shipping.

Acknowledgments. H.K. and I.P. *contributed equally to this project*. I.P. developed the lyo formulation screen and identified the ideal formulation for the crude *Taq* extract. H.K. optimized the qualitative functional assay. I.P. optimized and performed the qualitative functional assay to show that the lyophilized crude *Taq* extract maintains the polymerase activity upon reconstitution. H.K. designed and executed the 10-week temperature stability study (stress test). Q.M. and C.S. performed the preliminary temperature stability study for the non-lyophilized crude *Taq* extract. J.H. and B.N. provided the open-source *Taq* polymerase (pOpen*Taq*, FreeGenes #BBF10K_003493) construct and the DNALC protocols for the crude *Taq* extract preparation, providing us with a starting point. B.N. continues to provide scientific advice for this project. W.W. supervised ASPIRE interns in the early phase of this project. A.K. offered ongoing support and supervision of this project. H.K., I.P., and A.K. drafted the manuscript.

We would like to thank ASPIRE interns who have contributed to this work: Cameron Flowers, Kaethe Schaefer, Hanna Bidzan, Keylin Mejia, Rajath Manohara, Emily Ayala, Fiona Gonzalez, Kevin Lemus, Neo Puchane, Andy Coca, Nikita Motchenko, Paola Maldonado, Sahil Jethra, Sharon Garcia. We would also like to express our gratitude to the Pasadena Bio Collaborative (PBC) Incubator, especially to Dr. Wendie Johnston and Judy

Villacorte, for their support and for allowing us to use PBC's laboratory facility to perform the groundwork efforts and troubleshooting of this project even before our project formally started. We would also like to thank Christopher Castillo for his continued support of our Biotechnology training program. This work was supported and funded by the National Science Foundation (NSF) under award DUE 2100575: *Training the Skilled Biomanufacturing Workforce Through Innovative Internships in Protein Biomanufacturing*.

Disclosures. The authors declare no conflict of interest.

Supplemental Materials. Please see https://micronanoeducation.org/wp-content/uploads/2024/09/Supplementary-Figure-1-Doc-edit-returned.docx

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Cytokine Expression in HepG2 Cells and the Effects of Budesonide

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Abstract: Inflammation has been shown to play a crucial role in regulating both the development of cancer and the response to therapy. Several cytokines are associated with inflammation, chronic illness, and subsequent cancer. Anti-inflammatory molecules show promise as a preventative therapy for high-risk populations by targeting inflammation. Glucocorticoids are widely recognized for their potent anti-inflammatory properties, but their therapeutic utility is often associated by the development of substantial adverse effects with prolonged administration. Budesonide, a synthetic glucocorticoid, offers a more favorable therapeutic index. Characterized by reduced systemic bioavailability compared to other glucocorticoids, budesonide demonstrates reduced classic glucocorticoid-induced side effects. Budesonide has been successfully employed in the management of inflammatory conditions such as ulcerative colitis, autoimmune hepatitis, and asthma. This therapeutic profile positions budesonide as a promising candidate for further exploration in various disease states characterized by chronic inflammation. To utilize the anti-inflammatory potential of budesonide, we aimed to determine its suitability as a prophylactic or therapeutic strategy for liver cancer, specifically hepatocellular carcinoma, by investigating its inflammation-modulating effects. We treated liver cancer cells (HepG2) with Lipopolysaccharide (LPS) that induced inflammatory cytokines like tumor necrosis factor (TNF) α and examined the effect of budesonide to suppress the cytokine expression. Our study provides novel insights into the LPS-induced inflammatory pathway in HepG2 cells and assesses the therapeutic potential of budesonide.

Keywords: inflammation, cancer, budesonide

Introduction

Rudolf Virchow first proposed the correlation between inflammation and cancer in the mid-19th century. He noted that cancer often originates in areas of chronic inflammation and observed abundant inflammatory cells in tumor biopsies [1]. Since then, research has shown that inflammation is closely linked to all stages of cancer development and progression and the effectiveness of anti-cancer treatments [2,3,4,5]. Today, cancer-related inflammation is recognized as one of the hallmarks of cancer, with chronic inflammation being linked to an increased risk of malignancies and the progression of cancer in various types [6,7]. Given this relationship, harnessing inflammation [8] has become an important strategy for improving anti-cancer therapies. Clinical studies have highlighted the potent effects of non-steroidal anti-inflammatory drugs (NSAIDs), notably aspirin, in mitigating cancer risk [9]. High doses and long-term treatment with NSAIDs, however, lead to peptic ulcers, kidney diseases, and stroke [10,11].

During chronic inflammation, when the immune system is over-stimulated for long periods of time, it is generally treated with corticosteroids [12]. These medications suppress the immune system but can only be prescribed for a short time as they have serious side effects like weight gain, bone density loss, high blood pressure, etc. [13]. On the other hand, budesonide, a synthetic steroid of the glucocorticoid family has a high topical anti-inflammatory activity. Budesonide has been used orally for several immune-mediated gastrointestinal and liver diseases and as a nasal spray or by inhalation for allergic rhinitis, asthma, and chronic obstructive lung disease [14,15,16,17]. Budesonide has a high first-pass elimination by the liver (90%) with minimal systemic absorption and is therefore felt to cause fewer side effects than traditional glucocorticoids and to be generally well tolerated. Low systemic exposure to oral budesonide enables the use of budesonide as a prophylactic. Another advantage is that budesonide does not need dose tapering before discontinuation as it does not markedly affect endogenous cortisol production [14].

We selected budesonide for our study because the student who initiated the research has ulcerative colitis and uses budesonide to manage his inflammation. While a few studies suggest that budesonide may have potential for treating moderate to severe colitis [18] or preventing the progression of partially solid lung nodules [19], this study aimed to examine its effect on suppressing inflammation in HepG2 cells derived from a 15-year-old hepatocellular carcinoma patient.

In healthy individuals, the liver is constantly exposed to inflammatory stimuli from dietary sources and commensal bacterial products. It responds by producing acute phase proteins, complement components, cytokines, and chemokines and serves as a reservoir for various resident immune cell populations. [20, 21]. Traditionally viewed as a primarily metabolic and detoxification organ, we now understand the liver as a complex immune organ. Hepatocellular carcinoma (HCC), which accounts for 90% of liver cancer cases, is associated with chronic inflammation and fibrosis, often stemming from factors such as hepatitis B and C, along with alcoholic and nonalcoholic fatty liver diseases [22].

To mimic the inflammatory conditions prevalent in the liver, HepG2 cells were subjected to LPS stimulation, a well-characterized model of hepatic inflammation [23, 24]. HepG2 cells are known to secrete pro-inflammatory cytokines like TNF-alpha and IL-6 upon LPS stimulation [25] or hyperglycemic challenges [26]. HepG2 cells were selected as a suitable model system. By employing this experimental model, we sought to elucidate the therapeutic efficacy of budesonide in mitigating the inflammatory response characteristic of liver disease, with a specific focus on its impact on hepatocellular carcinoma.

Methods

Cell Culture

The human hepatocellular carcinoma cell line HepG2 was obtained from the American Type Culture Collection (ATCC, Maryland, USA). HepG2 cells were routinely grown in Dulbecco's Minimal Eagle Media (DMEM) media (Thermo Fisher) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 mg/ml) (Thermo Fisher). Cells were grown at 37°C in disposable T-75 & T-25 flasks in a humidified atmosphere of 5% CO_2/95% air. The cells were split every three days in a ratio of 1:4.

Treatments

Novus Biological lipopolysaccharide (LPS) from *E.coli* was procured from Fisher Scientific and dissolved in media at a concentration of 1mg/ml. Budesonide (97%) was purchased from VWR and dissolved in DMSO at a concentration of 100 mg/ml.

CCK-8 Cell Viability Assays

To comprehensively evaluate the effects of LPS and budesonide on cell viability, HepG2 cells were exposed range of concentrations of each compound independently, as well as in combination with both. The cell viability was tested by incubating the cells with Dojindo molecular technologies CCK-8 WST-8 reagent [27]. WST-8 is reduced by dehydrogenases in cells to give an orange-colored product (formazan), which is soluble in the tissue culture medium. The amount of the formazan dye generated by dehydrogenases in cells is directly proportional to the number of living cells. The absorbance of cells was read at 450nm on an ELISA plate reader from Biorad.

Cytokine Expression

To systematically characterize the cytokine response elicited by LPS and the subsequent modulatory effects of budesonide, we carried out a proteomic profiling approach utilizing Abcam's human cytokine antibody arrays (ab133997). The Abcam cytokine array analyzes 42 human cytokines simultaneously, and cytokine arrays function similarly to ELISA assays but utilize a membrane substrate instead of a plate. Capture antibodies are arranged in spots on the membrane, with each spot pair representing a different analyte/cytokine. Conditioned media that would contain the cytokines secreted by the cells under the four treatment conditions were collected, added to the membranes, and incubated overnight. The membranes were washed and incubated with paired biotinylated detector antibodies provided in the kit, and finally, they were incubated with streptavidin HRP. The blots were exposed to a chemiluminescent substrate provided with the arrays from Abcam. The blots were exposed to GE Amersham Imager 680.

Analysis Using Image J Software

Densitometric analysis was carried out with ImageJ open-source software (http://rsbweb.nih.gov/ij). ImageJ was chosen for its capability to manipulate contrast settings without distorting true density, setting it apart from other software. The scanned images were inverted, and background signals were excluded from each reading based on the presence of three intentionally blank spots printed on the membranes.

Mathematical Formulation

Excel (Microsoft) was used for mathematical calculations. The formula used for analysis was: $X(Ny) = X(y) * P1 / P(y)$ where: X(Ny) represents the spot "X" density on array "Y." X(y) is the signal density at the spot of interest. P1 signifies the mean signal density on the "+ ref" reference array. P(y) stands for the mean signal density of the positive control on array "Y." This methodology allowed for the semi-quantitative assessment of cytokine levels in the samples, with a focus on accuracy and consistent data analysis using specialized software and mathematical formulas.

Confirming Cytokine Array Results with Gene Expression Analysis

RNA extraction from the HepG2 cells was done using the phenol-free RNA extraction kit (VWR). RNA was quantified with a nanodrop spectrophotometer from Thermo Fisher. Cytokine expression levels were quantified by examining RNA expression through reverse transcriptase polymerase chain reaction (RT-PCR). For reverse transcription, 2 µg of RNA was used. ProtoScript II Reverse Transcriptase kit from New England Biolabs was used for cDNA synthesis. Real-time PCR was performed using a powertrack SYBR master mix from Thermo Fisher.

Pre-designed primers for gene expression analysis were obtained from Integrated DNA Technologies (IDT). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as the housekeeping gene for normalization. The PCR was run on Thermo Fisher's Quantstudio-3 real-time PCR machine. The cytokines that were analyzed were TNF-α, Tumor growth factor (TGF)-beta, Interferon gamma (IFN-γ), IL-15, Oncostatin (OSM-1), IL-2, and Granulocyte-macrophage colony-stimulating factor (GMCSF). PCR reactions for each sample with all the primers in every experiment were done in triplicate, and the fold changes were calculated using the Delta-delta ct method ($2^{-\Delta\Delta Ct}$) [28].

Results and Discussion:

LPS and Budesonide Not Toxic to Cells

To comprehensively evaluate the interactive effects of LPS and budesonide, a comprehensive experimental design was implemented. Cells were subjected to a matrix of treatment conditions, including both individual and combinations of LPS and budesonide concentrations within the specified ranges. HepG2 cells were incubated for 24 hrs. in the presence of varying concentrations of LPS -1 µg/ml to 40 µg/ml and budesonide - 10^{-3} M to 10^{-10} M. None of the tested concentrations affected cell viability, as indicated by the CCK-8 assay (results not shown). Replicated experiments demonstrated consistent outcomes. Based on these results and established literature, LPS and budesonide concentrations of 4 µg/ml and 10^{-7} M, respectively, were selected for subsequent investigations. The experimental design aimed to determine if a 24-hour LPS exposure would induce inflammatory cytokine production in HepG2 cells and whether a 10^{-7}M budesonide treatment could attenuate this response.

Minimal or No Cytokine Induction by LPS and Inhibition by Budesonide

Conditioned media from HepG2 cells which were untreated, treated with 4 µg/ml LPS, treated with 10^{-7} M budesonide alone or with LPS and budesonide for 24 hrs., was collected and used to probe Abcam cytokine antibody arrays. Figure 1 shows the images of the Abcam cytokine arrays obtained from the GE Amersham Imager 680 scanner.

In contrast to previous literature, our findings revealed an unexpected absence of detectable levels of several LPS-induced cytokines, including TNF-α, IL-1 beta, IFN-γ, and IL-6, as assessed by cytokine array (Figure 1). This discrepancy between our results and prior studies warrants further investigation.

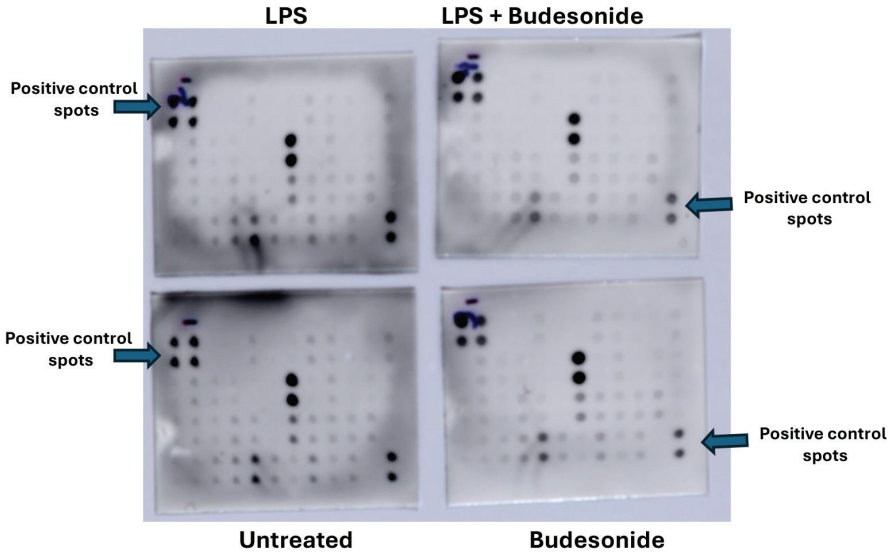

Fig.1: Abcam arrays were developed using a chemiluminescent substrate and exposed to GE Amersham imager 680 scanner to generate the images.

Given the absence of visually apparent cytokine expression differences, a quantitative analysis was performed. To accurately quantify cytokine levels, ImageJ densitometry software was employed to measure the signal intensities associated with antigen-specific antibody spots on the array. A fundamental principle underlying this analysis is the direct proportionality between signal intensity and antigen concentration within the sample. Relative differences in cytokine expression levels among treatment groups were determined by comparing signal intensities of individual antigen-specific antibody spots to the untreated control. By meticulously quantifying the signal intensity of each spot, we obtained a numerical representation of the corresponding cytokine abundance. This quantitative approach enabled a precise comparison of cytokine expression levels across different experimental conditions, facilitating the identification of subtle variations that might have been overlooked through visual inspection alone. Fold changes were calculated by comparing the signal intensities for each cytokine after background subtraction and normalization, as mentioned in materials and methods, such as mathematical formulation. Table 1: Lists the cytokines that show more than a 2-fold change in expression in LPS-treated cells when compared with untreated cells (row 1) and the cytokines that were suppressed by budesonide and LPS treatment when compared with LPS treatment alone (row 2).

Table 1: Shows the cytokines that were induced by LPS treatment and suppressed by Budesonide treatment. Fold changes after the normalization of the data are shown.

Cytokine	LPS treated/Untreated	LPS+budesonide/ LPS treated
IL-2	2.74	0.34
IL-1 alpha	2.50	0.50
IFN-γ	2.60	0.90
IL-15	5.60	0.20
GMCSF	2.01	0.58
IL-3	2.35	0.82
I-309	2.74	0.25

IL-1 alpha, IL-2, IL-3, IL-15, IFN-γ, GMCSF and I-309 which is small glycoprotein that belongs to the chemokine family showed fold change in expression above 2-fold though the induction of these cytokines were not visible on the arrays as the signal intensities were low. The induction of these cytokines was also suppressed by co-treatment of cells with LPS and budesonide.

Effect of Different Concentration of LPS on Cytokine Expression at the RNA Level

HepG2 cells were treated with different concentrations (2-20µg/ml) of LPS for 24 hours and analyzed for the expression of cytokines. Since the sensitivity of the Abcam arrays is low, we analyzed the cytokine expression at RNA level by RT-PCR. A comprehensive analysis of cytokine expression profiles was undertaken, specifically, the expression levels of TNF-α, IFN-γ, and OSM-1 (a member of the IL-6 family) as described in previous literature [24], as well as IL-15, GM-CSF, and IL-2, which exhibited significant induction in our cytokine array analysis. This focused study of a targeted cytokine panel allowed for a deeper understanding of the complex inflammatory response elicited by LPS and the potential modulatory effects of budesonide.

Figure 2 shows that 2-4µg/ml of LPS did induce the expression of Oncostatin (IL-6 family), IFN-γ, IL-2, and IL-15. Though at higher concentrations of LPS (8-20µg/ml) there was suppression in the expression of the cytokines as has been previously reported [29]. Cytokines GM-CSF and TNF-α showed increased expression at 2µg/ml LPS concentration and gave variable levels of expression at higher concentrations. TGF beta was not induced by LPS. Further experiments to analyze the suppression of cytokine expression were done using 4 µg/ml of LPS to induce cytokine expression and 10^{-7} M of budesonide to examine its effect on suppressing the induced cytokines [30].

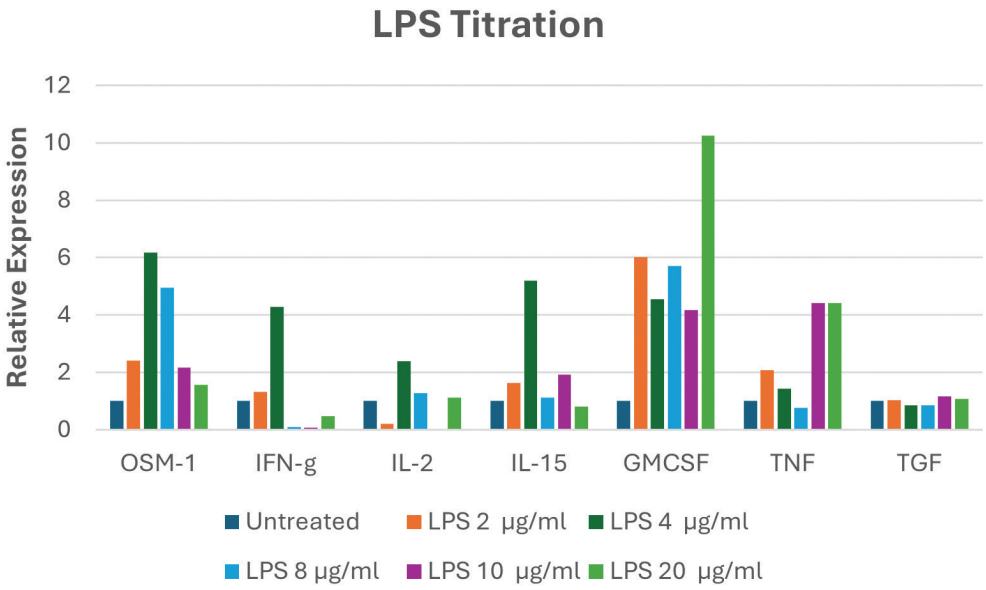

Fig. 2: Shows the induction of Oncostatin (IL-6 family), TNF-α IFN-γ, IL-2, IL-15, GM-CSF and TGF beta in HepG2 cells treated with LPS concentrations ranging from 2µg-20µg for 24 hours. The Y axis represents the expression level of the cytokines calculated by the Delta-delta ct method ($2^{-\Delta\Delta Ct}$).

LPS and Budesonide Impact Cytokine Expression

HepG2 cells treated with 4 µg/ml of LPS consistently showed a 6.86-fold increase in OSM-1 (a member of the IL-6 family), a 12.38-fold increase in IFN-γ, and a 4.23-fold increase in IL-15 levels (Figure 3). These results are averaged from five independent experiments. A paired t-test was employed to compare cytokine levels before and after treatment across all five experiments, with significance determined at $p<0.05$. Significant increases were observed for OSM-1 and IFN-γ expression, with p-values less than 0.05, as indicated by the asterisks in Figure 3. However, the p-values for IL-15 and GM-CSF were 0.08 and 0.6, respectively, indicating variability in their expression levels. TNF-α, TGF-beta, and IL-2 did not show consistent induction following LPS treatment and are therefore not included in Figure 3.

Treatment with 10^{-7} M budesonide alone did not induce the expression of IFN-γ, IL-15, or GM-CSF. However, budesonide treatment did result in an increase in OSM-1 expression (p=0.02). In our experiments, co-treatment with LPS and budesonide did not consistently suppress the induced cytokines OSM-1, IFN-γ, IL-15, and GM-CSF. Contrary to expectations, budesonide, in combination with LPS, led to an increase in IFN-γ (p=0.01) and GM-CSF expression, while IL-15 expression remained unchanged. This outcome differed from results observed in cytokine arrays, where budesonide and LPS co-treatment inhibited the expression of these cytokines. As we did not conduct a titration experiment with varying concentrations of budesonide and LPS, we cannot confirm whether a different concentration of budesonide might have effectively suppressed the cytokines analyzed in our study. We chose the 10^{-7} M concentration because previous studies reported that this concentration suppressed cytokine expression in colon cancer cells co-cultured with peripheral blood mononuclear cells (PBMCs) or in PBMCs stimulated with LPS [30], as well as in human lung epithelial cells [31].

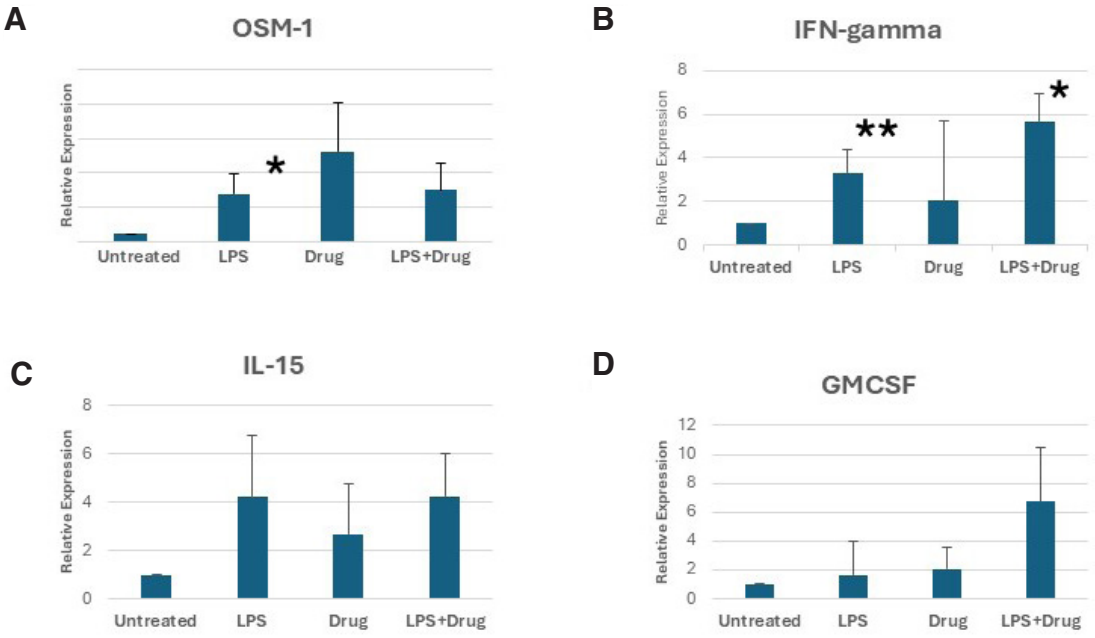

*Fig. 3: Effects of LPS, budesonide, and LPS + budesonide treatment on HepG2 for 24 hrs. The levels of (A) OSM-1, (B) IFN-γ, (C) IL-15, and (D) GMCSF were analyzed by RT-PCR. Results are presented as the mean and ±standard deviation of five independent experiments. * represents statistically significant differences in the level of cytokine expression between treatments compared to the untreated cells. *P<0.01, **P=0.008 vs. control group. The Y axis represents the expression level of the cytokines calculated by the Delta-delta ct method ($2^{-\Delta\Delta Ct}$).*

Conclusion

This study confirms that HepG2 cells in culture secrete OSM-1 and IFN-γ, IL-15, and GMCSF when treated with LPS. However, we were unable to replicate the results of Ma. C. Gutiérrez-Ruiz et al. [24] reported the induction of TNF-α and TGF-beta at the mRNA level and secretion of TGF-beta and IL-1beta cytokines. The results obtained from the analysis of Abcam cytokine arrays suggest that budesonide suppresses the induction of IFN-γ, IL-15, and GM-CSF at the protein level. The data in Figure 3 shows that adding budesonide and LPS together gives a different result at the RNA level. Co-treatment with budesonide and LPS appears to increase the expression of IFN-γ by twofold, which is consistent with previous reports indicating that glucocorticoids can induce certain cytokines depending on the cell type [32]. These contrasting reports suggest that cell type may influence inflammatory cytokine expression in response to budesonide. Budesonide has been reported to have variable effects on cytokine expression depending on cell type and co-stimulatory stimuli [33, 34, 35]. The analysis of cytokine IL-3, IL-1 alpha, and I-309 expression at the RNA level was not conducted. IL-2 showed inconsistent results in PCR.

The suppression of induced cytokines by budesonide remains unclear based on the current data. To more fully elucidate these effects, future research should include a broader range of budesonide concentrations. Furthermore, exploring budesonide's interactions with a variety of inflammatory stimuli capable of inducing cytokine expression could provide crucial insights into its potential therapeutic applications. Given the unclear and inconsistent cytokine response observed in HepG2 cells exposed to LPS and budesonide, it is plausible that this cell line may not constitute the most optimal model for investigating cytokine expression dynamics. A more complex experimental design, such as a co-culture system integrating HepG2 cells with primary immune cells like monocytes or PBMCs, could offer a more physiologically relevant environment to assess budesonide's anti-inflammatory efficacy. By including these refinements in future studies, a more comprehensive understanding of budesonide's therapeutic potential can be achieved. Our study offers novel insights into the intricate interplay between LPS-induced inflammation and the modulatory effects of budesonide within the context of HepG2 cells. Our findings illuminate the necessity for further investigation into the precise molecular mechanisms governing these effects. A deeper understanding of the signaling pathways involved, such as the NF-κB cascade, is imperative for unraveling the complexities of budesonide's therapeutic actions. By understanding these mechanisms, the development of more targeted and efficacious anti-inflammatory strategies could be developed. Moreover, our findings underscore the importance of carefully considering the limitations of cell-based models, such as HepG2 cells, in recapitulating the full spectrum of in vivo inflammatory responses. Future studies incorporating more complex models, such as co-culture systems or animal models, are warranted to bridge the gap between in vitro observations and potential clinical translation.

Ultimately, our research contributes to the growing body of knowledge surrounding the therapeutic potential of budesonide in inflammatory diseases while highlighting the need for continued exploration to fully realize its clinical utility.

Acknowledgments. This work was supported by the RCN-UBE: Cell Biology Education Consortium (CBEC) grant from the National Science Foundation (NSF) under award #1827066.

Disclosures. The authors declare no conflicts of interest.

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From Ground to Air: Developing a Drone Curriculum for Law Enforcement Education

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Abstract: Unmanned Aircraft Systems (UAS) have proven very beneficial for law enforcement activities and are used for search and rescue, surveillance, and crime scene reconstruction. As such, integrating this innovation into the law enforcement curriculum at two-year community colleges is becoming significantly important. This study surveyed 94 law enforcement officers and asked them to rank how vital 31 curriculum items were to include in a UAS certificate program. The researchers found three key conclusions from the top third-ranked curriculum items. First, the study participants thought regulations were critically important to include in the curriculum. "Federal and state UAS regulations" was the highest-ranked item in the survey, and "concepts assessed with FAA Part 107 licensing exam" ranked third highest. Learning to operate a drone is very important, with "basic flight skills," "intermediary flight skills," and "advanced flight skills" listed as separate curriculum items. The third key conclusion is that students should know how to conduct general missions, supported by high rankings for night missions, ethics, mission planning, risk management, and overwatch and command center techniques. This emphasis on practical drone operation skills underscores their growing importance in law enforcement training. It reflects the increasing need for a curriculum that thoroughly prepares students for real-world law enforcement scenarios, equipping them with the ability to effectively employ UAS technology in various operational contexts.

Keywords: drone, Unmanned Aircraft Systems (UAS), Uncrewed Aerial System, law enforcement, curriculum

Introduction

In recent years, there has been a notable rise in the use of drones within various industries, with significant uses in law enforcement. The top five uses of drones in law enforcement are surveillance, search and rescue operations, traffic monitoring, crime scene analysis, and crowd monitoring [1]. Drones are deployed in search and rescue missions to swiftly cover large territories and deliver immediate information to assist in finding individuals who are missing or criminal suspects [1]. Drones are used for traffic monitoring to observe traffic patterns, detect violations, and reconstruct accidents [2]. In crime scene analysis, drones provide an aerial perspective to capture comprehensive images and footage, aiding in evidence collection and analysis [3]. Additionally, drones are employed for crowd monitoring during public events or demonstrations to ensure public safety and security [4]. Drones have increasingly become essential components of police activities due to their ability to provide aerial surveillance, which aids in monitoring large areas efficiently and cost-effectively [5].

This study aims to provide faculty members in two-year college law enforcement programs with a comprehensive set of curriculum topics ranked in order of importance. A questionnaire was distributed to law enforcement agencies across the southeastern United States—including Tennessee, Florida, North Carolina, South Carolina, Virginia, and Georgia—to evaluate the significance of including 31 specific topics in a drone curriculum. This paper concentrates on the detailed discussion and analysis of the data collected from these responses.

Literature Review

What is a Drone?

The FAA defines a UAS as an aircraft operating without direct human control onboard or inside it [6]. The origin of the word "drone" is often linked to the DH.82B Queen Bee radio-controlled aircraft used by the British during World War 2 for target practice [7]. This paper will use the Part 107 definition, which describes an uncrewed aircraft weighing less than 55 pounds, excluding many military-grade planes like the Predator and Reaper. Most UAS following Part 107 rules are either fixed-wing, multirotor or a hybrid of both. Multirotor

drones use vertically oriented propellers to go up, like helicopters, while fixed-wing drones create lift by passing air over a horizontal wing, like airplanes. A recent study revealed that over 90% of public safety agencies employing drones opt for the multirotor variety [8]. Multirotor drones are popular because they can take off and land vertically, stay in one spot, and move well around obstacles. Typical payloads include red, green, and blue (RGB) cameras with high-zoom capabilities, low-light sensors, and infrared (thermal) imagers [9]. High-zoom cameras are crucial for law enforcement, helping with surveillance and covert operations. Low-light and infrared cameras are beneficial for nighttime tasks and search and rescue missions. Additionally, still pictures taken by these drones can be turned into 3D models of accidents or crime scenes using structure-from-motion software [9].

Part 107

Most non-hobbyist UAS activities fall under the governance of Part 107, occasionally supplemented by additional state and local regulations. Part 107 serves as the regulatory framework for operating UAS weighing less than 55 pounds. The regulation outlines the requirements for pilot certification, operational limitations, and aircraft specifications. These include passing an aeronautical knowledge test, restrictions on flying drones over people, conducting night missions, adhering to maximum altitude limits, and maintaining visual line-of-sight with the pilot [10]. This regulatory framework aims to ensure the controlled incorporation of drones into the national airspace system while still providing a large amount of operational freedom.

Certificate of Authorization

Alternatively, law enforcement agencies utilize UAS by adhering to Part 91 guidelines, which involve obtaining a Certificate of Waiver or Authorization (COA). A COA, granted by the FAA, permits certain public agencies to operate beyond the confines of the Part 107 rules. Operators flying under a COA are exempt from the requirement of possessing an FAA remote pilot certificate and are not bound by Part 107 restrictions, such as the 400-foot altitude limit. However, obtaining a COA involves a comprehensive safety case analysis encompassing operational procedures, pilot qualifications, and the specific use case of the drone. COAs grant greater operational flexibility than Part 107, allowing for activities like night flights or beyond visual line of sight under FAA-approved conditions [11]. From a law enforcement perspective, the FAA's Part 107 regulations for small-unmanned aircraft provide simplicity and accessibility, particularly beneficial for smaller departments or occasional drone use. The straightforward procedure for acquiring a Remote Pilot Certificate, along with clearly defined operational limitations, facilitates the legal deployment of drones in an efficient and streamlined fashion [12]. Nevertheless, restrictions on night operations, flying over people, and flights beyond visual line-of-sight may impede effectiveness in specific law enforcement scenarios. In contrast, a Part 91 COA provides greater operational flexibility, especially beneficial for emergency or sensitive law enforcement operations like surveillance or search and rescue missions. However, the COA application process is more time-consuming and necessitates a detailed safety case, which could pose challenges for smaller departments with limited resources [12].

Proliferation of Drone Use Across Industries

Drones have steadily gained popularity across different sectors owing to their diverse uses and benefits. A partial list of industries and applications includes healthcare, agriculture, construction, disaster management, defense, delivery, entertainment, photography, marketing, rescue, and research [13]. Agriculture, construction, and manufacturing are the leading industries deploying drone technology [14,15,16].

Agriculture

Drones are increasingly employed in agriculture for diverse purposes, including mapping soil properties, assessing crop health, targeted spraying of fertilizers and pesticides, livestock monitoring, and predicting crop performance [17,18]. Additionally, drones outfitted with multispectral sensors have shown considerable advantages in forecasting crop yields and analyzing spatial disparities across fields, thus enhancing the management of silage production for livestock feed [19]. Jarial (2023) highlighted the benefits of drones for precision irrigation, livestock tracking, crop disease surveillance, and aerial monitoring [20]. Moreover, drones have been employed to monitor crop biomass, growth, and quality, as well as to identify weed populations for targeted herbicide applications and improve resource management [21].

Construction

Drones have become an integral part of the construction industry, providing various uses such as site planning, surveys, inspections, safety assessments, progress monitoring, and precise volumetric data collection during excavations [22]. Using drones in the construction industry yields several advantages, including cost-effectiveness, diminished risk to personnel, and increased efficiency in data gathering, owing to their superior spatial and temporal resolutions in contrast to other remote sensing methods approaches [15]. Moreover, drones equipped with infrared cameras and LIDAR offer applications in construction sites, particularly for building inspection and monitoring [23].

Manufacturing

Incorporating drone technology into the manufacturing sector has resulted in notable progress. Drones have been employed across various manufacturing activities, such as inventory management, facility inspections, logistics, and production monitoring. These uses have enhanced operational efficiency, bolstered safety measures, and enabled real-time monitoring of manufacturing operations [16]. In the manufacturing industry, drones have been utilized for tasks such as payload lifting and transportation [24]. Additionally, Majeed et al. (2021) highlighted the application of drones in industrial gas sensing [25].

How Drones Are Used in Law Enforcement

In 2019, DRONERESPONDERS, a leading UAS public safety support organization, released the results of a comprehensive survey of drone use for first responders. A significant finding was that most public safety UAS programs are informal and lack the structure associated with more established technology and practices [26]. Subsequently, DRONERESPONDERS' parent organization, AIRT, conducted an extensive survey of more than 300 professionals in law enforcement, emergency management, and fire safety [8]. The study found that drones' most common practical applications were search and rescue, incident command and control, and crime scene investigation. Other applications included damage assessment, non-forensic mapping, surveillance, public information, structural fire response, SWAT support, special event planning, HAZMAT response, and wildfire response [8].

During 2018 and 2019, the Police Executive Research Forum (PERF), together with the U.S. Department of Justice's Office of Community-Oriented Policing Services and the U.S. Department of Homeland Security's Office for State and Local Law Enforcement, initiated a comprehensive initiative to explore the use of UAS technology in law enforcement. This initiative involved disseminating a survey among law enforcement agencies and hosting a two-day conference to address the challenges and opportunities related to police use of drones. The project highlighted three components, which involved conducting an informal survey of 860 law enforcement agencies across the country, interviewing police executives and staff in agencies with drone programs, and organizing a conference where police executives, federal officials, and other experts from across the country discussed various aspects of police drone usage [27]. The primary objective of this effort was to provide guidance and insights to police agencies interested in implementing their drone programs [28].

The PERF report primarily focused on the law enforcement application of drones. It noted that search and rescue was the primary application for drones, with over 90% of participants acknowledging its implementation in their drone activities. Furthermore, other types of missions where drones were extensively used, covering 80% or more of the respondents, included reconstructing crime scenes, investigating suspects, responding to disasters, and reconstructing traffic collisions. The report also outlined additional mission types such as HAZMAT response, bomb observation, fugitive apprehension, crowd monitoring, and surveillance [27]. Table 1 depicts the frequency law enforcement agencies reported using drones to support various mission types, as outlined in the PERF report [27].

Table 1. Common Purposes for Using Drones in Policing[a]

Drone Purpose	Percentage
Search and Rescue	91%
Crime Scene Photography and Reconstruction	85%
Investigating Armed and Dangerous Suspects	84%
Disaster Response	84%
Traffic Collision Reconstruction	81%
Hazardous Material and Bomb Observation	68%
Fugitive Apprehension	63%
Crowd Monitoring	51%
Surveillance	27%
Other	14%

[a]From PERF (2020), the percentage values of the surveys are given

Public Perception

The deployment of drones by law enforcement agencies raises important concerns about privacy and trust. It is essential for these agencies to engage with their communities before starting a drone program to build support and understanding of the purposes, techniques, timing, and places of drone usage [28]. Law enforcement agencies should develop detailed written guidelines specifying when drones can be used, their intended purposes, the kinds of data or footage that will be gathered and retained, methods for protecting this information, protocols for controlling access, and other pertinent factors. Securing community support and endorsement for the restricted application of drones is essential for maintaining trust-based solid relationships with community members while leveraging this highly beneficial new technology [28].

Privacy and Ethical Considerations

The use of drones by police for surveillance and law enforcement purposes has raised significant ethical considerations. The potential for drones to invade individuals' privacy and gather sensitive data has sparked concerns about confidentiality and consent [29]. Additionally, the deployment of drones for surveillance purposes has been associated with the normalization of ongoing subjugation and the politics of drones, raising questions about the ethical implications of their use [30]. Furthermore, ethical and sustainability aspects of civil drone use have been highlighted, emphasizing the need for systematic, structured guidance to examine and evaluate drone applications [31].

Studies on UAS Criminal Justice Curriculum

Integrating drone technology into the criminal justice curriculum presents a significant opportunity to enhance educational offerings and prepare students for the dynamic landscape of law enforcement. The potential benefits of incorporating drone technology into the curriculum include fostering spatial visualization skills and providing students with practical experience utilizing emerging technologies [32]. Integrating drone education can contribute to developing a more humanistic approach to policing, aligning with the nationwide trend toward community policing [33].

Alternative Views and Counterarguments

Drones offer innovative capabilities for law enforcement, such as enhanced population monitoring and surveillance [34]. However, existing research, including studies by [35] and [36], suggests that initiatives like Secure Communities have not consistently led to the anticipated safety outcomes. The deployment of

drones by law enforcement has also triggered counter-surveillance measures by activists, sparking debates over privacy and civil liberties concerns [36]. Moreover, the use of drones in law enforcement raises significant issues related to discrimination, surveillance practices, data security, and respect for human dignity. These challenges emphasize the need for careful consideration and the establishment of ethical guidelines in the deployment of drone technologies [37].

Summary and Knowledge Gap

The literature highlights the effectiveness of drones as tools in law enforcement, employed across various applications. Despite their efficacy, the technology is relatively new, with FAA regulations for UAS enacted in less than a decade. Consequently, a significant number of police departments do not have standardized policies or programs in place for the use of drones, primarily owing to the novelty of the technology [28]. Aside from the skills and knowledge necessary for operating drones in the national airspace, which are characterized by commercial aviators, law enforcement faces additional prerequisites and considerations [9]. Privacy concerns and fears of governmental overreach among the general public require law enforcement officers to be mindful of their public image and maintain transparency regarding drone policies, operations, and usage limits.

Moreover, constitutional considerations arise concerning how this fast-paced technology gathers evidence. Further, considerations arise concerning the storage and secure distribution of data collected by police. Overall, law enforcement must navigate these complexities to ensure responsible and effective use of drones.

Basri et al. [38] stress the importance of technology-driven education, underlining the need to integrate innovative learning tools into higher education. This emphasis aims to enhance the quality of the learning experience and adequately prepare students for the dynamic workforce ahead. Organizations frequently adopt new technologies by recruiting young technicians who possess the skills that the organization currently lacks [9]. The existing literature fails to address the specific recommendations from law enforcement agencies regarding the curriculum that should be taught to two-year college students in drone technology. This lack of information highlights the importance of directly consulting with law enforcement agencies to determine the crucial aspects of drone-related education that should be included in the curriculum for two-year college students. Clarifying these recommendations will enable educational institutions to better align their programs with the needs and expectations of law enforcement agencies, thus facilitating the development of skilled technicians equipped to meet the demands of modern law enforcement practices.

Methods

This study aims to provide law enforcement faculty members with a comprehensive set of curriculum topics and learning objectives for including drone technology in a two-year college certificate program. By equipping professors with these tools, the study aims to facilitate the development and implementation of effective drone education within law enforcement training programs.

Steps to Collect Data

The study aimed to achieve its objectives by examining the websites of police departments and county sheriff offices in six southeastern U.S. states. The email addresses were collected by visiting the websites of 2,300 police departments and sheriff's offices throughout Georgia, Virginia, North Carolina, Florida, Tennessee, and South Carolina. In instances where the email addresses for these officials were not provided, the research team sent an email to the general contact addresses requesting the contact information of the police chief or sheriff. Through this method, a list of 1,334 email addresses was created. Subsequently, an online survey was sent to these email addresses. The email text contained directions for the chief or sheriff to pass the survey along to the individual best equipped to respond to questions about their UAS program. If the agency did not have a UAS program, the email requested that the police chief or sheriff complete the survey themselves.

Survey Data Collection

The survey was executed via the online platform 'Qualtrics' and structured into three sections. The first section evaluated whether law enforcement agencies adhere to industry best practices, and the second section focused on counter-UAS operations. Both sections are directly tied to the recommendations made in the PERF report and are the focus of another publication. The third section of the survey focused on the appropriateness of various UAS in law enforcement curriculum items. The findings from this portion of the survey are the focus

of this paper. Ten questions about the general characteristics of their agency were included. The survey then presented 31 law enforcement UAS curriculum topics and asked the participants to indicate how important they were to include in a "UAS in Law Enforcement Certificate Program." The participants were given a four-point Likert scale and asked to rank their importance.

Once a draft of the survey was created, it was sent to two law enforcement officers with significant experience in UAS technology for review. Following their input, the survey was pilot-tested with 48 law enforcement offices from the email addresses collected for this study. The goal was to gauge participants' time to complete the survey and ensure it was free of any systematic issues. The data underwent analysis utilizing SPSS (Statistical Package for the Social Sciences).

Use of Artificial Intelligence

Artificial intelligence (AI) tools were employed to assist in organizing the authors' thoughts and performing initial editorial proofreading tasks in preparing this manuscript. The authors have independently verified all citations and statements of fact presented in this paper. The use of AI in this context served purely as an aid to enhance the clarity and coherence of our narrative and not as a substitute for the rigorous academic scrutiny traditionally applied in scholarly research.

Results

The survey was completed by 94 police department chiefs and county sheriffs. The distribution is shown in Table 2. Of the respondents, 83 of the surveys were completed by a representative of a police department and 11 of them from a county sheriff's office. Florida (n=20), North Carolina (n=26), and South Carolina (n=24) had the most responses, with (n=2) having notably the fewest. A one-way ANOVA statistical significance test was conducted to confirm whether the police departments and county sheriffs have any significant impact in each of the states. The result shows there was no statistical difference between the two roles.

Table 2. Survey Response Distribution

State	Police Departments	County Sheriffs	Responses (n)
FL	15	5	20 (21.3%)
GA	7	3	10 (10.6%)
NC	21	5	26 (27.7%)
SC	14	10	24 (25.5%)
TN	2	0	2 (2.1%)
VA	6	6	12 (12.8%)
Total	65	29	94 (100.0%)

Characteristics of Survey Participants

The survey instructed the police chiefs or sheriffs to forward the email to the most appropriate person to address questions about their agency's drone use. This study will refer to the individuals the police chiefs or sheriffs forwarded the survey to as "specialists." As shown in Table 3, the majority (88.3%) of the surveys were completed by specialists. More than half of the participants (57.4%) reported serving in suburban areas, with an additional 23.4% in rural and 19.1% in urban settings. A question was asked about the count of sworn law enforcement officials working for the agency. The responses were relatively evenly spread across the median number of approximately 100 officers. Less than half (47.9%) of the respondents indicated that their agency has a drone program. A third of the respondents' agencies had patrol officers regularly issued drones. More than 70% of respondents reported that their agencies own between 1 and 5 drones in their fleets, with a median of 5 certified pilots. Additionally, 89.4% anticipate an increase in certified pilots over the next decade. See Table 3 for a description of the agency's characteristics.

Table 3. Agencies' Characteristics

Category	Operational Scope	Frequency	Percentage
Agency position	Chief/Sheriff	11	11.7%
	Specialists	83	88.3%
Population density	Rural	22	23.4%
	Suburban	54	57.4%
	Urban	18	19.1%
Number of sworn officers at agency	0-50	25	26.6%
	51-100	26	27.7%
	101-150	13	13.8%
	151-200	8	8.5%
	201 Above	22	23.4%
Does the agency have a drone program?	Yes	45	47.9%
	No	49	52.1%
Number of certified drone pilots at agency	0	7	7.4%
	1-5	47	50.0%
	6-10	26	27.7%
	11-15	11	11.7%
	16 Above	3	3.2%
Do you expect the number of certified pilots to change over the next 10 years?	Yes	84	89.4%
	No	10	10.6%
Full-time drone unit personnel	1-3	11	11.7%
	7-9	1	2.1%
	10 Above	1	1.1%
Number of drones in agency's fleet	1-5	66	13.8%
	6-10	13	6.4%
	11-15	6	6.4%
Does your agency regularly have patrol officers issued a drone for routine shifts?	Yes	32	34%
	No	62	66%

Law Enforcement's Opinion on the Importance of Curriculum Topics

The law enforcement respondents were asked to rank the importance of 31 UAS curriculum topics. A four-point Likert scale was used, giving the option to rank their importance as 1) do not include, 2) somewhat important, 3) very important, and 4) must include. Participants also had the choice to select "I don't know," leading to their responses for that topic being excluded from the analysis. Table 4 outlines the mean and the standard deviation of the responses for each curriculum topic sorted by highest importance (3.6) to lowest (1.98)

Table 4. Curriculum Topics Ranking

#	Total Mean	Stan Dev.	Item
1	3.6	0.612	Federal and state UAS regulations
2	3.58	0.647	Basic flight skills
3	3.41	0.799	Concepts assessed with FAA Part 107 licensing exam
4	3.36	0.732	Intermediary flight skills
5	3.32	0.736	UAS search and rescue tactics and techniques
6	3.29	0.831	UAS night operations techniques
7	3.25	0.834	Ethics with UAS
8	3.25	0.816	How to request regulatory waivers from the FAA
9	3.13	0.726	Mission planning
10	3.09	0.69	UAS tactics for active shooter
11	3.08	0.824	Overwatch and command center techniques
12	3.04	0.821	Risk Management
13	3.01	0.842	Advanced flight skills
14	2.98	0.888	Aeronautical decision making
15	2.98	0.786	UAS tactics for surveillance
16	2.82	0.847	UAS tactics for crowd control
17	2.8	0.776	UAS tactics for hostage situations
18	2.78	0.964	Reading FAA sectional charts
19	2.75	0.871	How to collect thermal images from an infrared camera
20	2.73	0.879	Crew resource management
21	2.7	0.844	UAS radio communication techniques
22	2.66	0.831	Capabilities of different types of drones and manufacturers
23	2.66	0.799	Crime scene reconstruction
24	2.63	0.883	UAS tactics for bombs and explosive
25	2.62	0.862	Photography and cinematography skills
26	2.49	0.896	Traffic collision reconstruction
27	2.38	0.871	Counter UAS techniques
28	2.18	0.953	UAS tactics for prison security
29	2.07	0.785	Certification from a 3rd party validating flight skills
30	2.06	0.807	Certification from a 3rd party validating UAS management skills
31	1.98	0.88	How to create a UAS land survey

Normality of Data Sample

Figure 1 (A) provides the Gaussian bell curve, representing a theoretical normal distribution. The figure shows a relatively small standard deviation (.437) compared to the mean (2.86), with most of the data points clustered close to the average. This suggests low variability in the data. Additionally, the data is distributed normally, meaning that around 68% of the data points are within one standard deviation of the mean.

A

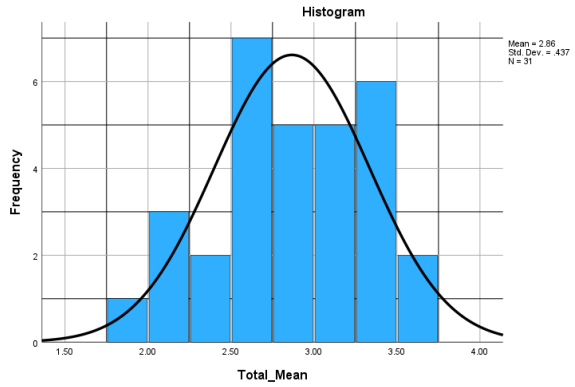

Fig. 1. (A) Gaussian Bell Curve of Mean

B

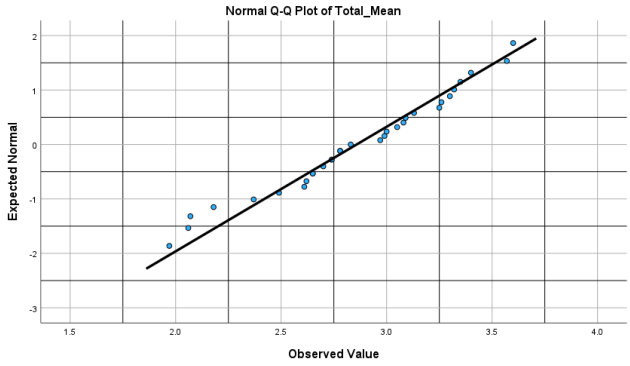

Fig. 1. (B) Normal Q-Q Plot of Mean

Figure 1 (B) presents a normal Q-Q (quantile-quantile) plot, showing that the data adheres to a normal distribution. This plot is used to assess how well the distribution fits the data. The data points roughly align with a straight line, which upholds the normality assumption and verifies that the data distribution conforms to the theoretical model.

The low variability around the mean implies that the mean is a good representation of the data. The Shapiro-Wilk test results and visual inspection of the curve and histogram back this conclusion. The test yielded a statistic of approximately 0.966 with a p-value of 0.426, suggesting that the distribution of mean values does not significantly deviate from normality. The means of the normal distribution indicates that the statistical premises for numerous parametric tests are met, enabling easy analysis and interpretation. This suggests that the data are consistent and reliable, ensuring that conclusions based on further statistical evaluations are well-founded.

The consistency and reliability of these statistical indicators allow for a high degree of confidence in the data set. It also suggests that any further statistical evaluations conducted using this data are likely to be accurate and reflective of the underlying phenomena being studied. The implication for our research is that the mean provides a reliable measure of central tendency suitable for further analysis and discussion concerning the broader study objectives.

Statistically Significant Differences in Curriculum Topics Based on Agency Characteristics

An essential part of the analysis was to identify if there are any statistically significant distinctions of opinion on the importance of the curriculum topics based on the characteristics of the agency shown in Table 3. The analysis of variance was conducted with SPSS statistical software, utilizing ten agency characteristics as factors concerning the thirty-one items intended for inclusion in the curriculum. Overall, there were few statistical differences based on the agency characteristics measured in this study. However, the following sections will explore the differences that were found.

Agency Position Differences in Ranking Curriculum Importance

Table 5 presents the statistically significant variable of items using the agency position as a factor. The statistically significant item measures the probability that the observed result is not attributable to chance. For this observation, aeronautical decision-making observed a statistically significant value of 0.005, and UAS night operations techniques observed a value of 0.013. The alpha level of a statistically significant variable used in this study is less than or equal to 5% (0.05). An F factor of 8.13 and 6.41 is observed. The F factor is used in the analysis of variance (ANOVA) to compare the variance between groups or to assess the overall fit of a model. A higher F value signifies a larger variance ratio within groups compared to variance between groups, indicating that the items account for a substantial part of the variance observed. The F-statistic ($F=8.13,6.41$; $p<0.05$) signifies that the difference is statistically significant. From our study, the specialists feel that aeronautical decision-making and UAS night operations (3.09 and 3.38, respectively) are more important to be included in the UAS curriculum than what the chiefs and sheriffs believed (2.33 and 2.75, respectively). Aeronautical decision-making, or "ADM," is a common expression in aviation and a mandatory topic in the Part 107 exam. Chiefs and Sheriffs may have undervalued the importance of this topic

due to their unfamiliarity with the phrase and its meaning. Similarly, the lack of experience with the difficulty of night operations may have caused the chiefs and sheriffs to see that as less important than the specialist.

Table 5. Statistically Significant Curriculum Items Based on Agency Position

Factor	Item	Mean	F Factor	Significance
Agency Position	Aeronautical Decision Making	Chief/Sheriff - 2.33 Specialist - 3.09	8.13	0.005
	UAS Night Operations Techniques	Chief/Sheriff - 2.75 Specialist - 3.38	6.41	0.013

Other Differences in Ranking Curriculum Importance

Table 6 lists other agency characteristics with a statistically significant ranking of various curriculum items. One example includes how the participants felt about "risk management" based on the agency's number of sworn officers. Agencies with between 51 – 100 officers ranked risk management the lowest, with a score of 2.62. Large agencies with more than 200 officers ranked risk management the highest, scoring 3.41. No pattern was observed between the different groups, and no meaningful conclusions could be drawn from the findings. It was observed that agencies who issued their patrol officers drones prioritized “ethics with UAS” and “risk management” over those that did not. The items significantly differ with an F factor of 7.37 and 6.42, indicating a greater variance ratio.

Table 6. Other Differences in Ranking Curriculum Importance

Factor	Item	Mean	F Factor	Significance
Number of Sworn Officers at Agency	Risk Management	0-50 (3.24) 51-100 (2.62) 101-150 (3.00) 151-200 (3.00) 200 Above (3.41)	3.58	0.009
Does your agency regularly have patrol officers issued a drone for routine shifts?	Ethics with UAS Risk Management	Yes (3.56) No (3.08) Yes (3.34) No (2.90	7.37 6.42	0.008 0.013
How many certified drone pilots does your agency have?	How to collect thermal images from an infrared camera	0 (1.86) 1-5 (2.85) 6-10 (2.64) 11-15 (3.0) 16 Above (3.0)	2.54	0.045
	UAS tactics for crowd control	0 (2.17) 1-5 (2.98) 6-10 (2.69) 11-15 (3.09) 16 Above (2.0)	2.59	0.042
Approximately how many drones under 55 pounds does your agency own?	Mission Planning	0 (2.50) 1-5 (3.17) 6-10 (3.46) 11-15 (2.83) 16 Above (2.67)	2.57	0.045
	UAS night operation techniques	0 (2.50) 1-5 (3.32) 6-10 (3.77) 11-15 (2.67) 16 Above (2.0)	3.18	0.017

The survey asked the respondents how many certified pilots their agency had. The responses were grouped as indicated in Table 6. The mean ratings for the curriculum topics “How to collect thermal images from an infrared camera” and “UAS tactics for crowd control” were observed to be statistically different based on the number of pilots an agency had.

The survey included a question addressing how many drones the agency has. The respondents were categorized into five groups, as shown in Table 6. “Mission planning” and “UAS night Operations techniques” had mean importance rankings that were statistically significant. The mean values for each curriculum topic based on fleet size appear to have a bell-shaped distribution.

Table 6 provides the statistics of various curriculum items with statistically significant importance rankings based on agency characteristics. While this analysis was necessary, no meaningful conclusions could be drawn from any individual variance identified. The real value of this analysis was to show that no agency characteristics significantly deviated from the average rankings of the entire sample. No agency characteristic deviated from the sample mean on more than two curriculum items. As a result, the researcher feels the rankings are stable and not significantly influenced by agency size, experience, location, or population density.

Conclusion

Conclusion from Top Third Ranking of Importance

To derive actionable conclusions from our study, we analyzed responses from law enforcement officers regarding 31 proposed curriculum items for UAS integration. These items were systematically ranked from highest to lowest based on perceived importance and segmented into three groups for detailed analysis. The top tier, comprising items with an average ranking of 3.00 out of 4.00 or higher, underscores the priority areas within the law enforcement UAS curriculum.

Our findings distinctly highlight the paramount importance of regulatory knowledge. The item "Federal and state UAS regulations" emerged as the most critical, with a high ranking of 3.60, underscoring its essential role in the curriculum. Furthermore, the "concepts assessed with FAA Part 107 licensing exam" also received significant emphasis, ranking third highest at 3.41. These results demonstrate a strong consensus among the participants on the necessity for thorough regulatory training in UAS programs.

Our analysis further reveals that law enforcement officers deem operational skills in drone usage highly crucial. The survey differentiated between "basic flight skills," "intermediary flight skills," and "advanced flight skills," all of which were placed in the top third of importance rankings. Specifically, "basic flight skills" were rated highest at 3.58, followed by "intermediary flight skills" at 3.36, and "advanced flight skills" at 3.01. This pattern suggests a robust foundational curriculum demand for drone operational skills. However, the diminishing importance with increasing skill level indicates a lesser perceived return on more advanced proficiencies.

Moreover, the ability to conduct general missions effectively emerged as another pivotal training area. High rankings for curriculum items such as night missions, ethics, mission planning, risk management, and overwatch and command center operations, all scoring an average of 3.00 or above. This underscores the necessity for comprehensive mission readiness. This finding aligns with the need for robust regulatory and flight training, confirming that survey participants prioritize a curriculum that equips students with technical skills and prepares them to effectively undertake diverse operational roles.

Conclusions from Lowest Third Rankings of Importance

Three key findings surfaced from the items deemed less important in the study's survey. First, participants placed a low value on third-party certifications for verifying flight and UAS management skills, with these elements receiving some of the lowest ratings—2.07 and 2.06, respectively. This suggests a prevalent skepticism among law enforcement officers about the effectiveness of third-party certifications in demonstrating the practical skills required for UAS operations in their duties.

Furthermore, the domain of photogrammetry received low importance ratings. Despite its technical relevance, the associated skills - crime scene reconstruction, traffic collision reconstruction, and land surveying - scored between 1.98 and 2.66. This suggests that photogrammetry has less immediate applicability or perceived value in everyday law enforcement operations than in more direct drone operational skills.

Most notably, the relatively low importance assigned to counter-UAS techniques, with a ranking of 2.38, starkly contrasts with the urgency highlighted in the PERF 2020 Report. The report calls for significant actions against the threats posed by harmful drones, a sentiment that appears not to have thoroughly permeated the local law enforcement training priorities. This discrepancy suggests a gap between national security concerns and the training priorities at the regional level, indicating a need for enhanced awareness and integration of counter-UAS strategies into training curricula.

Limitations and Future Research

This investigation is subject to several constraints that merit careful consideration. First, the survey encompassed a modest cohort of 94 law enforcement officers, a sample size that may not capture the full spectrum of perspectives on drone curriculum needs. Additionally, the survey's regional focus was confined to the Southeastern United States, which may not reflect the nuances or requirements of law enforcement agencies in other areas. Moreover, relying on self-reported data could introduce a bias toward socially desirable responses rather than an unvarnished portrayal of the officers' true views. The study also did not account for the potential of on-the-job training as an alternative to formalized educational programs, which could provide significant insights into practical applications of drone technology in law enforcement. These limitations underscore the necessity for extended research in these areas to bolster the findings presented here and ensure a comprehensive understanding of the educational needs for drone technology in law enforcement.

Acknowledgements. The authors would like to acknowledge Clemson Applied Technology Laboratory (CAT Lab) and the Nieri Department of Construction, Development, and Planning at Clemson University for their invaluable support and resources throughout this research. Your contributions have been instrumental in the completion of this study.

Disclosures. The authors declare no conflicts of interest.

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Community College Partners with Industry to Train Next Generation of Water Operators

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Abstract: In order to meet the dire need for certified water and wastewater operators, utilities in East Tennessee approached the local community college to develop a new degree program. In 2019, the Water Quality Technology (WQT) Program was launched at Pellissippi State Community College (PSCC), funded by the National Science Foundation. An industry advisory board worked closely with the faculty to provide students with the best academic and practical experience. Local utilities continue to support the program by hosting students for on-site field experiences and paid internships. Students who have completed the program have been employed in successful careers in the water and wastewater sector prior to graduation, and those who have attempted the state certification exams have surpassed average pass rates.

Keywords: water treatment, wastewater treatment, program implementation, program design, small programs

Introduction

Nationwide, water and wastewater operators are set to retire without a strong pipeline to fill their essential positions [1]. In Tennessee, up to 50% of the current workforce is set to retire within the next five to ten years, amounting to over 1,500 open positions [2]. Consequently, several utilities united to approach PSCC, the largest community college in the state. The utilities asked PSCC to create a new program specifically designed to train water and wastewater operators to help fill the void left by the retiring workforce. With funding from the National Science Foundation, PSCC created the WQT Program. Working in coordination with an advisory board, the college designed an interdisciplinary program to prepare students for the rigorous state certification exams, which historically have low pass rates. The program includes site visits and field experiences every semester and culminates in a 90-hour internship and capstone project. The inaugural cohort of students began in the fall of 2019, and more than a dozen graduates of the program are already fulfilling these much-needed roles in East Tennessee.

Methods

PSCC gathered an advisory board that included a dozen utility stakeholders to develop the courses and experiential learning for the WQT program. The original advisory board was comprised of individuals who approached the college with the proposal to begin the WQT Program: members of the Tennessee Association of Utility Districts (TAUD) and managers of utilities in the local area. The current advisory board consists only of managers and operators from local utilities. Based on the board's advice, the Deans of Engineering and Natural and Behavioral Sciences collaborated to choose the best course offerings available at PSCC to educate a well-rounded operator. By creating an interdisciplinary curriculum spanning natural sciences, mathematics, engineering, and water treatment, the chosen courses aim to meet the critical skills needed in a successful operator: theoretical and practical knowledge of water treatment, strong math skills, and professionalism.

A Program Coordinator, who is also the author of this current submission, was hired to develop course content and teach all nine water-specific courses. The niche nature of the subject presented challenges for the Program Coordinator, including time-intensive material creation with few traditional resources available. Past studies on small programs indicate that one barrier includes difficulty retaining faculty willing to perform all roles associated with running the program, in part due to heavy workloads or other implicit responsibilities within academia [3,4]. Additionally, the onus of voluntary but labor-intensive student mentorship, recruitment, and inclusion is placed on a small number of faculty members; in the case of WQT, one [5,6,7,8].

Table 1. Interdisciplinary Course Requirements for the Water Quality Technology Degree

Water Quality Technology	STEM Theory	Professional Skills	Technical Skills
Orientation to Water Operations*	Precalculus Algebra	Leadership Development	Industrial Safety
Regulations & Compliance	Precalculus Geometry	English Composition I	Fluid Mechanics
Water Facilities & Maintenance*	Chemistry I	Business & Technical Writing	Computers in Engineering
Water Treatment I*	Chemistry II		
Water Treatment II*	Microbiology		
Wastewater Treatment I*			
Wastewater Treatment II*			
Applied Skills for Operators Technology			
Water Quality Capstone*			

**Courses with substantial field experience component*

Program Outreach and Structure

PSCC collaborated with local utility representatives to create a program that was attractive to those already employed in the field. Recognizing the critical shortage of certified operators, many utilities offer tuition reimbursement for employees pursuing the WQT degree. As an added incentive, graduates often qualify for promotions and salary increases, making the degree a valuable investment for both the employer and the employee.

The degree program offers a unique advantage to state-certified operators: credit for their existing experience and certifications. Operators holding the highest level of state certification receive credit for all required water quality courses, while those with relevant safety training can receive credit for the industrial safety course. This accelerated path allows completion of the degree with roughly one year of coursework. To accommodate a variety of schedules, courses are offered online and at night. This flexibility, combined with the program's accessibility across the state, removes geographical barriers to degree completion for working adults.

Industry Engagement

The active advisory board ensures curriculum and course offerings align with industry demands, producing the most well-rounded and prepared graduates. This includes updating textbooks, bringing industry experts to share their experiences with students, and developing new courses to meet direct industry needs. For example, hiring managers on the advisory board identified two program-specific courses in the curriculum, Small Water Systems and Advanced Wastewater Treatment, that were not as useful for practical job applications as had been previously anticipated. These two courses were discontinued and replaced with an Applied Skills course, which addresses the most common requests from managers: applied laboratory experience, professionalism, computer skills, and advanced water-industry mathematics.

Integration of Employability Skills

PSCC has supported the program from its inception. A testament to this commitment is the WQT Leadership course taught by the college's Vice President, which is the only course she teaches each year. Coupled with the students' technical knowledge of the industry and treatment processes, including these managerial skills as part of their education is invaluable. The Program Coordinator reinforces professional development skills throughout the WQT curriculum. Students begin building essential job-search tools early in the program with a graded resumé assignment, which students utilize as they network at technical conferences and attend site

visits. Professional communication, including email etiquette and interview techniques, is also emphasized. In their third semester, students go through a mock job application and interview process, where advisory board members serve as the interviewers.

Integration of New Technology

The Program Coordinator also looks for the latest technologies in the industry to expose students to new lab techniques and computer software. Although PSCC's campus does not have its own on-site water or wastewater treatment facilities, the author's integration of theoretical technologies allows students to prepare for abnormal conditions before encountering them in real life. By networking within the ATE community, the author was introduced to an Augmented Virtual Reality (AVR) app developed at Eastern Iowa Community College. The AVR allows students to visualize and interact with equipment not available on campus, such as troubleshooting atypical issues in large pumps or simulating field sampling techniques. Additionally, in the Capstone Course taken in the last semester prior to graduation, the Program Coordinator employs the computer modeling software developed by Hydromantis: GPS-X. Students can "build" a treatment facility in the computer program and simulate various theoretical scenarios. This allows students to predict and prepare for emergency situations without having to experience them first-hand.

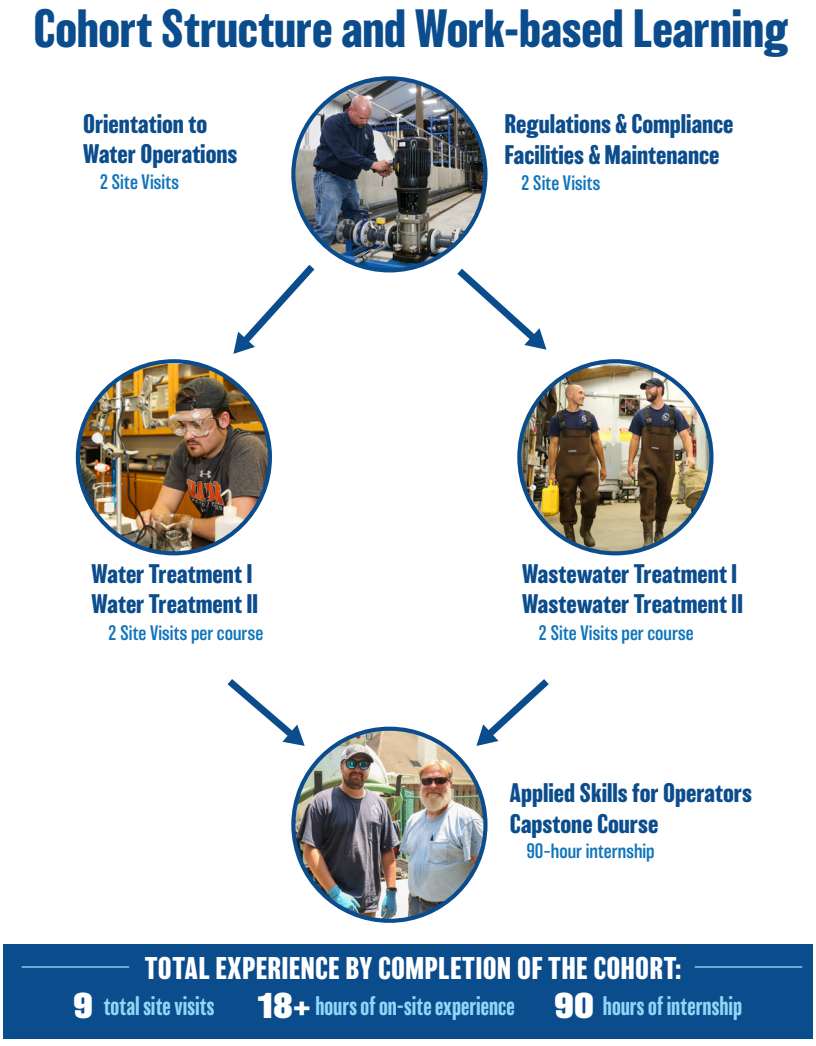

Fig. 1. Progression of the WQT Courses and Included Field Experience

Results and Discussion

The WQT Program successfully completed its fifth year and has already demonstrated a positive impact on the water and wastewater industry by cultivating a skilled workforce. Nearly two dozen students previously unfamiliar with the industry have secured full-time employment and advanced within their organizations as a direct result of the concepts learned through the program. Hiring managers consistently praise WQT graduates entering the workforce for their higher level of skills and knowledge compared to their peers. The program's blend of theoretical coursework and extensive site visits equips students with a comprehensive understanding of water and wastewater treatment processes.

To date, the WQT Program has produced 13 graduates with an Associate of Applied Science degree in Water Quality Technology. A remarkable 93% of students (13 out of 14) have passed the Grade III or Grade IV Tennessee Certification in either Water or Wastewater Operations, greatly exceeding the state's average pass rate of around 20 to 30 percent [9]. To expand the program's reach, the author is exploring ways to make the program more accessible to those outside the local area, including increasing online course offerings. Some early challenges in implementing this expansion include difficulty in coordinating field experiences and addressing lower student success rates observed in asynchronous online STEM courses like chemistry, microbiology, and mathematics.

Conclusions

Despite initial challenges faced by the Program Coordinator relating to workload, material creation, and coordination of field experiences, utilities have benefited from the program's achievements through the hiring of qualified operators. The key to the program's success is relying on the expertise and support of the local water industry, particularly the Advisory Board. By training the next generation of water and wastewater professionals, PSCC is directly addressing a critical community need. This program serves as the pipeline needed to replenish the workforce and train the future leaders of the water industry.

Graduates of the two-year WQT degree program have their choice of well-paying careers not only in the local area, but also nationally. Perhaps most importantly, students are feeling fulfilled in their careers and have found a rewarding way to support their families and protect their communities.

Fig. 2. Students Lisa Sforza (left) and Jaden Goodman (right) are shown at their treatment facilities. Both passed the highest levels of state certification in their field.

Acknowledgments. This work was supported by the National Science Foundation (NSF) under award 1800789.

Disclosures. The author declares no conflicts of interest.

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Establishing and Engaging Collaborative Partnerships for Sustainable Technician Training Project Implementation

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Abstract: Collaboration with industry and other partners is a critical component of many Advanced Technological Education (ATE) projects, including the Central Coast Partnership for Regional Industry-Focused Micro/Nanotechnology Education (CC-PRIME) project at Santa Barbara City College (SBCC). This project includes partnerships and collaborative efforts with various entities, including local industry, national ATE Centers, and regional business and community partners. Challenges around identifying partners and defining structures to engage with them on an ongoing basis are described as important lessons learned from the initial development of the project. Tools and resources are outlined for successful project implementation by connecting with industry through an existing and heavily utilized shared cleanroom facility, and National ATE Centers and other ATE-funded projects for access to shared resources and tools. Additional partners for potential awareness, outreach, and recruitment efforts are identified, and efforts to sustain collaborative initiatives with all project partners are outlined in the context of further expanding the local micro nanotechnology training opportunities in the future.

Keywords: micro nano technology, collaborative partnerships, industry partners, technician training, local workforce

Introduction

The Central Coast Partnership for Regional Industry-Focused Micro/Nanotechnology Education (CC-PRIME) [1] is a collaborative project between Santa Barbara City College (SBCC) [2], the University of California Santa Barbara (UCSB), and local industry partners. This National Science Foundation (NSF) Advanced Technological Education (ATE) project seeks to address a regional workforce development challenge, working with industry partners to develop technician training for micro nano technology and semiconductor manufacturing jobs in the area [3]. The initial project goals are to (1) build industry visibility and relations within the community, (2) provide SBCC students and faculty with training and experiences in manufacturing at the university cleanroom facility, and (3) to create a student educational pathway to acquire semiconductor manufacturing jobs, incorporating industry input [1].

The National Science Foundation ATE program targets improvements in the education of science and engineering technicians [4]. While its focus is on two-year institutions, projects are strongly encouraged to include partnerships between academic institutions and industry, as well as economic development agencies, in order to be initially successful and sustainable in the long run beyond the grant project period [4].

Working early on and directly with companies operating in the fields for which a particular training program is designed is critical for its long-term success. One main metric for success is if many of its graduates ultimately obtain jobs in the field or are able to utilize their newly obtained skills in their existing jobs, as may be the case for upskilling participants. That, in turn, means that the training must meet industry needs to a large extent. In order to ensure that, industry input and collaboration are needed from the beginning of the project [5], [6]; this includes the identification of need, a potential gap analysis, and the curriculum development or corresponding modifications [3], [7], [8].

The project design and its initial implementation involved collaborating with various partners, including industry, a cleanroom facility at a nearby four-year institution, national ATE Centers, and regional community partners [1]. Approaches to identifying and working with partners from industry and other entities that have been encountered during the development of this micro nano technology cleanroom training program are described below. This includes experiences working with various collaborators, their impact on the project, lessons learned, and efforts around sustaining those collaborations.

Methods

Identifying Potential Industry Partners

Identifying potential industry partners for a workforce development project can be challenging. Some companies may be identified easily due to their main line of work being directly aligned with the targeted workforce area, while others may be less obvious [9]. They may need technicians with similar skills, even though their main line of work may be in a different field or not directly related to the specific employee's job duties [3], [10]. For example, a company that does not manufacture or develop devices in cleanroom environments may still benefit from some of their technicians having an understanding of general cleanroom protocols and procedures for semiconductor manufacturing as they work with others in the field. In addition to leveraging existing collaborations or partnerships with industry, utilizing third parties in identifying new potential industry partners can be a valuable strategy. Some companies may be utilizing an existing shared facility or may be organized around industry trade or business organization, which could facilitate initial outreach to potential partners [1], [11], [12].

Once a particular company has been identified as a potential partner, reaching out to the right person at that company is an important next step. For most projects that involve curriculum development or modification, a subject-matter expert with corresponding expertise in the required knowledge, skills, and abilities is critical. General human resources contacts may be able to provide contact information for relevant subject-matter experts [5], [6], [8], [13].

Potential Partnerships Beyond Industry

Beyond the critical industry partners that are looking to hire trained technicians, additional partners may be able to provide valuable input into and assistance with the project. Existing government-initiated or government-funded centers, such as the Micro Nano Technology Education Center (MNT-EC) [14] or the Support Center for MicroSystems Education (SCME) [15], or industry organizations could provide assistance with gaining access to existing curriculum resources from other institutions. Local community business organizations, such as the Chamber of Commerce, could provide a venue for broader input and assist with outreach or community awareness campaigns [16]. Regional collaboratives assisting with employment data collection or corresponding data analysis could provide long-term employment data and projections that could be leveraged to validate an initial workforce development need and a potential subsequent project expansion [7], [17]. Regional or county workforce development boards or similar local government organizations could support a project in a similar way [18]. Once the initial training curriculum has been developed and implemented, local high school partners can play an important role in continuing efforts to create awareness and in outreach and recruitment efforts.

Collaborating and Interacting with Partners

As is the case with any partnership or collaborative effort, all parties involved will need to see value and gain at least some benefit for it to be sustainable long-term. While some activities may be more valuable to some partners than others, all partners will likely want to see ongoing value from participating in the project or be able to link activities to the objectives supporting the long-term workforce development goals [13], [19].

Most technician education projects will include some level of curriculum development or modification that requires industry input. Gathering critical industry input in an efficient manner and implementing it into the curriculum, as appropriate and necessary, can aid in supporting the common workforce development vision among the project partners. Regular check-ins with industry partners on their desired level of involvement in potential activities beyond curriculum development and their possible time commitment to the project can further support the collaborative nature of the interaction [13]. It is important to keep in mind that different

partners may come with different expectations or different constraints to the partnership, which may impact their level of involvement. The size of the company, the number of support staff or their availability, or additional constraints could impact overall time commitment or the availability for certain additional activities, such as tours or networking events [13].

Effective and efficient collaboration with industry partners is critical for the ongoing success of the project. One common model for facilitating regular interaction between faculty and industry representatives is an Industry Advisory Board (IAB), in which industry representatives and subject-matter experts advise the faculty on the desired learning outcomes and provide additional input into the project, as needed and desired. A variation of this advisory board model is the Business and Industry Leadership Team (BILT), which provides for deeper industry involvement in program leadership [20]. In this co-ownership model, industry members co-lead and co-design the project components and curriculum, potentially leading to stronger faculty-industry relationships and stronger student-industry connections [20], [21].

Common Goals and Challenges

An initial needs assessment carried out by an external evaluator can assist with identifying some of the specific industry needs [3]. This can be particularly helpful with respect to developing common ground for additional training needs, i.e., developing training that serves industry well without being too narrowly focused on a particular company's specific requirements that could otherwise be addressed through initial on-the-job training [10], [22]. Students will ultimately be best served completing a pathway that can lead to jobs with several industry partners rather than being specific to one or a very limited number of companies [3].

In addition to agreeing on and developing curriculum that will serve a sufficiently broad set of industry partners, other common challenges could be around employment growth predictions, community awareness and outreach efforts, and student recruitment avenues.

Results and Discussion

Identifying Potential Industry Partners

The CC-PRIME project evolved out of ongoing interactions between community college and university faculty and staff, and local industry representatives through the Center for Science and Engineering Partnerships [1], [23]. All of the project's initial industry partners are regular users of the NanoFab facility at the University of California Santa Barbara, resulting in regular and ongoing interaction between NanoFab staff and industry representatives [11], [12]. In addition, long-standing collaborative projects between university staff and community college faculty provided for initial conversations around micro nano technology-related job opportunities in the area [23]. It is in these conversations that NanoFab users from industry relayed their need for technicians in micro nano technology and semiconductor-related fields, along with their difficulty in recruiting corresponding talent, both from within as well as from outside of the region [3], [23], [24]. These connections, in turn, enabled initial conversations between industry representatives and faculty around industry's training needs. The project's initial IAB then grew out of industry users of the NanoFab facility, which facilitated finding additional industry partners with a vested interest in aiding in the development of regional technician training. Most of these companies are small- to medium-sized; some have their own cleanroom facility, while others rely exclusively on the NanoFab facility for cleanroom use [1]. Even some of the larger companies with their own cleanroom facilities have connections to researchers and staff at the University of California Santa Barbara who are known to NanoFab staff [11], [12]. While these companies operate in varying fields, they share a common need for cleanroom technicians or micro nano technology/semiconductor technicians. Recruiting these companies via the shared NanoFab facility greatly facilitated the initial engagement, which otherwise would have required personal connections or individual outreach to each company.

Table 1. Main Project Partners and Roles

Project Partner	Acronym	Role/Facility
Santa Barbara City College [2]	SBCC	Community College; Project Lead
University of California, Santa Barbara [25]	UCSB	R1 Research Institution; Project Subawardee
NanoFabrication Facility [12]	NanoFab	UCSB Research and Industry (Shared Use) Cleanroom Facility; Technical Project Staff

Potential Partnerships Beyond Industry

The initial training that was developed for the CC-PRIME project includes significant hands-on training time in a cleanroom, thus requiring access to a facility that most community colleges do not have. While the NanoFab facility at the nearby University of California Santa Barbara campus was instrumental in identifying industry partners, it is exclusively used by university researchers and industry, and is not available for instructional use beyond tours [11]. With the aid of NanoFab staff and the Center for Science and Engineering Partnerships, two additional cleanroom facilities at the university were identified that can be utilized for instructional purposes [26], [27]. Facility and staff availability for training then determined the most suitable facility to be at the California NanoSystems Institute located at the university. This model of building instructional facility access time into projects that require training in a specific facility that is not available at a community college can be effectively implemented by utilizing appropriate facilities at nearby 4-year institutions [1], [23].

In developing and implementing an Advanced Technological Education (ATE) project, the greater ATE community can be an incredibly valuable resource. Existing ATE centers can be an excellent resource for connecting to existing programs in similar fields, sharing best practices, accessing previously developed curricula, and networking. The Support Center for MicroSystems Education (SCME) at the University of New Mexico was instrumental in initiating the CC-PRIME project by sharing existing curricula and best practices and providing training for project staff and faculty [15]. Additional curriculum and best practices resources were provided by the Nanotechnology Applications and Career Knowledge Center (NACK) at Pennsylvania State University [28]. The Micro Nano Technology Education Center (MNT-EC) at Pasadena City College provided valuable Knowledge, Skills, and Abilities resources, as well as faculty professional development and networking opportunities [14], [22]. Being able to take advantage of the resources available in the greater ATE community through these national and resource centers facilitated initial project development and implementation in significant ways [1].

The National Institute of Innovations in Technology served as another resource with respect to assessing the project's KSAs [22]. Its National Talent Hub portal enabled us to link course learning outcomes to required skills listed in local employers' job postings [10]. This information is then utilized to identify potential curriculum adjustments for current or additional training pathways.

Local business and community partners can provide additional benefits to a project targeting a new workforce development pipeline in the region. A local Chamber of Commerce can serve as a hub to connect to additional companies with similar workforce needs and assist with networking, community awareness, and outreach events. Joining the Santa Barbara South Coast Chamber of Commerce Tech Roundtable enabled project leads to network with potential additional industry partners, take advantage of marketing resources to improve visibility and community awareness, as well as pursue additional collaborative funding opportunities for scaling efforts [16].

Similarly, a Chamber of Commerce, a Workforce Development Board, or a related local or regional entity can assist with visibility, awareness, and outreach efforts, also connecting to additional community partners. For CC-PRIME, the Santa Barbara County Workforce Development Board allowed us to make connections to local community organizations working with non-traditional and re-entry students potentially interested in technician job opportunities in the micro nano technology sector [18]. Further support for scaling and pursuing associated funding efforts can also be obtained through a Workforce Development Board.

Once the initial course offerings are in place, ongoing student recruitment into the program may become more and more important. Depending on the type of course offerings and whether or not they are part of an existing pathway, forging new or growing existing relationships with local high schools can have a positive influence on student recruitment and pathway development, as well as overall visibility and outreach efforts. Depending on the local educational environment, including potentially existing collaborative agreements with school districts, availability of instructors and necessary facilities or equipment, one might be able to explore offering some career technical education courses as dual enrollment courses at local high schools.

Table 2. Additional Resource Partners and Roles

Resource Partner	Acronym/Location	Role
Center for Science and Engineering Partnerships [23]	CSEP University of California Santa Barbara	Collaborative Hub for Science/Engineering Education Projects
Education and Industry Working Partners Project [13]	WPP Bellevue College	National ATE Project
Micro Nano Technology Education Center [14]	MNT-EC Pasadena City College	ATE Center
Nanotechnology Applications and Career Knowledge Center [28]	NACK Pennsylvania State University	ATE Resource Center
National Talent Hub [10]	NTH National Institute for Innovation and Technology	Job Portal and Analytics Provider
Santa Barbara County Workforce Development Board [18]	County of Santa Barbara	Local County Business Services Provider
Santa Barbara South Coast Chamber of Commerce [16]	Cities of Carpinteria, Goleta, and Santa Barbara	Local Business Organization Network
Support Center for MicroSystems Education [15]	SCME University of New Mexico	ATE Resource Center

Collaborating and Interacting with Partners

For the development of the initial cleanroom training boot camp at SBCC, we followed an IAB model to work with our industry partners, mainly to align better with their available time and personnel resources and desired level of involvement than what would have been required for following a BILT model [20], [21]. Regular meetings with IAB members at least once per semester, with additional follow-up meetings in smaller groups or one-on-one, as needed, have proven to be important, especially in the initial project phase [13]. Facilitating the initial connections between industry and faculty and keeping IAB members in the loop on the project's progress and the implementation of their input was integral to having industry partners continue

to support the project [3]. The generated buy-in then allowed for additional collaboration and interaction, such as networking opportunities for faculty and students, industry facility tours, and industry participation in outreach and community events, such as the High Tech Pavillion at SBCC's Science Discovery Day. Some of our IAB members have been very active in participating in additional activities, while others have not, for a variety of reasons, such as other work demands, limited support staff availability, timing constraints, etc. Other industry partners, on the other hand, have exclusively participated in networking and outreach events or tours without actively participating in the IAB [29], [30]. We have found that engaging different industry partners at different levels, trying to meet their specific needs, and working with their constraints can result in different yet valuable and ongoing relationships that ultimately serve both partners, as well as the students and the community. Resources from the Education and Industry Working Partners project at Bellevue College have proven to be very valuable in developing and adapting these connections with industry. The Working Partners project's workshops and partnership models toolkits can be utilized to successfully frame some of this work [8], [13], [19], [31]. The recently developed Working Partners rubric can assist in gaining clarity on desired levels of industry involvement and in assessing the ongoing engagement appropriately [13], [32].

In addition to the Working Partners models and resources, our external evaluator's initial needs assessment with industry partners provided valuable insight, not only into their employment and associated training/ curriculum needs but also into their desired level of participation in the project [3], [19].

Common Goals and Challenges

The initial needs assessment identified significant overlap in technician training needs for the various industry partners, as well as common challenges with regard to hiring technicians [3]. Technician-level training did not exist locally prior to the development of the initial cleanroom training, and recruiting and especially retaining technicians from outside of the area has proven very difficult due to the overall lack of available, affordable housing in the region [7], [17], [24]. Despite the significant overlap in training needs among IAB members, building consensus for an initial training that would serve the local micro nano technology industry broadly without going into training on specific instrumentation desired by one or very few companies proved challenging. Being able to draw from existing resources with long-standing track records of successfully training students in broadly applicable concepts and curriculum, such as the available SCME curriculum, ultimately aided in generating the necessary buy-in and consensus among IAB members [3], [15], [22]. The external evaluator’s industry needs assessment also aided in highlighting the commonalities among industry partners’ training needs [3]. As additional training modules and courses are being developed, some of the more specific training asks can be explored further.

In cases where industry partners are primarily small, start-up, or newly established companies, awareness in the local community about these companies and associated job opportunities can be lacking. Santa Barbara County is such an example. It hosts more than 45 companies, most of which are small to medium-sized and which operate in the micro nano technology and semiconductor application space [1], [7], [9], [33], [34]. At the same time, some of the larger companies that operate in the defense application sector have been in the area for a long time and are relatively well-known in the community; most of the smaller ones that are relatively new are not. There is a significant lack of awareness in the community about these companies that operate in the integrated photonics, infrared, microelectronics, microfluidics, biotechnology, and medical imaging fields, and the job opportunities, especially at the technician level, that are available [1], [9]. As additional training and student pathways are developed, this lack of awareness is likely to lead to recruitment challenges, especially in the early stages of program roll-out. Being able to draw from industry partners who are willing and able to engage in networking events and facility tours for faculty and students, as well as larger outreach events to the community, has helped with increasing awareness about these companies and associated job and career opportunities [29], [30]. Collaborating with community partners, such as the local Chamber of Commerce and the County Workforce Development Board, has provided additional opportunities to engage in community outreach and awareness campaigns and events [16], [18], [35]. Once additional student pathways and training are implemented, the next step is to take these outreach and awareness efforts to the local high schools, again drawing on industry partners to assist.

Additional or ongoing funding may be necessary to carry out some of these outreach and awareness efforts, along with additional curriculum and student pathway development, as well as potentially emerging equipment needs. Being able to show strong industry support is critical for additional funding proposals to address some of these challenges. In addition to our strong industry partner support for obtaining subsequent funding,

some of the mentioned additional partners, such as the Workforce Development Board and the Chamber of Commerce, have proven to be valuable partners in pursuing subsequent external funding opportunities, especially for outreach, networking, and marketing efforts [16], [18]. These entities have also been able to assist with gathering broader regional and state-wide data on future employment trends in related fields to further guide the conversations around expanding technician-level training efforts [7], [17].

Conclusion

In working with industry and other partners on developing and expanding this initial micro nano technology training, several important key points have emerged:

Leveraging and Expanding Existing Partnerships

Existing collaborations through the Center for Science and Engineering Partnerships facilitated the initial discussions to envision a workforce development effort such as the CC-PRIME program. The critical piece, however, that enabled us to implement the initial project with significant and broad industry buy-in and support was the connection to the shared NanoFab facility [1], [11], [12], [27]. Being able to draw on NanoFab staff expertise and direct connections to facility industry users had a significant positive impact on developing initial industry connections and getting the project started. It is clear that it would have taken much longer to develop these industry connections and relationships without access to a shared-use facility and its existing staff connections [1], [29], [30].

Being able to connect to an existing shared use facility, potentially located at a four-year institution, can aid significantly in making initial industry connections or expanding to additional ones.

Establishing and Engaging Partnerships beyond Industry

Beyond the critically necessary industry partners, valuable additional collaborators can be both at the local/regional as well as national levels. Existing national ATE Centers or long-standing resource/support centers can provide valuable curriculum and KSA resources, networking opportunities, and information about best practices. For our initial project, the Support Center for MicroSystems Education (SCME), the Micro Nano Technology Education Center (MNT-EC), and the Nanotechnology Applications and Career Knowledge Center (NACK) all provided extremely valuable information and resources, including curriculum and KSAs, to develop and implement the initial project phases [1], [14], [15], [22], [28].

Another national resource that is available through the NSF ATE community is the Education and Industry Working Partners project, which provides various tools, resources, and workshops around industry involvement and engagement efforts [8], [13], [19], [31]. The resources and guidelines available through the Working Partners project, along with some of its workshops, have been particularly helpful in assessing, documenting, and expanding our collaborative efforts with industry [32].

At the local and regional level, the Chamber of Commerce and the Workforce Development Board have been able to assist with visibility and marketing, outreach and recruitment efforts, along with making connections to additional industry partners, all of which have benefited these workforce development efforts in the community [16], [18].

Utilizing and adapting existing curriculum resources and known best practices is often a successful strategy when developing new training pathways. The existing ATE Centers have been shown to serve as excellent hubs for enabling these types of connections and providing corresponding resources. Existing city or county entities dedicated to overarching workforce development efforts can provide additional support at the local level, focused on increasing awareness and outreach.

Ongoing Partner Involvement

Regular interaction and communication with key partners are important for ongoing engagement [31]. Regardless of whether an IAB model or BILT model is followed, all partners in the collaborative effort will need to see ongoing value in their contributions, with some of their views getting incorporated into the project, if feasible and agreed upon [13], [20], [21]. The specific commitment level may vary from one partner to the next but should be aligned with the agreed-upon expectations and the project goals [13]. We have found an initial needs assessment, e.g., through an external evaluator, to not only aid in assessing training needs but also in developing a common framework for project participation from each partner [3]. In addition,

the Education and Industry Working Partners project has valuable tools, resources, and rubrics available for engaging industry partners, assessing and improving the partnership [8], [13], [19], [31], [32]. These resources and tools have had a significant positive impact on our ability to gauge and assess industry engagement with the ultimate goal of continuing the partnership long-term and sustainably.

Future Directions

Being able to leverage connections through an existing shared-use facility with industry, as well as curriculum, tools, and resources from national ATE Centers, have been critically important in being able to launch the initial micro nano technology cleanroom training and have reduced the planning phase significantly. Current efforts are in progress to expand this training to include more in-depth semiconductor fabrication technician training modules, in order to provide more robust pathways for local students to obtain technician jobs with our local industry partners. Resources and tools from ATE-funded projects, such as the SCME, NACK, and the Working Partners Project, are aiding in these efforts [13], [15], [28]. Ongoing collaborations with regional entities focused on workforce development continue to assist in local outreach campaigns to increase community awareness, all in an effort to broaden the micro nanotechnology training opportunities and corresponding student educational pathways in the region [16], [18], [35].

Acknowledgements. This work was supported by the National Science Foundation under ATE award 2100405 (Central Coast Partnership for Regional Industry-Focused Micro-/Nanotechnology Education (CC-PRIME)).

Disclosures. The authors declare no conflicts of interest.

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Analysis of the Impact of Train-the-Trainer Workshops on Robotics Education

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Abstract: In this paper, we present the results of the evaluation conducted for six train-the-trainer workshops on intelligent industrial robotics that were organized over three years from 2021 to 2023. The workshops targeted STEM faculty of community and technical colleges and high schools. The workshops included factory tours, industry speakers, and hands-on activities on industrial robots and vision system programming. Evaluation of the effectiveness of the workshops was measured using surveys at the end of the workshops, as well as pre-and post-intervention assessments. A six-month follow-up survey was conducted to assess the impact of the workshops on students. Results show that most participants reported that their knowledge of intelligent industrial robotics increased and that the knowledge gained from the workshops is applicable to their work. In addition to that, statistical calculations show that 3,572 ± 1,286 students were impacted by the workshops six months-to-one year after the workshop completion with a confidence level of 90%.

Keywords: industrial robotics, collaborative robots, train-the-trainer workshops, evaluation, Industry 4.0

Introduction

In 2020, and for the first time in history, the electronics industry became the largest market for industrial robots [1], taking over that position from the automotive industry [2], [3], which dominated the market throughout modern history. The most recent report by the International Federation of Robotics shows that the demand for industrial robotics grew rapidly, vertically and horizontally, and has become a major element in multiple industries (Table 1 below). Industries that were almost absent in the last 20 years have become major markets for industrial robots, such as the metal and machinery, chemicals, and food industries.

Table 1. Market Share of Industrial Robotics by Industry

Industry	2022 [4]	2016 [2] [3]
Electronics / Electrical	28.4 %	36.9 %
Automotive	24.6 %	41.7 %
Metal and Machinery	11.9 %	11.6 %
Plastics and Chemicals	4.3 %	6.5 %
Food	2.7 %	3.3 %
Other industries	28 %	~ 0 %

Intelligent industrial robotics, also known as collaborative robots, constitute one of the technologies that emerged as a defining feature of Industry 4.0 [5]. They are industrial robots that are equipped with sensory and perception systems that allow them to work collaboratively with human operators without the need for safeguarding. Despite the great potential for collaborative robots to improve production by as much as one million folds [6], [7], they have been shy in penetrating the industrial robotics market due to hesitation by industries to deploy the robots without safeguarding. Consequently, they only occupied 10% of the industrial robot market share in 2022 [8].

There are over 100 definitions of "Industry 4.0" that were reported in the literature [9], but they share the same set of enabling technologies that include, among others, advanced robotics, the Internet of Things (IoT), and 3D printing. A term that can be used interchangeably with Industry 4.0 is "Smart Manufacturing," which was more commonly used in the United States and was officially introduced in U.S. Congress in Congressional Bill S.1054. The National Institute of Standards and Technology (NIST) defines Smart Manufacturing Systems as systems that are "fully-integrated, collaborative manufacturing systems that respond in real time to meet changing demands and conditions in the factory, in the supply network, and in customer needs." One of the keywords in the NIST definition is "collaborative," which describes human-machine collaboration, one of the major applications of incorporating Artificial Intelligence (AI) in the industry [10].

One of the factors that contributed to the rise in the demand for industrial robotics by industries other than the manufacturing industry is the outbreak of the Covid-19 Pandemic, which resulted in a sharp increase in the need for industrial robots for no-contact material handling in applications beyond the manufacturing industry [11]. The main operational advantage of collaborative robots over traditional robots is the significant flexibility that can potentially be achieved through the less restrictive standard safety requirements. For example, for operating traditional robots, safety standard ISO 10218-2 requires robot safeguarding using physical barriers or electro-sensitive protective equipment or other means that are compliant with the standards IEC 61496-1 and IEC 62046 [12], [13], [14]. While for collaborative robots, the principal standard is the ISO/TS 15066 standard, which guarantees operator safety by limiting robot speed and distance and restricting the momentum of the robot such that any collision with an operator will not result in an injury without the need for any physical safeguarding [8].

Workshops and seminars are commonly used effective tools for faculty development and professional development [15]. One of the objectives of the workshops discussed here aims to answer the need for a trained workforce that can handle collaborative robots in advanced manufacturing. The train-the-trainer workshops that were developed included speakers from industry, industry tours, and hands-on training on collaborative robots and robot vision systems. The workshops were funded by the National Science Foundation's Advanced Technological Education program.

Methods

Six workshops were organized in the summer of 2021, the summer of 2022, and the summer of 2023. The target population for the workshops was educators of Science, Technology, Engineering, and Mathematics (STEM) in community and technical colleges and high schools, primarily in the states of Tennessee and Alabama, and neighboring states. Three workshops were offered in Chattanooga, TN, two in Bessemer, AL, and one in Smyrna, TN. The research was designated as exempt from IRB oversight by the Institutional Review Board (IRB) of the University of Tennessee at Chattanooga.

Each of the workshops was two days long and included speakers, factory tours, and hands-on training on intelligent industrial robotics. All training was conducted on-ground, a FANUC CR-7iA/L collaborative robot unit was the primary training unit that was used for the workshops at Chattanooga State and Motlow State Community Colleges, and a Sawyer collaborative robot by Rethink Robotics was the primary training unit used for the workshop at Lawson State Community College in Bessemer, AL. Other industrial robot units that were used or demonstrated during the training include Motoman HP3JC, ABB IRB 140, KUKA KR5sixx R650, and Fanuc S-430i. Pictures from the workshops are shown in Fig. 1.

Fig. 1. Photos from the Intelligent Industrial Robotics workshops that took place in the summers of 2021, 2022, and 2023 in Bessemer, AL (top), Chattanooga, TN (bottom left), and Smyrna, TN (bottom right).

To align with best practices, three methods were used to evaluate the effectiveness of the workshops:

1. Participants are directly assessed by pre-workshop and post-workshop assessment exams, which include assessments of technical knowledge and terms in the fields of industrial robotics and vision systems.
2. Indirect assessment through post-workshop evaluation surveys that included multiple-choice questions and open-ended questions.
3. Six-month Follow-up Survey to asses the impact of the workshops on students.

All evaluations were administered by the independent evaluator. To comply with federal requirements on research ethics, participants had the option to decline to answer any question on the surveys anytime [16].

To recruit participants, the project team advertised the workshops to local and regional secondary and post-secondary institutions nationwide through ATECentral, the project website, emails, and other means.

Results and Discussion

Effectiveness of Recruitment and Outreach Efforts

To measure the effectiveness of the outreach and recruitment efforts, Table 2 shows the location, state, educational level, and sex of applicants who applied to the workshop. As the table shows, a total of 93 applications were received from 13 states to participate in the workshop. A very large percentage of applicants indicated that they hold a PhD or a Masters degree as their highest educational level (76.3 % of total applicants). A small number of applicants indicated that they hold a high school degree as their highest educational level (6.5 % of total applicants). Females constituted 36.6 % of applicants. The results show that the recruitment efforts were successful in outreach to different groups.

Table 2. State of Residence, Sex, and Highest Educational Level of Applicants

Demographic	Number of Applicants
Applicants by State	Applicants came from 13 states: Alabama, Florida, Illinois, Indiana, Louisiana, New Jersey, New York, Ohio, Pennsylvania, Tennessee, Texas, Washington, West Virginia
Applicants by Sex:	
Female:	34
Male:	58
No answer:	1
Applicants by highest degree achieved:	
PhD or Doctoral	26
Masters +	45
Bachelors Degree	8
Associate Degree	8
High School Degree	6
Total:	**93**

Of the 93 applications that were received, 47 applicants participated and completed the workshops. The demographic distribution of the workshop participants is shown in Table 3 below.

Table 3. Demographic Data for Workshop Participants

Demographic	Number of Applicants
Participants by State	Participants came from six states: Alabama, Florida, Illinois, Indiana, Louisiana, and Tennessee
Participants by Sex:	
Female:	24 (51 %)
Male:	23 (49 %)
No answer:	1 (2 %)
Participants by Home Institution Type:	
Four-Year Institution	2 (9 %)
Two-year Post-Secondary Institution	20 (43 %)
High School	23 (45 %)
Participants by Race:	
American Indian	1 (2 %)
Asian	0
Black or African American	21 (45 %)
Hispanic	1 (2 %)
White	22 (47 %)
No answer given	2 (4 %))
Total participants:	**47 (100%)**

Table 3 shows that the number of participants from underrepresented groups in engineering (American Indians, Blacks, Hispanics, and Women) was considerably higher in the workshops than the national average for the engineering workforce.

The presence of these underrepresented groups in engineering fields continues to be a major concern in the United States. According to the American Society for Engineering Education (ASEE), in 2019 women constituted only 14% of the engineering workforce in the U.S. Racial and ethnic underrepresented groups (Native Americans/Native Alaskans, Hawaiian/Pacific Islanders, Blacks, and Hispanics) constituted only 13% of the engineering workforce [17]. In the workshops of this project, women constituted 51% of the participants. Furthermore, 45% of participants identified as Black. In 2022, a question on the veteran status was added to the workshop applications, for which four participants (8.5 %) identified as veterans.

It was shown in a previous publication [7] that the four underrepresented groups identified above constitute a smaller percentage of the workforce in engineering compared to their population ratios, as can be seen in Table 4, which shows data taken from the National Center for Science and Engineering Statistics (NCSES) for the year 2019.

Table 4. Underrepresented Groups in Engineering in the United States, Data for 2019 [7] [18]

Demographic Group	Percent of Engineering Workforce	Percent of U.S. Population
American Indian/ Native Alaskan, other races, and individuals with more than one race	< 1.60 %	1~ 2 %
Black or African American	5.4 %	12.5 %
Hispanic or Latino	7.9 %	18.5 %
Female	13.8 %	50.8 %

*Numerical values were taken from Tables 1-2, 9-2, and 9-3 of the NCSES report for 2019 and then converted to percentages.

Effectiveness of the Workshop Activities

In total 44 out of the 47 participants responded to the pre-workshop technical assessment exam, and 41 responded to the post-workshop technical assessment exam and survey. Results of the pre-and post-intervention assessments and the post-intervention survey are shown in Table 5. The results show that the average score of participants significantly improved after completing the workshops with an average score of 84 % compared to 23.1 % before going through the workshop.

Table 5. Results of Measurement Tools Used for Assessment of Workshop Effectiveness

Measurement Tool/ Metric	Total Responses	Average Score
Pre-Workshop Technical Assessment Exam	44	23.1 %
Post-Workshop Technical Assessment Exam	41	84.0 %
Post-Workshop Evaluation Survey Multiple Choice Questions:	**Total Responses:**	**Strongly Agree or Agree:**
My skills/knowledge increased as a result of participating in this workshop.	29	27
Workshop activities were appropriate and reasonable in the time allowed.	29	26

Open-Ended Questions: The surveys included open-ended questions to accurately capture participant opinions about the learning achieved from the workshops. Some of the comments received for the open-ended question: "What is your major takeaway from this workshop?" include:

- "More like this one to allow additional time to grasp skills."
- "The materials presented were broken down to assure understanding by all levels of knowledge of workshop attendees."

Comments received for the question: "Would you recommend this workshop to your colleagues? Why or why not?" include:

- "Yes, I will be able to integrate the materials in my classroom, and I gained community resources."
- "It opened my eyes to real-world experiences my students will face. It was a challenge to do and learn."

From the assessment tools and the post-intervention survey results shown above, and from the demographic data of participants shown in this section, we can conclude that the workshops achieved their objectives of introducing intelligent industrial robotics to STEM educators particularly from under-represented groups.

Assessment of the Impact of the Workshops on Students Using Statistical Tools

A follow-up survey was sent to the participants about six months to one year after completing the workshops to assess the long-term impact of the workshops. The term "impact" here is defined as the effect of the training that the faculty member received as a result of this project on their students. Therefore, only the students who were directly impacted by the trained faculty member in his/her classroom were of interest in the follow-up survey.

Five of the 47 participants responded to the follow-up surveys (10.6 %). The low response rate is attributed to several reasons, among which is that some faculty members may have changed jobs during the period between the end of training and the time the survey was sent. In addition to that, the surveys were sent by the independent project evaluator, with whom the participants only had electronic contact. Furthermore, research studies show that the recent trends in increased overloads of emails received per day have caused individuals to respond to a smaller fraction of emails per day with smaller responses [19], and response rates to surveys have generally been on the decline in the last few years and are observed across almost all disciplines [20]. Therefore, it was important for the follow-up survey to be short and consume the least amount of time possible to complete it. The survey's primary focus was to quantify the number of impacted students, as shown in reference [21]. A question was added to the survey in 2023 that asks for the classification of the institution to identify minority-serving institutions. Three of the five survey respondents indicated that their institutions are listed as Predominantly Black Institutions (PBI), which are defined as institutions with more than 40 % of their student body identifying as Black. One respondent indicated that their institution is a Historic Black College (HBCU). Four respondents indicated that they implemented what they learned from the workshops in their classrooms.

The total number of students impacted six months to one-year after the workshop was completed, as indicated by the survey respondents, was at least 379 students (one respondent indicated that over 25 students were impacted, another indicated that 300 were impacted, and the remaining three responses were: 54, "none", and "several"). Therefore, for the five respondents, and taking "several" to be equated to three, on average about 76.4 students with a standard deviation of 126.9 were impacted by each trained instructor six months to one year after completing the workshop.

To determine the total number of students impacted at a 90% confidence level, we apply the rules of mathematical statistics for a population size of 47 and a sample size of 5 respondents, which shows that the margin of error is 36%. Therefore, it can be stated with a 90% confidence level and 36 % margin of error that 80 % of the instructors that went through the training used the training in their classrooms.

Furthermore, it can be stated that the number of students per instructor impacted by the workshops is 76 students per instructor with a 90% confidence level and a margin of error of ±36% or that 95 students per instructor were impacted by the workshops but at a ±40% margin of error (this is due to the reduction in the sample size since four of the five respondents indicated that students were impacted). Consequently, using the average number spread over the larger sample approach, we can estimate with a confidence level of 90% that 3,572 students ± 1,286 were impacted by the workshops six months to one year after the completion of the workshop. The results of the statistical assessment are shown in Table 6.

Table 6. Impact of the Workshops on Students

Statistical Quantity	
Average number of students impacted per instructor	76.4
Standard Deviation	126.9
Total number of students impacted	3,572
Margin of Error	±36%
Confidence Level	90%

Challenges and Lessons Learned

Some of the challenges that were faced in this project include the high differences in levels of educators that participated in the workshop, the limit on the level of accuracy in collecting demographic data due to change in entries by the participants, the low response ratio for the six month follow up survey, and a limit on equitable access to training resources for some rare cases. For the first challenge, the trainers used hands-on exercises with different levels that were given simultaneously to participants. The second challenge arises because demographic data is collected when individuals apply to the workshop, and when they come to participate in the workshops. Some participants choose to answer on one of the two occasions and decline to answer (or change the answer) on the second data collection occasion. This discrepancy could be reduced by only collecting demographic data once in the application form for the workshops. One way to increase the response rate to the six-month follow-up surveys is to give the responsibility of sending participants survey links to the workshop trainers rather than to the project evaluator. Another way of increasing the response rate is to reduce the number of questions to one or two questions only or to provide an incentive for completing the survey.

Collaboration with Other Advanced Technological Education Communities

One unique aspect of this project was the collaboration with other centers and projects within the ATE community. The lead student on this work received training on technical paper writing by the NSF- Micro and Nano Technology Education Center (MNT-EC). The project evaluator and a Principal Investigator participated in the DIE in ATE program, a reflective action project created by the NSF- EvaluATE Center. The collaboration with the EvaluATE center resulted in an action plan that included, among others, conducting Diversity, Equity, and Inclusion (DEI) professional development training for the PI team and methods to capture accommodation needs for participants with special needs such as hearing or mobile impairment. For example, in some workshops, the hands-on training and lectures took place in rooms with high ceilings, which caused a hearing difficulty for participants with hearing impairment. To account for this challenge, questions on accommodation needs were added to the workshop application forms to help prepare proper and equitable access for all participants.

Conclusion

An assessment of the effectiveness of six train-the-trainer workshops on intelligent industrial robotics is presented here. The workshops included three parts: factory tours, industry speakers, and hands-on training on collaborative robots and robotic vision systems. The effectiveness of the workshops was measured using pre- and post-workshop technical assessment exams and a post-workshop survey that included multiple-choice and open-ended questions. In addition to that, the impact of the workshops on students was measured using a follow-up survey that was sent to participants six months to one year after completion of the workshops. A total of 93 persons applied to the workshops from over 13 states, of whom 47 participated and completed the on-ground workshops from six different states in the U.S. Voluntary Pre- and Post- workshop assessments and evaluations were completed to assess the effectiveness of the workshops. Results show that the participants' knowledge of industrial robotics significantly improved after the workshops, and the vast majority indicated that the training will be used in their home institutions. The estimated total number of students that were impacted by the workshops was 3,572 students ± 1,286 students with 90% confidence

Acknowledgments. This work was supported by the National Science Foundation- Advanced Technological Education program under award numbers 2000651, 2000776, 2000685, 2000682, 2325500, and 1841783

Disclosures. The authors declare no conflicts of interest.

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RCNET's Girls In STEM: Insights and Recommendations for Future Projects and Community College Programs

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Abstract: The labor market demand for individuals with STEM skills and degrees continues to grow in the United States and other countries. However, the gap between men and women in these fields persists, with fewer women participating in STEM education or pursuing STEM careers. For years, programs have been implemented nationwide in school and after school to engage girls, foster interest in STEM, and increase participation in STEM education. However, these programs are often grant-funded, time-limited, and lack long-term sustainability. In 2020, the Regional Center for Nuclear Education and Training (RCNET), housed at Indian River State College, launched a Girls in STEM program. This program initially engaged three high school girls to work collaboratively to research, create, and disseminate short videos interviewing women working in STEM. Now, as college students, they share their perspectives on the project, its impacts, and recommendations for the future of this work.

Keywords: girls in STEM, engagement, gender gaps, barriers, funding, sustainability, gender bias, community college, career development, advocacy

Introduction

Girls participating in Science, Technology, Engineering, and Mathematics (STEM) education play a crucial role in diversifying and enriching these career fields. Unfortunately, the historical gender gap in STEM persists, with fewer girls pursuing careers in these areas compared to boys [1-3]. However, efforts to encourage and support girls in STEM have been increasing, aiming to break down stereotypes and barriers that may discourage them from entering these fields. To better understand the barriers, this article centers on the experiences of three women who participated in the Girls in STEM Project through the Regional Center for Nuclear Education and Training (RCNET) (https://gonuke.org) from 2020-21. The Center, housed at Indian River State College in Fort Pierce, Florida, was established in 2011 with the support of NSF award #1104238 and re-supported in 2016 with award #160058 to address the critical nuclear workforce demands in a unified and systematic way [4].

In the Girls in STEM project, the girls interviewed women in STEM fields to create and disseminate videos via social media. They participated in multiple STEM education experiences to help raise awareness and build interest in STEM among girls their age. They also participated in panel discussions at several national conferences. This project builds on the success of STEM role model interventions described by González-Pérez et al. [5] but was led and implemented by the girls who participated in the program. This experience, in part, has motivated these girls, now college students, to become STEM champions and role models for other girls in STEM. In early 2024, these women shared their perspectives on STEM education during and after participating in the RCNET Girls in STEM Project. This article summarizes how these experiences shaped their decisions about education and careers, empowered them to teach others about the value of STEM, and strengthened their 21st-century skills. Building upon these experiences, recommendations are included for future projects and practical steps community colleges can take to expand opportunities for girls and women in STEM.

The work of programs such as Girls in STEM is critical in reducing and eliminating the gender gaps in STEM. This is important because, in the United States, the demand for employees in science, technology, engineering, and math continues to grow. According to the US Bureau of Labor Statistics, employment in STEM occupations is projected to rise by 10.8% between 2022 and 2032, creating a high demand for employees compared to the 2.8% growth in all occupations. Additionally, wages in these occupations are higher, with a median salary of $97,980 for STEM occupations compared to $44,670 for all occupations in 2022 [6]. Meeting this demand remains challenging, with only 32% of bachelor's degrees awarded in the United States being STEM majors [7] and a gender gap that has persisted for years [7, 8]. This gap is reported by the US Census Bureau [7] in STEM Bachelor's degrees, employment in STEM Fields, and earnings in STEM Fields, with women lower than men in every category (Table 1). Similarly, IPEDS [8] reports the bachelor's degree gap as it has persisted from 2012-2022 with little change (Figure 1). While overall degree attainment in STEM has increased, the gap between men and women persists [8].

Table 1. US Census Data Comparing Women and Men in STEM Fields[a]

US Census Bureau Category	Women	Men
STEM Bachelor's Degrees	24.7%	39.4%
Working in a STEM Field – Non-STEM Major	6.6%	20.5%
Working in a STEM Field – STEM Major	15.3%	37.1%
STEM Worker Earnings – Non-STEM Major	$76,230	$87,380
STEM Worker Earnings – STEM Major	$82,190	$98,870

[a]Adapted from US Census Bureau - From College to Jobs: Pathways in STEM (2021) [7]

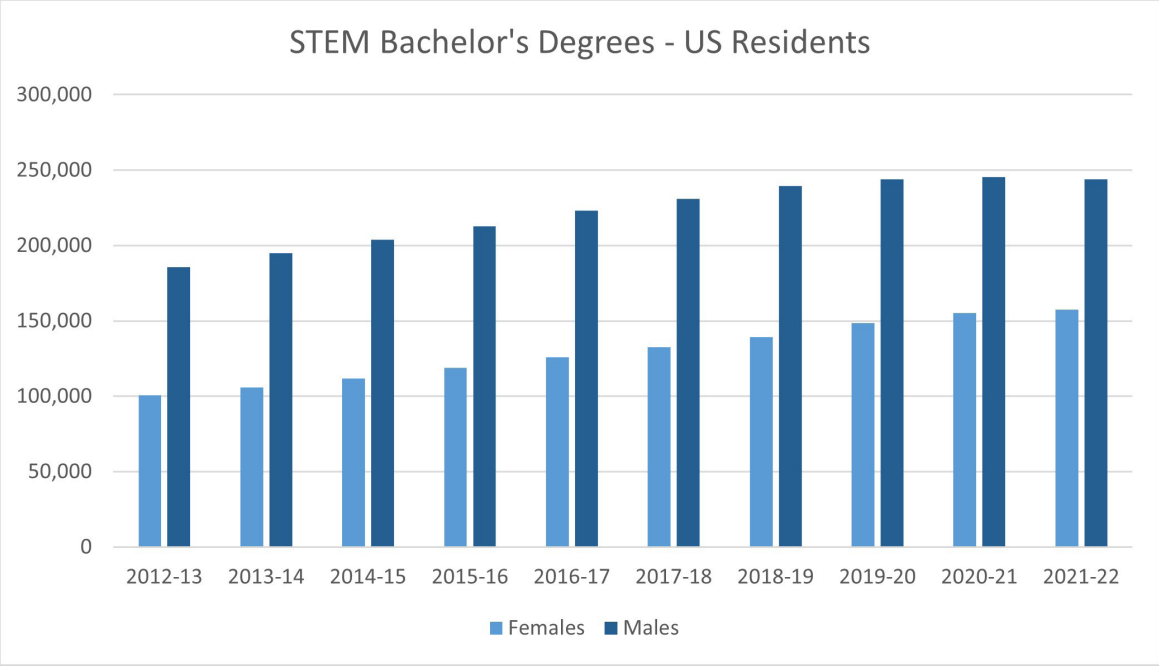

Fig. 1. IPEDS - Distribution of science, technology, engineering, and mathematics (STEM) degrees/certificates conferred by postsecondary institutions by sex of student: Academic years 2012-13 through 2021-22 [8].

Multiple factors can influence and support girls' participation in STEM, including external factors such as school culture, teachers, family, and peers [1, 2]. Internal factors can also motivate girls to engage in STEM education, including enjoyment, curiosity, self-regulation, and subject-embedded interest [1]. These factors, described as STEM self-efficacy by Falco and Summers, are improved by positive learning experiences [3]. Programs that create opportunities for exposure can enhance these factors by creating knowledge and opportunities for exploration. Many initiatives worldwide promote STEM education and careers for girls [1, 5]. These initiatives often involve outreach programs, mentorship opportunities, and highlighting the achievements of women in STEM fields to inspire and encourage young girls. Additionally, educational institutions and companies are working to create inclusive environments where girls feel welcome and supported in pursuing their interests in STEM.

The impact of RCNET's Girls in STEM project was deemed valuable with a small shadow, as noted in the Impact Allies RCNET 2022 Evaluation Report [4]. Additionally, the evaluation indicated that the project was valuable, but the time was limited to two years and was small-scale. Summarizing the biggest overall takeaway, RCNET PI Kevin Cooper noted that grants and other funding supporting this work must be restructured, recommending at least a 20-year funding cycle to move the needle in any given region. The initiative created opportunities for outreach to girls and tools for K-12 teachers to introduce STEM concepts, companies, and careers. Direct benefits to the participants were also identified, including professional development and skill building in creating videos and public speaking skills through sharing their experiences with others. Even though, as Cooper noted, conference audiences did not always believe the unfiltered/uncoached perspectives of the girls. With these results, the evaluation noted that efforts like this need continuous effort and funding to have an impact. It is not enough to create and post the content; engagement with girls must happen consistently with multiple communication points and engagement to create a high impact. Videos are one component that builds awareness, but that needs to be coupled with strategies such as direct outreach and in-person engagement to have a broader effect [4].

Methods

The RCNET's Girls in STEM initially engaged three high school girls in identifying and interviewing women working in STEM fields. These interviews were used to create video content shared on the YouTube platform RCNET's Girls in STEM channel [9] (https://bit.ly/4dlnHvv). Additional video content features interviews with the program participants and 2022 conference presentations at conferences, including the Advanced Technology Education Principal Investigators' (ATE PI) Conference and the High Impact Technology Exchange Conference (HI-TEC), providing an overview of the program and participant insights. The YouTube channel includes 15 videos that have nearly 400 views.

An interview in early 2024 with these initial RCNET Girls in STEM participants provided insights on the relatively low number of views, lessons learned about content distribution, and recommendations for future projects like this. Additionally, the qualitative interview analysis was coded using the ATLAS.ti (https://atlasti.com/) analysis software, identifying several themes about the successes, challenges, and lessons learned through this type of intervention and their direct impact on the three participants. ATLAS.ti is an online data analysis tool that utilizes OpenAI to customize data coding and insights. The results and discussion section summarizes those themes, recommendations for future programs, and strategies for community colleges.

Results and Discussion

The RCNET Girls in STEM participants (hereafter referred to as participants), now all in college, were interviewed in early 2024 and shared their perspectives on the project, success, challenges, lessons learned, and recommendations for the future of this work. These themes are summarized here and include observations directly related to the project and reflections on their broader experience as girls participating in STEM education.

Successes

Participants shared their experiences with adults and professionals in STEM fields through conference presentations at ATE PI and HI-TEC. They remarked that these experiences impacted adults by showing them the importance and impact of a young person's perspective in sharing the message about the importance of STEM versus the message from an adult. As the participants identified and connected with women in STEM occupations, they created and expanded their professional networks by building relationships with industry professionals, exploring careers, and managing projects. Even though the outreach and recruitment were challenging, the girls expressed that part of the positive impact of these videos was the in-person, on-site component that showed the women in their workplace, which made the work accessible and relatable.

As they learned more about STEM education and careers, the participants said they felt more empowered to share information with their peers, friends, and family. The experience helped them move from a basic understanding of STEM to a more confident stance that STEM is cool and interesting. They all continue to share information about STEM now that they are in college and consider that a positive outcome of the project. They are STEM champions, and in many ways, they have become lifelong STEM ambassadors and role models so critical to girls' success in STEM. While only one of the program participants is currently enrolled in a STEM major, all three expressed the value of this program in helping them understand their education and career path choices in and out of STEM. The research they did set them up to continue to ask questions, seek information, and make informed choices.

Challenges

Participants identified challenges specific to the project, including difficulties in cold-calling and recruiting STEM professionals to participate in the video interviews, researching and learning about different STEM fields, developing scripts with engaging content, and promoting the content. Noting that, while we are in the digital age, videos informing teenagers about education pathways are challenging to promote as this population utilizes platforms such as YouTube to destress and unwind. These challenges were also identified as learning opportunities, and they supported these participants in building confidence, fostering creativity, and expanding their knowledge. Additionally, as with many educational endeavors in 2020-2022, the COVID-19 pandemic had a limiting impact on this project. The participants noted that their inability to travel and access worksites hampered their ability to collaborate fully and produce the videos. While the three of them could collaborate virtually, they felt that creating the videos virtually would not have had the same impact.

Even though the participants feel empowered to share their knowledge about STEM with other girls and young women, they often find resistance or lack of interest, with some peers using words like "weird" or "nerdy" to describe STEM education. The participants attributed this to multiple factors, including the perception of math and science as challenging courses, resistance to asking for education support such as tutoring, the ongoing stigma of the difficulty of STEM courses, or gender imbalances in STEM courses. Teachers are essential in exposing young people to STEM education and careers. While most of their teachers supported their pursuit of STEM, participants noted that many teachers were busy with or focused on other things, including meeting students' basic needs, discipline issues, and the general day-to-day teaching work, making it challenging to provide meaningful support. The participants have also encountered gender bias in their STEM courses in high school and college, reflecting that they felt they had to work harder than the boys in their classes and do better on exams to be taken seriously. They felt this inhibits many women and girls from pursuing STEM education and careers. Participants noted that overall, schools struggle to emphasize STEM pathways enough, and opportunities like STEM clubs may be stigmatized, if they exist at all, discouraging participation.

Lessons Learned

Programs like this are intended to strengthen or deepen career exploration, leading to more informed choices about high school and college courses. The girls expressed that they did not even really understand what STEM was when they first started, having a vague idea going into the project but not a deep understanding of the different career paths. This project helped change that for them and gave them confidence as they entered college and made choices about their next steps. Two participants had previous exposure to STEM through their parents and understood what it meant, including a base knowledge of the equity gaps between men and women. Even so, they felt the project taught them more about the disparity between men and women in STEM fields and broadened their understanding of inequity in the workforce in general. In addition to the learning curve about STEM, there was a learning curve in building a professional network, outreach, and

creating video content. In projects like this, it takes persistence and effort to get the message out; even though the videos were posted on YouTube, the participants felt that more could have been done through other social media platforms to create a broader impact.

Recommendations for Future Projects

Based on their learning in this project, challenges, and experience in STEM education, the participants make the following recommendations for future projects.

- Engage students at a younger age through fun activities that expose them to STEM education and careers in an accessible way. Exposure in multiple ways breaks down barriers and supports participation in STEM education and careers. It is important to communicate this message in various ways, including print media representing girls and women engaged in STEM activities and sharing content across multiple social media platforms. While this project utilized YouTube, the girls recommended branching out into other platforms to broaden the reach of the content.
- Raise awareness by creating and distributing the message that not only is it okay but essential for girls to pursue STEM education and careers. This message has more value when peers deliver it and can reach students in ways that are different from the influence of the message delivered by teachers, parents, mentors, and other adults. While adults need to support this work, it has more power if it is led and delivered by girls. Their direct engagement in the research and outreach strengthened the authenticity of what these girls learned and gave them the credibility to share with their peers in a more powerful way than if the PI had done all the work. This representation and experience is critical for girls to see themselves in STEM.
- Organizational structures such as STEM Clubs can help educate students and normalize women in STEM by sharing examples that create exposure, providing mentoring, and connecting students with similar interests. These clubs can be an essential support structure if the message includes all interested, not just those with higher aptitudes. Schools must find a way to support these clubs and destigmatize participation in order to create the community needed to expand STEM participation.
- To build capacity and reach, programs like RCNET's Girls in STEM must be funded and sustained for a longer period. Exposure must start early (elementary and middle school) and be continuous. Doing this for a few years for a few classes is not good enough. Funding models and sustainability strategies must be built to sustain the work for much longer periods. If adequately funded, peer outreach, activities, and media can work together to support early and ongoing exposure, creating interest and participation.

Recommendations for Community Colleges

As Girls in STEM become women in STEM, community colleges play a vital role in recruiting and retaining students in two-year degree pathways that can lead to many next steps in STEM education and the workforce. If girls learn about STEM in elementary and middle school and participate in STEM activities in high school, there must be a seamless transition as they enter higher education. This transition can take many forms, including partnering with K-12 schools to create girls in STEM programs spanning K-14, intentionally creating pathways, and planning tools for a student's next step. Beyond these programs, there are specific things community colleges can do to support girls and women in STEM.

- Increasing the number of females participating in STEM education will require institutions to address academic sexism and harassment. Understanding the data is critical; institutions should analyze enrollment, persistence, and assessment data to identify equity gaps, including gender, and to help administrators and faculty identify areas for improvement [10, 11]. This data and other factors can be used to inform the development of a gender equity plan that focuses on inclusive recruitment and retention strategies, increasing enrollment of females in STEM programs, creating a welcoming environment, and empowering students (both female and male) to create long-term change in this gender gap [10, 11]. The plan should also include implementing professional development for faculty, staff, and students that raises awareness of gender and other biases in STEM [12]. Along with engaging students, the gender equity plan should examine hiring practices and create tools such as gender bias training for hiring managers/committees, identify strategies for recruiting and retaining female faculty, and empower female faculty as role models [11].

- Build a sense of belonging. Similar to the idea of a STEM club mentioned above, colleges can create a sense of belonging by building connections to support networks for women in STEM create spaces and structures that provide resources and support [10]. This can include strategies such as creating a STEM Navigator position or peer mentors, responsible for connecting students to trained STEM mentors, advisors, tutors, and wrap-around support services, including childcare, housing support, scholarships, and other assistance. Equally important is the cultural change of destigmatizing these resources. As the RCNET Girls in STEM participants noted, accessing help must be normalized, or it will just be another contributing barrier to the challenges for all students in these programs [11].
- Engage employer partners in this work. Work with local and regional employers to identify internships and other work-based learning opportunities that can provide girls and women with the opportunity to work alongside women in STEM careers and strengthen their professional network [10]. As part of this strategy, colleges should provide clear expectations about the experience, information to promote awareness of gender bias, and feedback mechanisms for students that provide data for continuous improvement [11]. In Career and Technical Education programs with a STEM focus, recruit women working in STEM fields to participate in Industry Advisory Committees. Engaging these women in developing and revising curriculum, recruitment and retention strategies, and job placement will support a welcoming and inclusive program culture for female students.

Conclusion

While there have been decreases in gender disparities in STEM education and careers, significant gaps persist. Interventions that provide exposure to and engagement in STEM education for girls are critical to decreasing and eliminating the gender gaps in education attainment, career pursuit, and wages in STEM careers. While adults such as parents, teachers, and mentors are essential to this work, empowering girls as leaders is also incredibly powerful. Programs like RCNET's Girls in STEM have the potential to build STEM champions, empower women to pursue STEM careers, and create life-long STEM ambassadors. These women become the adults who will continue to share the message about the importance of girls and women participating in STEM. As these projects are designed and implemented, it is crucial to learn from these programs, adjust approaches, and seek support for long-term funding to build the capacity needed to sustain and grow these efforts over time. Long-term sustainable funding and the seamless connection to higher education would strengthen these efforts and continue to change the culture of STEM education, moving toward a more inclusive ecosystem and increasing STEM participation overall.

Acknowledgements. This work was supported by the National Science Foundation (NSF) under award #1600558.

Disclosures. The authors declare no conflicts of interest.

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A Workflow for Creating Narration for Voice-Over Presentation Using Commercially Available Artificial Intelligence

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Abstract: This rapid communication presents a multi-step workflow for recreating existing course lectures using artificial intelligence (AI) and natural language processing (NLP). The workflow encompasses audio extraction from original lectures, transcript refinement via ChatGPT and human proofreading, audio regeneration through text-to-speech, closed captioning, presentation recreation with AI-generated content, and the development of supplementary resources like study guides and AI chatbots. The implemented approach leverages AI to enhance educational accessibility and personalization while balancing automation with human oversight. Potential benefits include tailored learning experiences and data-driven decision-making. However, ethical considerations surrounding AI biases, intellectual property, privacy, and misinformation must be carefully addressed before deployment. Overall, the workflow demonstrates AI's transformative potential in education.

Keywords: artificial Intelligence, Generated PowerPoint, human-created, online asynchronous, workflow

Introduction

When professors are unavailable to teach online courses, it creates a big challenge for educational institutions. This paper introduces a comprehensive AI-assisted workflow to revise or recreate course materials when the original instructor isn't available. The process involves extracting audio from existing lectures, refining the transcripts using speech recognition and natural language processing, and generating new audio through text-to-speech technology. Additionally, closed captions are added for accessibility, and presentations are recreated with updated audiovisual content. Supplementary learning resources are also developed using AI-generated content. This workflow allows institutions to effectively repurpose and refresh course materials, ensuring that students can continue learning seamlessly, both online and offline. The AI-generated materials can be customized to meet the needs of individual students or entire classes, enhancing personalized learning and improving understanding and knowledge retention. By addressing the issue of instructor unavailability, this approach highlights the transformative potential of AI and natural language processing in education. It paves the way for more efficient, accessible, and personalized learning experiences that keep pace with technological advancements in education.

Literature Review

AI in online education has come a long way, incorporating advanced technologies like web-based systems, humanoid robots, and chatbots to make learning more engaging and effective [1]. These advancements in AI and related technologies are transforming not just education, but also how professionals are trained and operate in various fields [2]. The combination of AI and wireless network technology has significantly transformed online engineering courses, showing how impactful AI can be on educational platforms [3]. Various AI applications, including chatbots, robotic assistants, and even holograms, are now helping teachers and students, enhancing the overall effectiveness of the educational system [4]. Such interactive tools not only enhance learning but also prepare students for real-world applications of their knowledge [5]. AI has also proven beneficial in streamlining processes within educational institutions. It has shown potential in optimizing student enrollment, improving retention rates, and providing organizational guidance and support to students [6]. In the K-12 sector, the idea of a "Turing Teacher" has been explored, focusing on the key features necessary

for effective AI-powered teaching tools [7]. Most AI technologies in education have centered on developing intelligent tutoring systems, virtual laboratories, and assessment tools, which help create more interactive and efficient learning experiences [8]. Regarding voice synthesis, research suggests that a speaker's enthusiasm can affect social cues, cognitive load, and learning transfer in multimedia learning environments [9]. Some studies recommend using human-recorded voices over synthetic ones for virtual agents, emphasizing the value of human-like interactions in educational settings [10]. Overall, integrating AI into online education presents numerous opportunities to improve learning outcomes, increase student engagement, and personalize the educational experience. By leveraging AI technologies effectively, educational institutions can create dynamic and interactive learning environments that cater to the diverse needs of learners.

Methods

A stepwise workflow has been created to generate AI voice-over presentations based on traditional human voice-over presentations. Figure 1 provides the steps for the workflow.

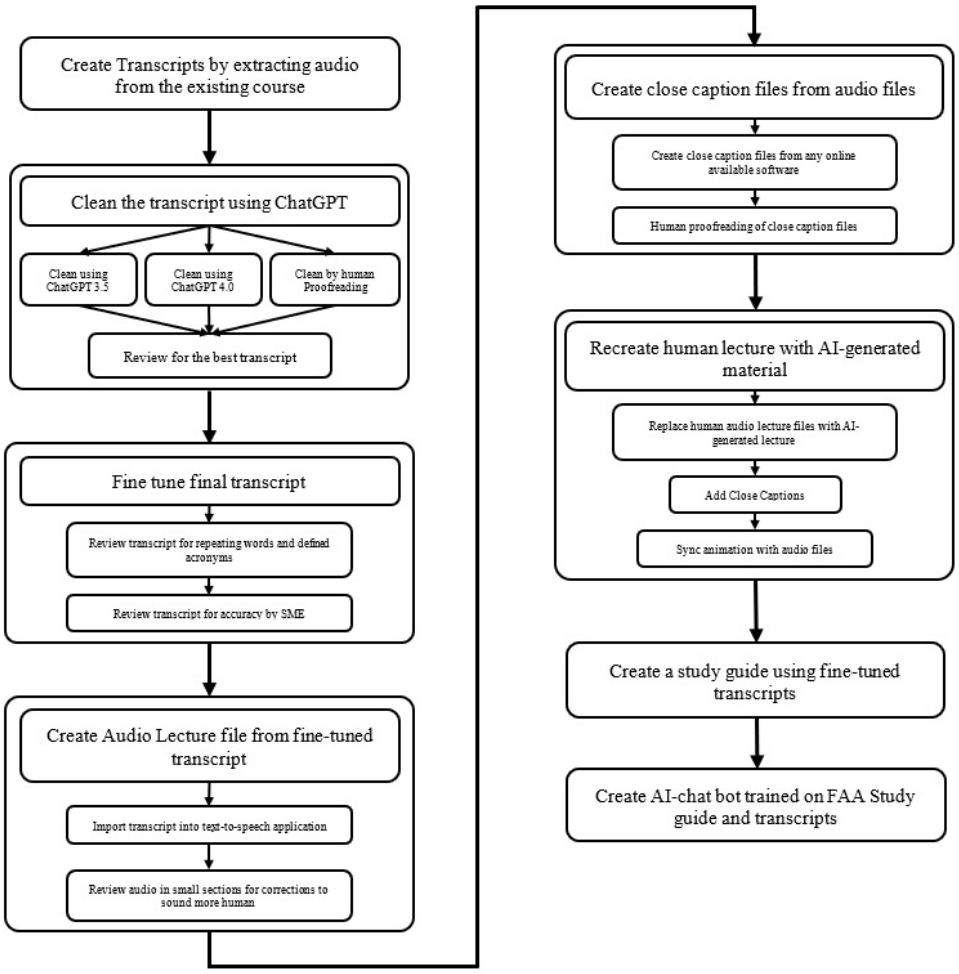

Fig 1. Stepwise workflow for creating AI-generated workflow

This workflow uses a multi-step approach to recreate course lectures with artificial intelligence and natural language processing techniques. The process includes (1) extracting audio from the original course and converting it into text transcripts, (2) refining the transcripts with ChatGPT 4.0 and human proofreading, (3) generating new audio files from the refined transcripts, (4) creating closed captions, (5) recreating the course presentations with AI-generated content, (6) developing a study guide, and (7) creating an AI chatbot based on the study guide.

1) Audio Extraction: Audio files were extracted from the existing course and converted into text transcripts using the online transcript converter (The researchers found KwiCut to be a reliable online transcript converter for this workflow, but many other converters are available commercially). The transcripts were then refined with the assistance of ChatGPT.

2) Transcript Refinement: Transcript accuracy and relevance were improved through two approaches: (a) Refining the transcript with ChatGPT 4.0, using a simple rephrasing prompt for each slide to enhance clarity and coherence while preserving core content and (b) Human proofreading of the transcripts for further refinement. This combined AI-assisted refinement and human oversight process ensured a highly accurate and contextually relevant transcript.

3) Fine-tuning and Proofreading: The final transcript generated by ChatGPT 4.0 underwent human proofreading to ensure content relevance and accuracy. Repeated words and predefined acronyms were identified and eliminated. A Subject Matter Expert (SME) thoroughly reviewed the transcript for accuracy before proceeding to audio generation.

4) Audio Generation: The transcript was imported into an online text-to-speech application. While many applications are available, the researchers used "ElevenLabs" for this workflow. ElevenLabs has over two dozen AI voices and can clone the original instructor's voice. This application was used to create the narration for the PowerPoint presentations. The researchers found that longer transcripts must be divided into smaller sections to avoid mispronunciation and synthetic slurring.

5) Closed Caption Creation: Closed caption files were generated using the online close caption generating software (KwiCut used in this study), extracting captions in the SRT format. The SRT files were uploaded into presentation software and manually refined to ensure error-free captions.

Results and Discussion

Presentation Recreation

The AI-generated audio files and closed captions were seamlessly added to the presentations, replacing the original human voiceovers. Animations were resynchronized with the new AI narrations to create a smooth and engaging audiovisual experience.

Study Guide and Chatbot Development

A detailed study guide was created using refined transcripts, providing students with valuable resources to understand the course material better. An AI-powered chatbot was also developed based on this study guide and the transcripts. This chatbot helps students get answers to their questions when the professor is not available. The researchers used AskAI to easily create a chatbot trained with this study's transcript and other relevant texts. Example: The following two hyperlinks demonstrate the original voiceover provided by the instructor [11] and the recreated presentation generated through AI [12] (Link #1: https://demo-drone-course.s3.amazonaws.com/course1/human-voice-presentation/story.html; Link #2: https://demo-drone-course.s3.amazonaws.com/course2/ai-voice-presentation/story.html).

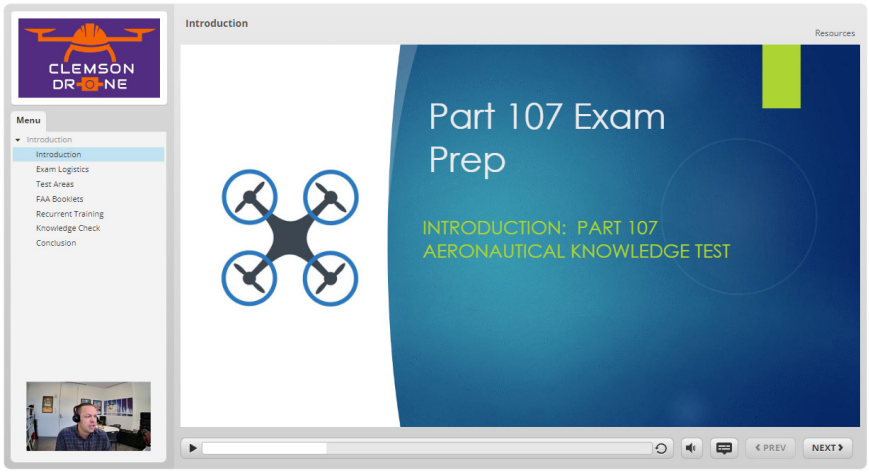

Fig. 2. (A) Human Tutor teaching in Zoom class

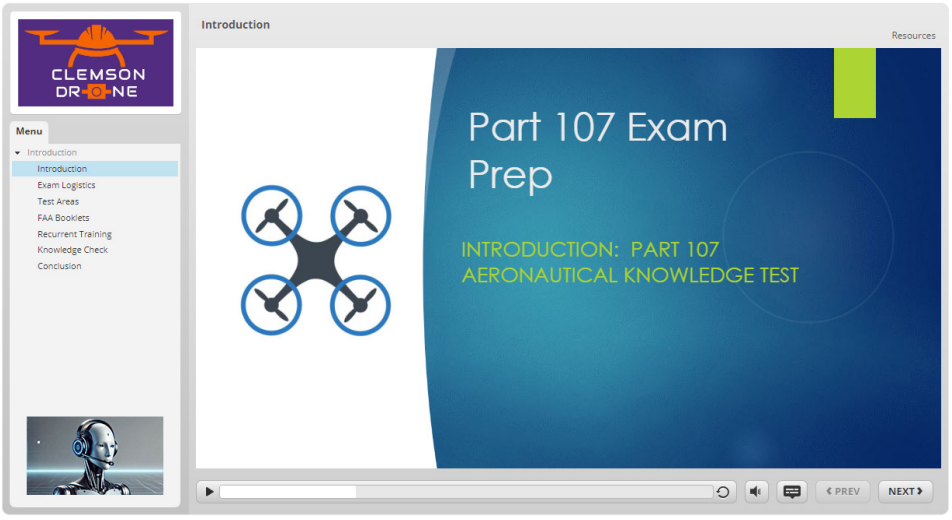

Fig. 2. (B) AI Tutor teaching in Zoom class

Benefits of the AI-Assisted Course Recreation Workflow

The AI-assisted workflow proposed here brings several benefits that can improve the educational experience and support informed decision-making. One key advantage is its ability to create personalized learning resources that match each student's unique needs, preferences, and learning styles. Using AI-generated content, materials like study guides and chatbots can be adjusted in real time, making learning more engaging and interactive for students. Another important feature is the workflow's capability to generate closed captions and audio narrations, making course content more accessible to students with different abilities and learning preferences. This ensures inclusivity in the learning process. Furthermore, including human proofreading and subject matter expertise guarantees that the course content is accurate and relevant, maintaining high standards of educational quality.

Potential Ethical Considerations

While the AI-assisted course recreation workflow offers many benefits, professors and educational institutions must consider the ethical implications before using this technology. One major concern is that AI-generated content could unintentionally reinforce biases or spread false information, especially if the training data or algorithms are biased. It's crucial to have strict measures to ensure that AI-generated content is accurate, fair, and unbiased. This includes having human oversight and fact-checking procedures to verify the content. Additionally, there are issues regarding intellectual property rights and giving credit to the original creators. As AI technology advances, questions arise about who owns and should be credited for AI-generated content. Institutions need clear policies to protect the rights of content creators and experts while acknowledging AI systems' role in content creation. Addressing these ethical considerations is essential for responsibly integrating AI into educational practices, ensuring that it enhances learning experiences while upholding fairness, accuracy, and respect for intellectual property rights. The AI-assisted course recreation workflow offers several advantages in terms of time, cost, and applicability.

Time-wise, this approach can significantly speed up updating or recreating course materials when an instructor is unavailable. Instead of starting from scratch, existing lectures can be quickly transformed into new, polished content. Cost-wise, while there may be initial investments in AI tools and software, the long-term savings could be substantial. Universities won't need to hire as many temporary instructors or spend as much time and money on manual content creation. In terms of applicability, this method is versatile. It can be used across various subjects and course types, making it a flexible solution for many educational institutions. The ability to easily generate closed captions and multiple language versions also increases accessibility for diverse student populations. However, there are considerations to keep in mind. The quality of the AI-generated content will depend on the quality of the original material and the effectiveness of the AI tools used. There may also be a learning curve for staff to become proficient with the new technology. Additionally, while AI can handle

much of the work, human oversight is still crucial. Time must be allocated for proofreading, fact-checking, and ensuring the content meets educational standards. Overall, this workflow presents an innovative approach to course content creation that could save time and money while maintaining educational quality, though it requires careful implementation and ongoing human involvement to be truly effective.

Conclusion

This research shows that AI can be a powerful tool for recreating and updating online course materials. By using a step-by-step process that combines AI technology with human oversight, universities can quickly refresh their courses when instructors aren't available. The key takeaway is that this method can save time and money while maintaining quality. It allows personalized learning experiences and makes content more accessible through features like closed captions and AI chatbots. However, it's important to remember that AI isn't perfect. Human experts still need to check the content for accuracy and fairness. There are also ethical concerns, like protecting intellectual property rights and avoiding bias in AI-generated material. Overall, this approach demonstrates how AI can transform online education, making it more efficient and adaptable. However, using this technology responsibly and in ways that truly benefit students and educators is crucial.

Acknowledgments. The authors would like to acknowledge Clemson University and the Clemson Applied Technology (CAT) Lab for supporting this research. It was only through their support that this research was possible.

The authors would also like to acknowledge the use of the AI large language model ChatGPT 4.0 in the drafting of this rapid communication for grammatical assistance. All facts and statements presented in this paper were independently verified.

Disclosures. The authors declare no conflicts of interest.

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Value-Creation Evaluation Framework for Evaluating NSF Advanced Technological Education Projects

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Abstract: Evaluation is a required component of NSF Advanced Technological Education (ATE) funded projects, consortia, and centers. Unfortunately, evaluation is an area of proposal development that may not receive adequate attention. Evaluation uses a language foreign to most technical faculty members, primarily classroom teachers with heavy teaching loads focused on their primary area of technological expertise. To help faculty overcome this language barrier requires putting evaluation in terms technical faculty can understand and creating a framework and visual model to help them develop a mental picture of the evaluation process. This paper describes a "Value-Creation Evaluation Framework" that has proven effective in evaluating ATE projects. A description of the application of this model to a series of ATE Projects will show how a well-constructed theory of change coupled with a value-creation evaluation framework can be successfully used for ATE project evaluation. This paper can provide insight into program evaluation for administrators, faculty, and grant professionals as they prepare ATE proposals for submission to NSF.

Keywords: evaluation, ATE, value-creation framework, Mentor-Connect

Introduction

NSF's ATE Program is 30 years old, having been created by the Scientific and Advanced-Technology ACT of 1992 with initial federal budget appropriation in 1993. The mission of NSF's Advanced Technology Education Program, as stated in the current ATE solicitation (NSF 21-598), is:

> "With a focus on two-year Institutions of Higher Education (IHEs), the Advanced Technological Education (ATE) program supports the education of technicians for the high-technology fields that drive our nation's economy [1]".

Each year, NSF's ATE Program receives grant proposals from eligible organizations, primarily two-year colleges, that educate the nation's technical workforce. Submitted proposals undergo a peer review process; NSF Program Officers review them and, if recommended for funding, a final review by the NSF Division of Grants and Agreements. A requirement for all ATE-funded work is that the research be evaluated. Project descriptions must include a subsection titled "Evaluation Plan," and the ATE Solicitation specifies the type of information necessary to adequately describe the plan. The ATE Solicitation also includes a directive that "there must be clear alignment between the evaluation plan and the project's intended outcomes, activities, and deliverables" [1].

To a Principal Investigator and Project Team who are focused on their project vision and the resources, activities, and personnel that will be required to achieve project goals and objectives, creating an evaluation plan can be a daunting task. An experienced ATE evaluator can assist the project team in developing an effective evaluation plan and meeting the proposal requirements outlined in the Solicitation.

Methods

Laying the Groundwork

This article assumes that the Principal Investigator (PI) has sought and found an evaluator for the proposed project. The first task facing the evaluator is to communicate the importance of evaluation in ATE grants. The value of including evaluation goes well beyond the NSF-imposed requirement for evaluation. The evaluator can describe the ways that evaluation will help the PI implement and execute a successful project. However, taking a longer view of grants, the ATE Solicitation states that if a subsequent proposal is submitted, the first

section of the second proposal must be entitled "Results from Prior NSF Support" [1]. In this section, the PI must show that the prior proposal produced significant results. In terms of Intellectual Merit and Broader Impacts, according to the two NSF Merit Review criteria, a proposal must show how the prior work improved technician education and impacted the technical workforce, thus laying the groundwork for a new proposal. Evaluation data is used to support claims made, and evaluation findings will point to the next steps needed to further technician education.

A potential barrier in working together and to effective evaluation planning and implementation is communication. The PI and evaluator will often speak using different vocabularies. The PI, usually a technical faculty member, will use a vocabulary linked to their technical discipline, while the evaluator will use terminology appropriate to evaluation science. Terms like "formative evaluation," "summative evaluation," "mixed methods," and "qualitative versus quantitative evaluation" may not be familiar to technical faculty and hence, have little meaning to members of the project team.

Another challenge is visualizing how the evaluation will fit into the proposed project. Visualization techniques like logic models have been used to link project activities to outputs to outcomes. Logic models are most often represented as tables but are word-based and thus may not easily convey evaluation processes and procedures to technical faculty. An alternative technique to logic models is the use of "road maps" as a visualization tool. Road maps are similar to flowcharts in that they use blocks to represent essential elements and arrows to indicate important interactions between project components. Technical faculty will be familiar with flowcharts and diagrams, such as circuit schematics that are similar to road maps.

Evaluation frameworks can also effectively overcome communication/language and visualization barriers. An effective framework shows how project activities and evaluation processes can be used to determine the "merit" or "worth" of the project being proposed. Most individuals will be familiar with the term "value," but we will differ in how to quantify it. An evaluation framework focused on value creation can be used to visualize and measure the merit or worth of a project.

A Value-Creation Evaluation Framework

Evaluation often stops short of answering the "so what?" question. What is the impact of the investment that a grant-funded project represents? How can an evaluation plan focus on this critical question? As an early-career project evaluator and building on years of experience as a STEM technician educator and NSF ATE grantee, the author developed a framework incorporating evaluation methodologies to measure a project's merit or worth (value). This value-creation evaluation framework was adapted from a conceptual framework developed by Etienne Wenger, Beverly Traynor, and Maarten de Laat for communities of practice. Their value-creation evaluation framework is described in a paper entitled "Promoting and Assessing Value Creation in Communities and Networks: A Conceptual Framework" [2]. Their framework measured value creation in terms of five-cycles:

1. Immediate Value: Activities and interactions
2. Potential Value: Knowledge capital
3. Applied Value: Changes in practice
4. Realized Value: Performance improvement
5. Reframing Value: Redefining success

The following is a brief introduction to Wenger's five levels of value creation.

Cycle 1 – Immediate Value – Activities and Interactions: "Activities and interactions can produce value in and of themselves. One can get an answer to a question, a solution to a problem, or help with a challenge [2]. Immediate Value is created or produced *during an activity.*

Cycle 2 – Potential Value – Knowledge Capital: "Not all value produced is immediately realized. Activities and interactions can produce 'knowledge capital' whose value lies in its potential *to be realized later* [2]." This can include changes in perspective or attitude. Potential Value is created *as a result* of an activity.

Cycle 3 – Applied Value – Change in Practice: "Leveraging capital requires adapting and applying it to a specific situation. Looking at applied value means identifying the ways practice has changed in the process of leveraging knowledge capital [2]." Applied Value is produced *after* an activity.

Cycle 4 – Realized Value – Performance Improvement: "New practices or tools are not enough, even when applied. It is therefore important not to simply assume that improved performance is the case when people change their practice, but to reflect on what effects the application of knowledge capital is having on the achievement of what matters to stakeholders [2]." Realized Value is the *desired product or outcome* of an activity.

Cycle 5 – Reframing Value – Redefining Success: "The last cycle of value creation is achieved when social learning causes a reconsideration of the learning imperatives and the criteria by which success is defined. This includes reframing strategies, goals, as well as value [2]." This cycle is a critical step for continuous improvement as the members of the project team seek to optimize activities, strategies, and methodologies. Reframing Value results from *reflection on an activity* and the evaluation data collected during Cycles 1-4.

Value-Creation Example

The following example will illustrate the use of the value-creation evaluation framework. Suppose your goal is to take better photos of your grandchildren during summer vacation and to produce a photo book to remember the time together. A first step would be to research available cameras, select the best camera to meet your needs and budget, find the best price at a local camera store or online, and purchase the camera to best meet the stated goal. This creates *Immediate Value*.

Having purchased the camera, *Potential Value* resides in the photographer's ability to use it to take photos at some future point. That Potential Value may or may not be realized.

Applied Value is created when the camera is actually used to take summer vacation photos of family members and document the time spent together. This Applied Value is created when the camera is actually used.

Once a library of photos has been taken, one of a number of software programs can be used to create a photo book documenting the vacation, which can then be printed. This achieves the goal. So, producing the photo book represents *Realized Value*.

The final cycle is to use self-analysis of the process and constructive feedback from family members about the photobook to determine what may be done to improve future vacation photobooks. For example, next time more candid photos than posed photos would make the photobook more realistic and meaningful. This will improve the process and creates *Reframing Value*.

The photo book example is hypothetical. Numerous examples may be found in everyday life. How many times has a book been purchased but never read, seeds purchased but never planted, or a workshop attended, and the workshop notebook with guides for improvement never again opened? Good intentions without follow-through prevent the achievement of goals. Evaluation provides an accountability tool that can help turn good intentions into reality.

A Case Study

To understand how the value-creation evaluation framework works, an evaluation of a series of linked ATE projects will be used to illustrate the application of the Value-creation framework. Over the past decade, this framework has been used to show the merit, worth, and return on investment of Mentor-Connect: Leadership Development and Outreach for ATE, a series of special projects funded by the National Science Foundation's Advanced Technological Education Program (DUE 1204463, 1501183, 1840856, and 2227301). Referred to as Mentor-Connect, this initiative is responding to a documented need to promote the achievement of the goals of the NSF ATE Program.

Prior to 2012, NSF's ATE Program had a preliminary proposal program. Prospective Principal Investigators had the option of submitting a five-page preliminary ATE proposal for review. Program Officers and selected reviewers would read the proposal and offer suggestions for strengthening the proposal prior to the final submission of a full proposal. When the preliminary proposal program was discontinued, most prospective grantees were faced with accessing and interpreting the NSF solicitation and associated grant policies and procedures on their own. Some prospective grantees decided it was much too daunting a task and did not even try.

By 2012, the ATE Program was 20 years old, but only one-third of the nation's 1,200 two-year colleges had received grant funding from the ATE Program. Conversely, approximately 800 two-year institutions had not received an ATE award. Furthermore, of the 1,200 two-year institutions, approximately 260 were small rural colleges [3] that often lacked the grant expertise and experience needed to compete in the competitive ATE grant program at NSF.

Several seasoned ATE grantees who had been providing ad hoc mentoring assistance for grantees in the ATE Program were encouraged by ATE Program Officers to create a mentoring program to assist new-to-ATE institutions with preparing and submitting competitive new-to-ATE proposals. Elaine Craft, based at Florence-Darlington Technical College, SC, led this effort. The first Mentor-Connect award, DUE 1204463, was designed to create a regenerative, sustainable process for peer mentoring for the ATE Program. The program focused on proposal development and leadership capacity-building among those who educate technicians in high-technology fields that drive our nation's economy. To achieve this goal, the Mentor-Connect Program was designed to [4]:

- Fill a void created by the elimination of the preliminary ATE proposal review process.
- Address the fact that roughly two-thirds of community colleges have not received an award from the National Science Foundation (NSF) ATE Program in the last ten years.
- Better manage the growing number of requests received by ATE Center Principal Investigators and NSF Program Officers related to grant proposal development and project management.
- Develop grant writing skills among community college faculty who lack grant development staff (or sponsored research officers) at their institution.

Mentor-Connect project objectives were to:

- Attract and prepare new and diverse cadres of ATE Principal Investigators (PIs) from community colleges.
- Transfer knowledge from experienced PIs to less-experienced or new PIs.
- Prepare experienced ATE PIs to serve as peer mentors.

Three subsequent grants (DUE 1501183, 1840856, and 2227301) enabled the Mentor-Connect Project to continue cohort mentoring over the past decade. Ten cohorts of two-year colleges have completed the ATE funding cycle, Cohort 11 proposals are currently in the review cycle, and Cohort 12 began proposal development in January 2024.

Mentor-Connect Roadmap

The theory of change for the Mentor-Connect Project is very straightforward. From a pool of applicants, a cohort of 20 mentee colleges would be selected. Each mentee team would consist of two faculty members, one being designated as the Principal Investigator (PI) and the other as the Co-Principal Investigator (co-PI). Each college would be assigned a mentor to guide them through the proposal development/submission process. The mentorship program (intervention) includes instruction and technical assistance in addition to mentoring. The intervention begins with an intensive three-day workshop that includes an overview of the ATE program, a description of essential proposal components, a mock panel review of actual ATE grant proposals, and a presentation of elevator speeches that allow each Mentee team to describe their project idea. Some workshop participants liken the workshop experience to "drinking from a fire hose," and it reflects the steep learning curve that exists when technical faculty begin preparing their first NSF ATE grant proposal.

As proposal development begins, the mentoring process is supported by webinars that are focused on specific aspects of proposal development, e.g., evaluation, forms, and budget and budget justification. Also supporting the mentoring process are proposal development resources accessible on the Mentor-Connect.org website. Online resource materials are designed to answer frequently-asked questions. Mentors and mentee teams communicate primarily through email messages and telephone/online conference calls. Nine months of mentoring culminates with the submission of proposals in early October. The target metric set for each cohort is an 80% submission rate (16 submissions by 20 mentee teams). Through the first ten cohorts, it was envisioned that nearly all of the proposals would be in the Institutions New-to-ATE proposal track within the ATE Program.

As Mentor-Connect selected the first cohort of Mentee colleges in 2012, the roadmap shown in Figure 1 was used to visually represent the project. In the left-center of the roadmap are blocks representing "Mentors" and "Community Colleges (Mentee teams)," and the double arrow depicts the interaction (i.e., mentoring) between the mentors and mentees. Three "value investments" support the mentoring process: Grant-writing Workshops, Webinars, and Website Resources. All three value investments support Mentors and Mentees during the nine-month mentoring process.

On the right side of the roadmap is a large block labeled "ATE Community." The arrow between Community Colleges and the New-to-ATE Projects block depicts the proposal submission process for our Mentees. Successful proposal submission provides entry to the ATE community.

The arrow at the bottom of the roadmap depicts the regenerative nature of the Mentor-Connect Program. It is envisioned that Mentees who receive a New-to-ATE award will move up to an ATE project award, gain experience with ATE awards over time, and then ultimately become mentors themselves for future cohorts. This is the regenerative, capacity-building nature of Mentor-Connect.

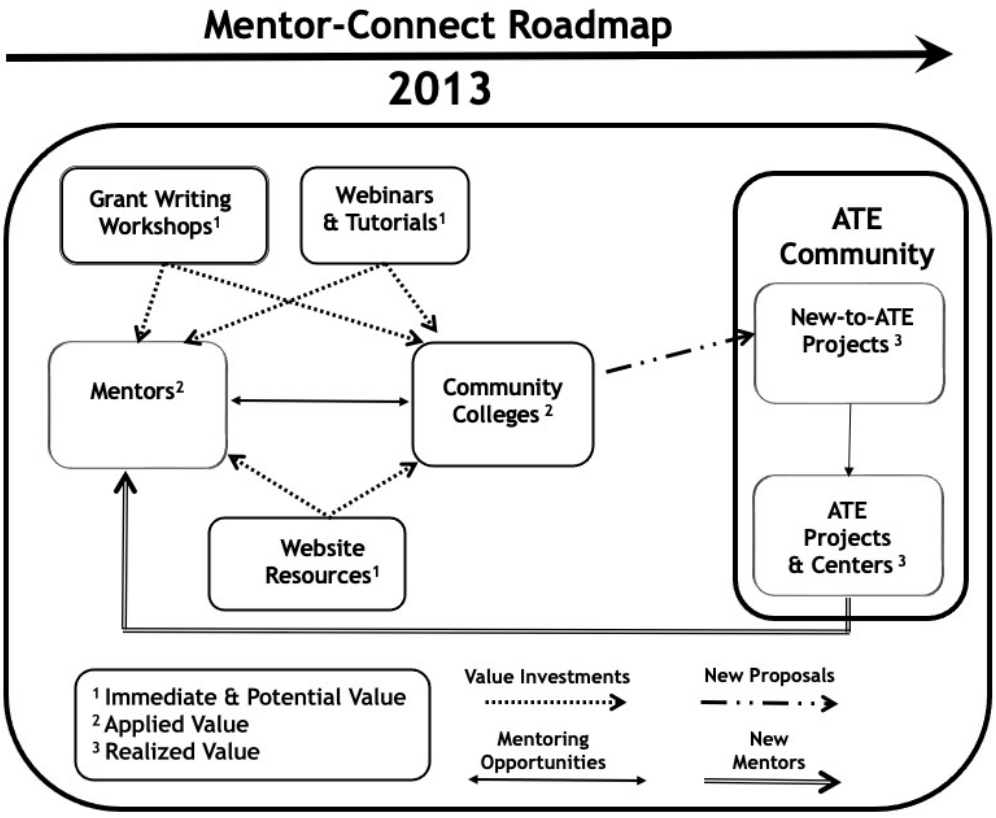

Fig. 1. The first Mentor-Connect Roadmap, circa 2013.

Evaluating the Mentoring Process

Immediate and Potential Value

Evaluating the cohort selection process is mainly quantitative: the number of teams accepted into the cohort from the applicant pool. As shown in Figure 2, the number of applications varied from a low of 23 applicants (Cohort 9) to a high of 37 applicants (Cohort 6). The overall acceptance rate is approximately 70%. Those not accepted into a cohort were offered a one-on-one mentoring session to discuss how their project idea and their Mentor-Connect application might be improved and to explore other programs that may be a better fit for assistance in meeting their goals, e.g., the MentorLinks Project for program improvement.

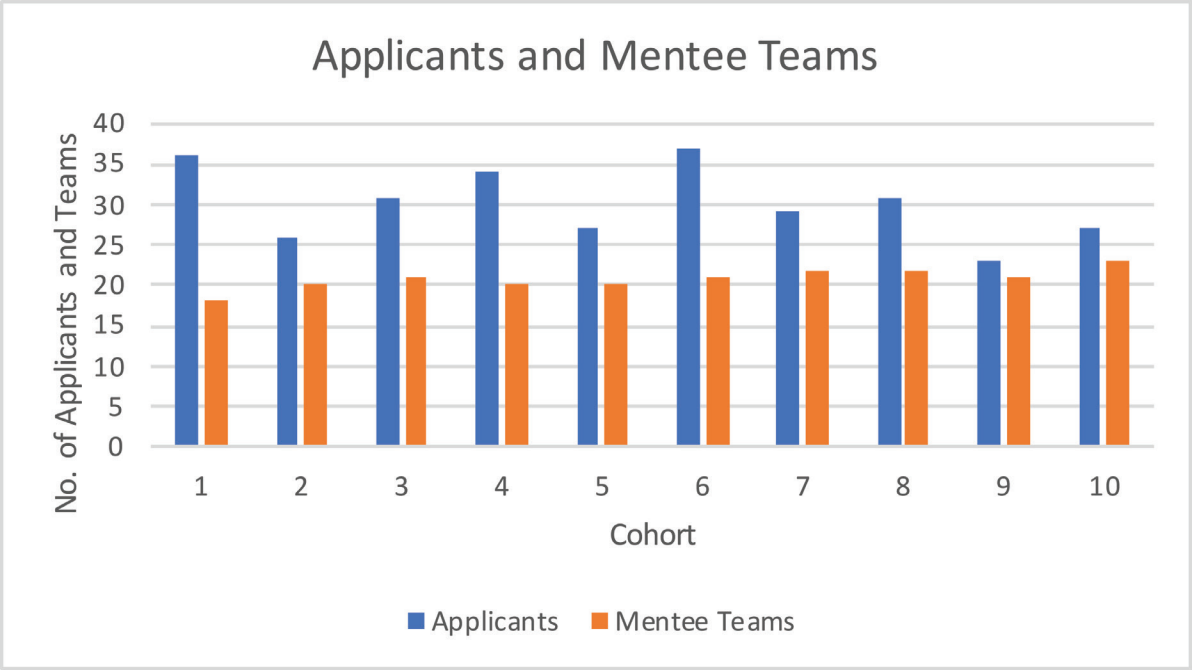

Fig. 2. The number of applicants and mentee teams per cohort.

Evaluating the two Mentor-Connect in-person workshops relied mainly on post-workshop surveys, and the surveys yielded "Immediate Value". Over the years, this data has been consistently positive. Workshop participants have been very pleased with the workshop content and rated all aspects of the workshop as "High Value" or "Very High Value." However, the data has become less valuable as an evaluation tool over the years since the data have not significantly changed from year to year. Suggestions such as the need for more Mentor-Mentee work time have been addressed while being attentive to the need to balance information transfer with Mentor-Mentee interaction and budget constraints on workshop length.

One question on the post-workshop survey pointed to "Potential Value." The last question on the survey asks, "On a 10-point scale with '10' representing 'very confident,' how confident are you that you will be able to use the workshop material to begin developing an ATE proposal?" Leaving the workshop, most participants are very enthusiastic and were very confident that the workshop material, along with mentoring, would result in ATE proposal submission.

Likewise, post-webinar surveys were administered at the end of each webinar. Over the years, the results of these surveys have shown a high level of satisfaction with the webinars. Most participants view the information presented as vital for preparing their proposal in the days ahead. Quick Reference Guides based on each webinar help participants find needed information without having to listen to the whole webinar a second time. Comments on participant surveys and questions asked during and at the conclusion of webinars guide content improvement to prepare subsequent cohorts of prospective grantees better.

Applied Value

The mentoring process is monitored through four progress reports sent out and collected by Emery DeWitt, Mentor-Connect Project Manager. These progress reports identify mentee teams that may require intervention by Mentor-Connect staff to address specific problems or issues as they arise. However, some problematic situations involve internal issues at a college. The project team and mentors do not intervene but strive to equip participants with leadership skills to help them resolve issues they encounter at their college. These data measure "Applied Value."

Creating "Applied Value" depends on a cadre of experienced mentors. To date, 34 different individuals have served as mentors. Having a stable group of mentors is critical to generating "Applied Value" in the Mentor-Connect Program. Each August, individual mentor interviews are conducted via phone, or more recently via Zoom, with each mentor currently mentoring in the cohort. During the interview, mentors are asked for suggestions on how to improve the workshops, webinars, and resources to help them better mentor their mentee colleagues. Mentors are also asked to describe their progress in mentoring each of their mentee teams and are asked to predict whether their mentee teams will be able to submit an ATE proposal in October. Cohort mentoring generates "Applied Value."

Realized Value

Following the submission deadline, mentee colleges are asked to voluntarily submit copies of their ATE proposals to Mentor-Connect. Using these proposals and by searching the NSF awards database, data is collected to document the number of proposals submitted, proposal numbers, amount of the request, and proposal type. The target metric for Mentor-Connect is an 80% submission rate for each cohort. This data measures "Realized Value."

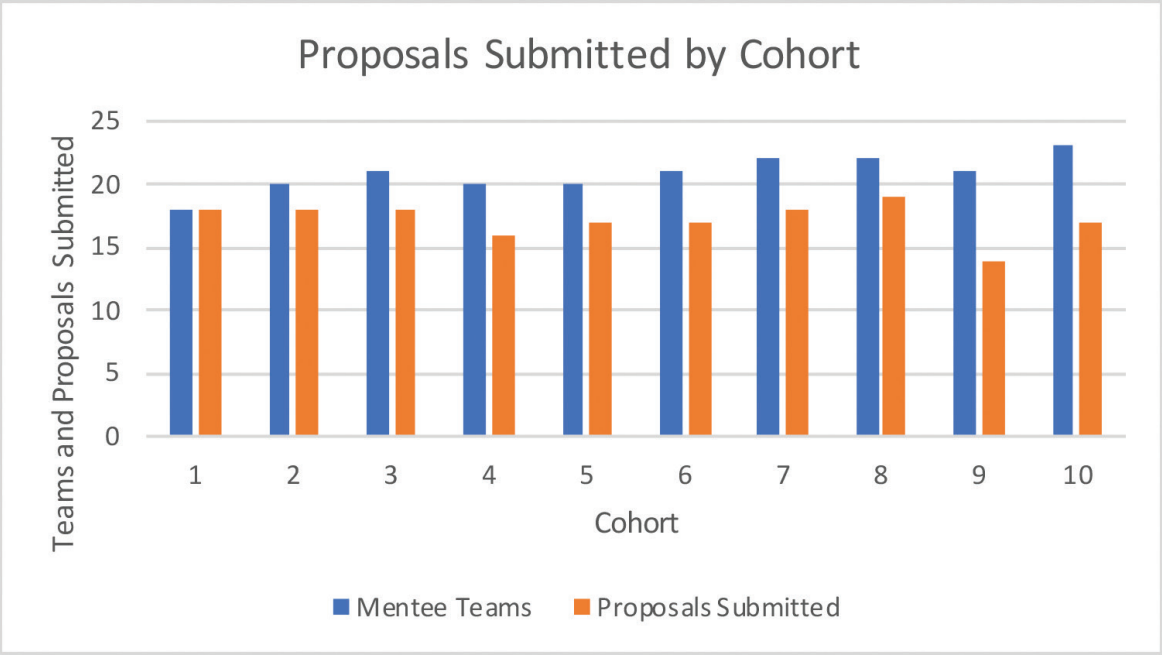

Fig. 3. Number of New-to-ATE proposals submitted by cohort.

Figure 3 shows the number of submissions per cohort. Only Cohort 9 fell below the 80% target, most likely a result of the disruption caused by the COVID-19 pandemic. During the pandemic, faculty were under increased stress as they converted their courses for online delivery while simultaneously working on their ATE proposal. Over ten cohorts, the Mentor-Connect project has achieved an 83% submission rate.

Reframing Value

Beginning with Cohort 2, evaluation data prompted the Mentor-Connect Leadership Team to reframe what success looks like. The following changes were made to Mentor-Connect processes and mentor activities. Reframing aims to increase the value generated in different activities in the Mentor-Connect Project.

Cohort 2: Feedback from mentors indicated that another face-to-face meeting between mentors and mentees during the summer would enhance the proposal development process, provide an opportunity to make changes, and get some mentee teams back on track prior to submission. Hence, a second, one-day, in-person workshop was added, and this workshop is now held each July just prior to the annual High Impact Technology Exchange Conference (HI-TEC). An important outcome of this workshop is the creation of a timeline for the final two months leading up to the proposal submission deadline, typically the first Thursday in October.

Cohort 6: Initially, the Mentor-Connect Leadership Team selected mentors from among the most experienced grantees in the ATE grantee community. This approach worked to launch the project but was not optimal for expanding participation over time. To address the growing need for more and better-prepared Mentors, a formal Mentor Fellows Internship Program was implemented in conjunction with Cohort 6. Mentor Fellows were selected through a formal application process. Those selected to be Mentor Fellows were each assigned to shadow and work with an experienced mentor for one year. In addition, Mentor Fellows participate in all Mentor-Connect grantee preparation activities throughout the year to inform them of current NSF ATE guidelines and expectations while enhancing their grantsmanship skills. Based on their mentor's recommendation and observation of the Mentor Fellow by members of the Leadership Team, it is then determined which Mentor Fellows who successfully completed an internship will be invited to serve as a mentor the following year. The Mentor Fellows Program builds mentoring capacity for the ATE Program and Mentor-Connect Project and thus supports the "regenerative" part of the Mentor-Connect Project.

Cohort 7: For earlier cohorts, only one hour of mentoring via phone was provided to those mentees whose proposals were "Declined" (not funded). Some ad hoc mentoring was also provided by the mentor who had been working with the mentee team. For Cohort 7, Mentor-Connect expanded mentoring services to those whose proposals were Declined by adding two additional mentoring assistance programs: Second-Chance Mentoring and Moving-Up Mentoring. Second-Chance Mentoring provides colleges that submitted New-to-ATE or ATE Project proposals that were Declined with additional help in responding to reviewer feedback and strengthening their project idea and proposal for re-submission the following October. Moving-Up Mentoring is for those colleges who have received a New-to-ATE award, have completed or are nearing completion of the project, and want to "move up" to an ATE project. Both of these mentoring initiatives provide a dedicated mentor for one-on-one mentoring and serve to encourage continued participation in the ATE Program

Cohort 9: The ATE Program began restructuring the funding opportunity for ATE Centers in 2018. With this restructuring came the expectation that ATE Centers would support other ATE grantees with discipline expertise and resources and mentor educators and encourage them to submit ATE grant proposals. Recognizing the primary role of ATE Centers as leaders in specific disciplines of advanced technological education and Mentor-Connect as a leader in specifics of NSF ATE grant proposal preparation, Mentor-Connect has partnered with ATE Centers to provide Co-Mentoring. Participating ATE Center designates persons from their Center to serve as co-mentors. A co-mentor has the option of preparing for this role by completing a Mentor Fellows internship. Co-mentor Fellows prepare to support the mentoring objectives of their ATE Center with no expectation that they will serve as Mentor-Connect Mentors. Co-Mentors support technician educators in the discipline and can rely on Mentor-Connect to support their work with grant proposal development education, allowing prospective grantees to benefit from both of their expertise. Co-mentoring also supports ATE Centers in other ways such as providing educational programs for their participants on the ATE Program, grant funding opportunities, and proposal development strategies. Co-mentoring increases "Applied Value."

Post-Cohort 9: Beginning with Cohort 10, Mentor-Connect broadened the definition of new-to-ATE to expand eligibility for cohort mentoring and build more capacity for seeking NSF grants at two-year colleges. New-to-ATE now includes college teams where faculty members on the team have not previously served as PI or Co-PI on ATE grants. This enables a college that has been a previous ATE grant recipient an opportunity to work with Mentor-Connect to increase ATE funding to the college and expand the number of faculty with grantsmanship knowledge and skills to advance technician education at the institution and beyond. New-to-ATE faculty where the college is not eligible for the Small Project for Institutions New to ATE funding track are guided in preparing competitive proposals for the ATE Projects funding track.

Post Cohort 10: Beginning with Cohort 11, a pre-mentoring initiative was implemented. Some applications to the Mentor-Connect Program have significant project description weaknesses or are not aligned with the goals of the ATE Program. Rather than rejecting these applications, a limited number of hours of pre-mentoring assistance is offered to these applicants and provides an opportunity for the college to strengthen their project idea or other elements of their application, which will allow them to participate in the Mentor-Connect Cohort and prepare a competitive ATE proposal. If the applicant is not able to address these issues, a referral may be made for the college to apply to another NSF Program. Most recipients of pre-mentoring have been able to adjust project idea, were accepted into the Cohort, and became prospective ATE grantees. Pre-mentoring intervention has increased the number of mentees accepted into a Cohort, and the number of prospective ATE grantees, thereby increasing "Immediate Value."

Post Cohort 11: A new initiative called “PI 101" was implemented. PI 101 is mentoring new PIs to help them implement their newly funded ATE project. This mentoring helps new PIs to become familiar with NSF procedures, e.g., complying with reporting requirements. Instruction and advice on NSF expectations, elements of project implementation, and management of federal grant funds are designed to reduce the frustration that most new PIs experience and will make implementing their project less stressful. By reducing the stress in their first project, it is hoped that PIs will be encouraged to move up to a complete ATE project in the future. Increasing the likelihood that a PI will seek another grant increases “Potential Value.”

Current Roadmap

Reframing has enhanced the Mentor-Connect Project to create additional value. Adding Second-Chance Mentoring, Moving-Up Mentoring, Co-Mentoring, and the Mentor Fellows initiative to the Mentor-Connect Roadmap shown in Figure 1 yields an expanded Mentor-Connect roadmap shown in Figure 4. The Second-Chance Mentoring block at the top of the roadmap is for Mentees who have had their first or second ATE proposal Declined and who are revising it for future submission. The Moving-Up Mentoring block at the bottom of the roadmap depicts Mentees who are preparing to move up from a small, New-to-ATE project to a larger ATE project. The Mentor Fellows block in the lower left corner represents the Mentor Fellows Program that prepares members from the ATE community to become future Mentor-Connect Mentors. And finally, Co-Mentors were added to the Mentor block on the left side of the roadmap. The changes to the roadmap are a direct result of the Mentor-Connect Leadership Team using evaluation data over ten cohorts to "Reframe" Mentor-Connect processes and procedures to maximize the value created.

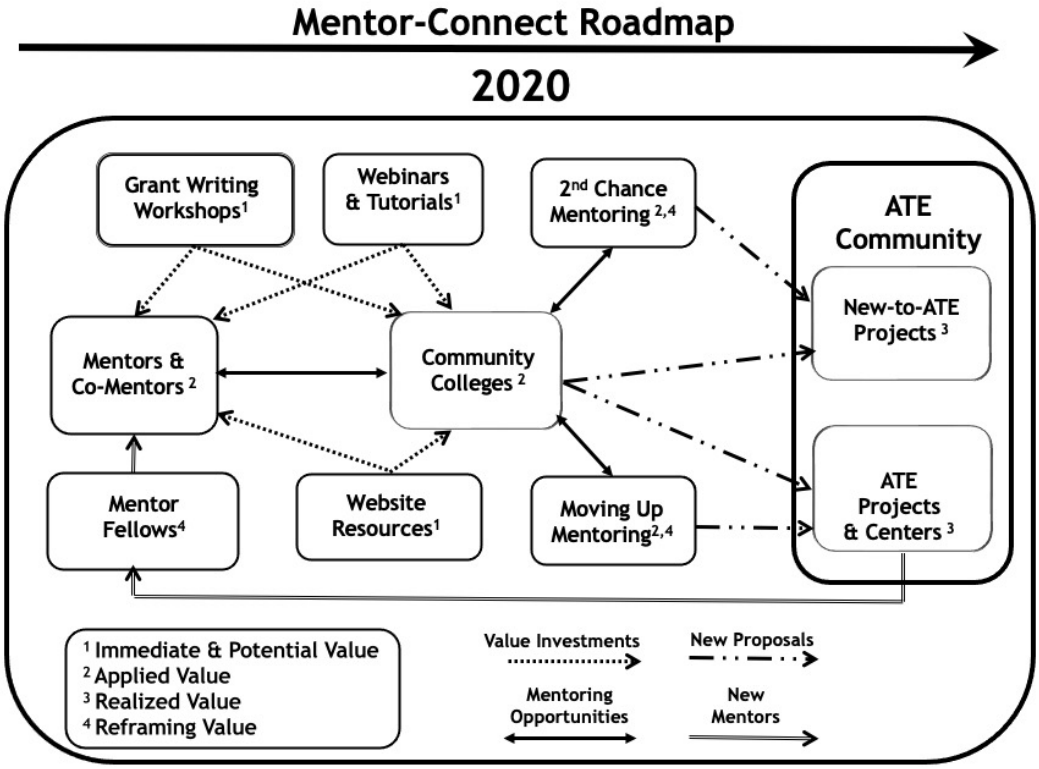

Fig. 4. The Mentor-Connect Roadmap, circa 2020, shows the addition of Second-Chance Mentoring, Moving-Up Mentoring, the Mentor Fellows Program, and the addition of Co-Mentors.

Evolution of Mentor-Connect

The table shown in Figure 5 summarizes the evolution of the Mentor-Connect Project from its beginning in 2012 to its current structure in 2024. The table shows the progression in terms of value creation from Immediate Value and Potential Value to Applied Value to Realized Value and Reframing Value. Over the past decade, the Leadership Team has continuously improved Mentor-Connect processes and procedures, resulting in a mentoring program that has produced a significant return on investment to the ATE Program at NSF.

Mentor- Connect Strategy	Immediate and Potential Value	Applied Value	Realized Value	Reframing Value
Initial Theory of Change (circa 2013)	Mentee teams In Cohort: new-to-ATE colleges Workshops/ Webinars Website Resources Mentors	Mentoring Proposal development Leadership development	New-to-ATE proposal submitted to NSF (Metric: 80% submission rate for New-to-ATE proposals)	Add another in-person Workshop Add additional webinars Expand/update website resources Implement Mentor Fellows Program Implement Second-Chance and Moving-Up mentoring Implement co-mentoring with ATE Centers
Current Theory of Change (circa 2023)	Mentee teams In Cohort: new-to-ATE colleges and new-to-ATE faculty Workshops/ Webinars Website Resources Mentors Mentor Fellows Second-Chance and Moving-Up Mentee teams Co-Mentoring with ATE Centers	Mentoring Proposal development Leadership development	New-to-ATE proposal submitted to NSF ATE project proposals submitted to NSF (Metric: 80% submission rate for New-to-ATE proposals) Mentor Fellows become Mentors	Expand Mentee teams from two to four members: Include an administrator and grant professional Expand the definition of eligible teams to include faculty new-to-ATE PI 101 Mentoring Pre-Mentoring applicant intervention Expand/update website resources

Fig. 5. The reframing of the Mentor-Connect Program from 2013 to 2023.

Results and Discussion

Longer-Term Impacts of a First ATE Grant Award

Evaluation outcomes for Mentee colleges do not stop with the submission of an ATE proposal and subsequent ATE grant award. Seventy-one percent of mentee colleges receive New-to-ATE awards and become members of a very welcoming and supportive ATE community. For many mentee colleges, New-to-ATE awards are transformative; the award is the catalyst for changing the grant culture within the institution. The result has been the implementation of procedures and policies that support both pre-award and post-award grant activities for faculty-led grants. The problem-based-learning scenario of developing a project fulfills a vision for preparing and submitting a competitive grant proposal, becoming a grantee, and implementing a project that develops leadership skills among STEM faculty and technician educators who complete this journey, especially if they continue to seek and implement additional ATE grant awards. Leadership skills are evident as individuals lead within their college, across the ATE Community, and beyond, including becoming a Mentor for prospective ATE grantees.

Funded Proposals

The success of Mentor-Connect is reflected at interim milestones as new educators are connected to the NSF ATE Program and culminate with the success that a funded proposal represents. Data are available for the first 10 Cohorts. Cohort 11 proposals are being awarded in 2024, and prospective grantees in Cohort 12 are currently developing proposals for submission in October 2024.

Out of 164 New-to-ATE proposals submitted, 117 were funded, with an overall success rate of 71%. Figure 5 shows the number of proposals submitted and awards received by the cohort. The success rate for New-to-ATE proposals ranged from a low of 47% for Cohort 8 to a high of 88% for Cohort 6.

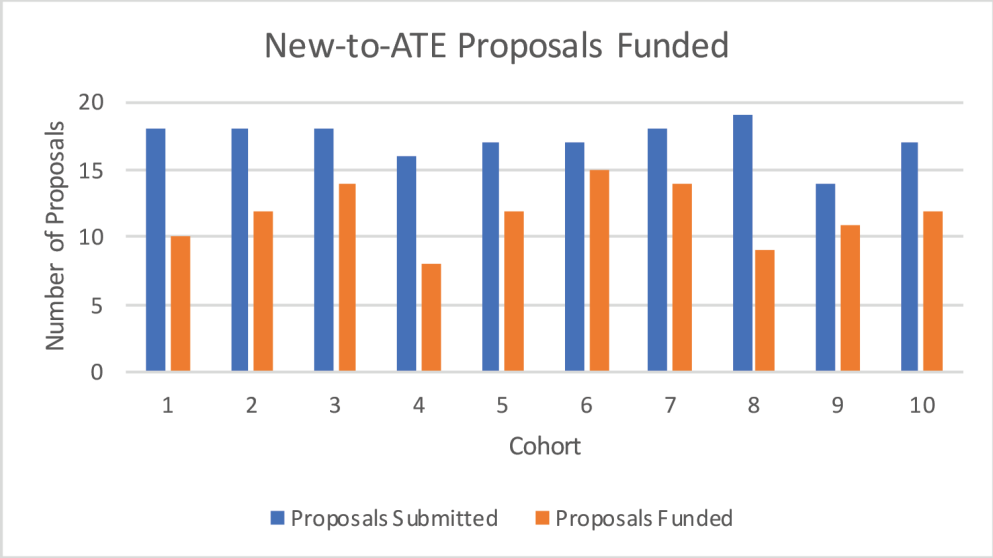

Fig. 6. The number of New-to-ATE proposals funded per cohort.

Cohort 1-10 New-to-ATE awards total $30,063,197. However, the value to the college goes beyond the dollars received from NSF. This first ATE award can be the catalyst for change within an institution. The award will increase awareness of the availability of federal funding. It can increase visibility for the program and college within the community and foster new and strengthen existing industry and community partnerships. Faculty become involved in a national community of technology educators and establish their networks with colleagues from other institutions. And, the experience of getting their first grant can stimulate submission for future grant proposals using the knowledge gained from their first successful proposal.

For evaluation purposes, each mentee team that submitted an ATE proposal is asked to voluntarily share their panel reviews with Mentor-Connect. The panel reviews indicate both weaknesses and strengths in the proposal. As an aggregate of proposals, reviewer comments may indicate areas of proposal development that should be highlighted in Mentor-Connect workshops. The reviewer ratings are also analyzed for "spread." That is the range between the highest rating for a proposal and the lowest rating it received. One finding was that the spread in ratings increased during the pandemic when NSF changed from in-person panels to "virtual panels." This suggests that it is harder for virtual panels to influence and moderate extreme perspectives on the merits of a proposal.

Site Visits and Outcomes Harvesting

To determine the impact of receiving their first ATE award, site visits were used to implement what we called "Outcomes Harvesting." By visiting each mentee college near the end of their project and interviewing the Principal Investigator, project staff, administrators, and grant personnel, outcomes from the NSF award could be "harvested." Approximately 40 mentee colleges in Cohorts 1-4 were visited prior to 2020 when COVID-19 forced travel limitations. For Cohorts 5-7, site visits were conducted virtually using Zoom.

One of the important findings during an in-person site visit after a first-time grant is being implemented is the degree to which the institution is developing a "culture-of-grants." A culture of grants can be defined in many ways. For the purposes of this paper, it is defined as "the set of shared attitudes, values, goals, and practices that support grants;" attitudes that grants are an asset to educational programs and can be a catalyst to improving educational programs and student learning, values that drive faculty and administrators to invest time and effort in seeking educational grants, goals that include educational materials development, laboratory improvement, and faculty enhancement, and, practices that encourage and support faculty as they step beyond their role as a classroom teacher.

Site visits have indicated a range of cultures related to grants. Some two-year colleges have no grant professionals on staff and do little to encourage faculty to seek grants. Other institutions have embraced grants as enablers for program improvement and faculty development. Some institutions now include faculty in grant planning and advisory committees, whereas before becoming ATE grantees, grant seeking was predominantly administrator-driven.

However, the best indicator of the development of a culture of grants is to track the grant activity following an institution's initial ATE award. Evaluation of this metric requires periodic "mining" or "searching" the NSF awards website by Mentee institution. Has the institution received a subsequent NSF ATE award, that is, have they "moved up" from a New-to-ATE project to an ATE project, consortium, or center award? Has the institution also received an award from another NSF program, e.g., an S-STEM or Hispanic-Serving Institution Program grant?

Mining the NSF awards website in November 2023 for Cohorts 1-10 awards identified that in addition to the 117 Mentor-Connect mentee colleges that have received New-to-ATE awards on their first try, totaling $30,063,197, an additional 25 mentee colleges received 25 second-chance, New-to-ATE awards totaling $8,010,850. Of these 142 colleges that received New-to-ATE awards, 36 have moved up to larger ATE awards totaling $34,536,793. These colleges also applied for and received 20 S-STEM awards totaling $22,247,095 and 32 other NSF program awards totaling $24,904,967. Since participating in the Mentor-Connect Program, 142 mentee colleges have received approximately $120M in grants from NSF programs.

Faculty Leadership Development

Community college culture sees the role of faculty as classroom teachers. Activities outside the classroom are often viewed as unnecessary and thus are rarely recognized or supported. Teaching loads are heavy, ranging from 18 contact hours/week to as many as 24 contact hours/week. With the associated office hours, preparation time, and assessment of student learning, most faculty view their jobs as more than full-time. So, when a faculty member is asked to participate in grant-related activities, their response may be less than enthusiastic. Even if provided with some "release time" for completing grant work, faculty too often lack a vision for grants, are unable to see the potential benefits, and do not pursue the opportunity.

Fortunately, there are faculty members who do see the advantages of grants. The motivation is usually a desire to improve student learning and to provide real-world experiences in the instructional program. For those faculty, the return on investment can be significant. For example, attending the Annual ATE Program Principal Investigators' Conference can be an eye-opening experience for faculty that expands their vision for technician education and builds their professional network.

Faculty leadership development through the Mentor-Connect Program is "leadership development by doing." Getting a proposal through an institution's internal approval processes and implementing a funded project requires faculty members to work outside of their comfort zone as they interact with other departments within in their institution, faculty at different colleges, and Program Officers at NSF.

Examples of faculty leadership development include faculty at Northland Community and Technical College (Unmanned Autonomous Vehicles), Sommerset Community College (Additive Manufacturing), Central Oregon Community College (Electric and Hybrid Vehicles), Snow Community College (Agriculture), and Northwest State Community College (Scaling Online and Hybrid Instruction). These are just a few examples of ATE grants serving as catalysts for faculty leadership on a state, regional, or national level.

A visible measure of faculty leadership development in Mentor-Connect mentees is demonstrated at the ATE Connect sessions at the ATE Principal Investigators Conference. In 2013, 10 Mentor-Connect mentee colleges participated. In 2023, the number of participating Mentor-Connect mentee colleges had grown to 55 colleges. There were a plethora of Mentor-Connect buttons worn by conference participants who are part of the Mentor-Connect family and Mentor-Connect stickers on posters at the two ATE Connects sessions. In addition, mentees also participated in conference sessions and breakfast roundtables and served on conference planning committees. Numerous program sessions are provided at the annual conference each year by new and repeat ATE grantees who first engaged with the ATE Program as Mentor-Connect Mentees.

Conclusion

Coupling a straightforward theory of change with a descriptive visual tool and a value-creation evaluation framework provides a robust way to monitor and evaluate an ATE project as well as communicate an effective evaluation plan to project team members and stakeholders. The theory of change describes how the project will get from inputs and activities to outputs, outcomes, and finally to project goals. A visual tool such as a roadmap presents the theory of change in a graphical format that can be easily understood and remembered. A value-creation framework defines types of "value" that will be created, measured, and analyzed so that the project team can make improvements to the project. It helps answer the "so what?" question that is of utmost importance to funding agencies.

The Mentor-Connect example illustrates how a well-defined theory of change coupled with a roadmap and value-creation evaluation framework can be used to determine the merit or worth of an ATE project. The goal of the Mentor-Connect Project is to help two-year colleges that have not received awards from the NSF ATE Program to submit competitive ATE proposals. Over 10 cohorts of mentee colleges and 208 mentee teams have received mentoring, with 83% submitting New-to-ATE proposals. Of the submitted proposals, 117 colleges (71%) received awards.

The impact of the Mentor-Connect Program goes beyond getting an ATE award. "Outcomes Harvesting" based on in-person and virtual site visits has uncovered outcomes beyond receiving their first ATE award. Outcomes include the development of a culture of grants to support future grant activity and faculty becoming leaders in technician education as a result of being a part of the larger ATE community. Colleges have become more adept at seeking, receiving, and managing grant funds. Partnerships have increased and become more meaningful, and colleges have garnered recognition and respect as NSF grantees. Mentees have moved up to larger ATE projects and even consortium and center grants. Regeneration and capacity-building among two-year colleges to engage with the NSF ATE Program is being realized as past mentees now serve as mentors for prospective grantees in each subsequent cohort of colleges.

Acknowledgments. This work was supported by the National Science Foundation (NSF) under awards 1204463, 1501183, 1800678, and 2227301.

Disclosures. The author declares no conflicts of interest.

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Exploration of a Bridge Program to Increase Student Understanding of Emerging Technological Fields

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Abstract: Bridge programs are common interventions colleges implement to improve student recruitment, retention, and performance. Key components are typically specific content instruction, tutoring, mentoring, and college orientation. This paper provides the results of a short-duration summer bridge program designed to increase student awareness of emerging technological fields in engineering technology (ET), specifically the semiconductor and data center industries. High school students in the summer bridge program were provided with information about NOVA's ET programs, participated in hands-on activities around topics important to semiconductor and data center operations (DCO) technician careers, and met industry representatives through industry site tours. Student data includes participant changes in understanding of ET educational and career pathways, knowledge of OSHA and industrial safety, understanding of college success skills and strategies, and interest in ET careers. Results of the study demonstrated that students of all subgroups (e.g., gender, grade level, race, ethnicity) exhibited equivalent improvement in their understanding of ET education and career pathways while student outcomes in OSHA and college success skills varied by subgroup. Based on these results, the use of a short-duration bridge program is one mechanism for post-secondary institutions to increase awareness of emerging technologies and educational pathways to support careers in those technologies.

Keywords: bridge programs, student programs, engineering technology, semiconductor, data center operations, emerging technologies, industry site visits

Introduction

Overview

Many higher education institutions aim to increase recruitment and retention in STEM through voluntary, institution-sponsored, co-curricular opportunities, such as bridge programs. With a rapidly increasing workforce within multiple engineering technology fields, community colleges are uniquely situated to help fortify the workforce pipeline for engineering technology (ET) fields such as data center operations, semiconductor manufacturing, and biotechnology [1]. However, community colleges often struggle to raise awareness and sustain recruitment strategies. Bridge programs are often aimed at increasing the participation of groups historically underrepresented in STEM. They offer a unique opportunity to diversify and broaden the pool of applicants in an industry that struggles to recruit and maintain an ever-expanding workforce [2].

According to a recent report from the Semiconductor Industry Association, as a result of incentives of the CHIPS (Creating Helpful Incentives to Produce Semiconductors) and Science Act, national semiconductor manufacturing is expected to increase by 200% by 2032. This increase in manufacturing is expected to attract $646 billion in capital investments. To meet the rising demands, experts highlight the importance of broadening the STEM pipeline and investing in scientific research to match the investments in semiconductor production and R&D [3]. A 2022 report from Deloitte [4] indicates that more than one million skilled workers will be needed by 2030 to solve the global semiconductor workforce shortage.

The Data Center Operations field is currently experiencing a rapid increase in demand for positions. Researchers estimate North America will see a net growth of approximately 85,000 jobs in data centers over the next few years. Of these jobs, 85% are in operations and maintenance of the centers themselves [5]. While

the number of jobs in data center operations continues to rise, the number of qualified staff available to fill these positions does not appear to be keeping pace. I-Masons estimate that there will be upwards of 100,000 vacant jobs in data center operations in the coming years [6]. In a survey of data center owners and operators, 1 in 5 respondents identified the difficulty of hiring and retaining qualified staff as the single largest challenge moving forward [7-8].

To address the growing demand for qualified DCO staff, community colleges have initiated outreach programs in collaboration with industry partners [9-10]. As associate degrees are outpacing the 4-year colleges in enrollment of engineering technology, it is logistically sound to situate engineering technology programs within [11]. Furthermore, engineering technology comprises one of the largest groups of STEM students enrolled in community colleges nationwide. Researchers have identified community colleges as a significantly underutilized pool of STEM talent [12]. However, there remains a considerable lack of awareness among students and faculty regarding the distinctions between engineering and engineering technology and the legitimacy of the engineering technology field. This study seeks to bridge this gap in the literature by exploring student perspectives on engineering technology to enhance outreach efforts.

Research Motivation

The NOVA Summer Bridge Program was designed to increase student awareness of emerging technological fields in engineering technology (ET) and recruit students from underrepresented backgrounds in STEM and ET. To examine the efficacy of the multiple mechanisms of the initiative across all of the participants, we sought to answer the following two research questions:

RQ1. What are student perceptions that the bridge program improved their understanding of the ET field, ET-related career competencies, and college success skills?

RQ2 - What are the differences across demographic groups regarding students' understanding of the ET field, ET-related career competencies, and college success skills?

Methods

Summer ET Bridge Program Design

The summer ET bridge program was designed to raise awareness of engineering technologies and omitted remedial coursework and college coursework like those found in more traditional bridge programs. The bridge program was hosted at four of NOVA's campuses over two years, and students were provided transportation between campuses during their program. The program took place over eight days in the summer and served rising seniors and recent graduates, emphasizing recruiting traditionally underrepresented students in ET fields. The program's primary activities consisted of a combination of hands-on learning and industry site visits. Hands-on learning activities included modules on pneumatic controls and industrial controls, with students controlling valves, sensors, and logic controllers to investigate closed systems. During year 1, students completed cabling and splicing labs using ethernet cables and received an introduction to motor and process control using NOVA's Amatrol workstations. During year 2, the program utilized LJ Create's tabletop pneumatics trainer, industrial control trainer, and virtual circuit builder with their integrated learning labs. This change allowed NOVA to offer the program at multiple sites while standardizing the curriculum. In both years, students completed a day of instruction onsite at an operational data center. Activities at the data center varied depending on the maintenance and operation schedules of the facility/ All labs and hands-on instruction were intended to introduce the types of skills a technician would need to be effective in the modern engineering technology workplace.

Through participation in the program, students also had the chance to earn two credits, a one-credit College Success Skills course (SDV 101) and a one-credit Industrial Safety course emphasizing the Occupational Safety and Health Act (SAF 130). Both courses are required for ET credentials at NOVA and serve to accelerate student's trajectory through the NOVA system if they continue on with the degree program. A more detailed guide of the bridge program's components is depicted in Table 1.

Table 1: Bridge Program Components

Component	Description
NOVA Campus Introduction and Resources	Tour NOVA campuses and meet with representatives from Student Services, Admissions, Financial Aid
College Success Skills Course SDV 101 – 1 credit	Required course for all NOVA students focused on topics such as Academic Planning and Career Exploration, Goal Setting, Communication, Financial Literacy, and Time Management
Industrial Safety OSHA 10 SAF 130 – 1 credit	Required course for all ET credentials that emphasizes safety standards, the Occupational Safety and Health Act (OSHA), and its rules and regulations (OSHA 10).
Introduction to Engineering Technology and Data Centers	Hands-on instruction and labs on topics such as electronic relays, programmable logic controllers, mechatronics, pneumatics, industrial control, and power distribution.
Data Center Tour	Tour of the STACK Infrastructure data center with discussion on critical topics and DCO career pathways
ET Tour	Tour of the Micron Technology's Manassas fabrication facility and discussion on ET career pathways

Program and Sample Demographics

Across the three offerings of the program, once in 2022 and twice in 2023, 34 students participated in the program. Students were either rising seniors or about to enter college, with a majority of students being rising high school seniors (N=22). While most participants identified as male (N=21), roughly 38% of the bridge programs participants identified as female. Similarly, while a plurality of students identified as Asian (N=11), 50% of the participants self-identified with races or ethnicities that are underrepresented within STEM. Table 2 shows the complete demographic breakdown of students who participated in the summer bridge program.

Table 2: Summer Bridge Program Participants

Participant Demographics	N	Percent
Gender		
Male	21	62%
Female	13	38%
Race/Ethnicity		
White	6	18%
Asian	11	32%
Black or African American	5	15%
Hispanic or Latino	8	24%
Multiracial	4	12%
Grade		
11th	22	65%
12th	12	35%
Total	34	100%

[a]*Percentages will not add to 100 since applicants could choose more than one race/ethnicity*

Results and Discussion

Data Collection

Data was collected from students at the end of the bridge program via an online survey. The survey consisted of 27 questions with both Likert-scale and short-answer responses. The researchers left the room while the survey was conducted to mitigate the influence of the researchers on the study's results. The survey consisted of multiple sections: program evaluation, student perceptions of industry skills focused on OSHA 10, college success skills, and understanding of the broader ET field. Likert-like responses were rated on a 5-point scale, from Strongly Disagree (1) to Strongly Agree (5).

Data Analysis

Researchers utilized independent sample t-tests to examine the differences in scores across three sets of questions, examining student preconceptions of engineering technologies, student knowledge of engineering technology-related industry skills, and various college success skills. Within the sample, independent sample t-tests were run to examine differences in understanding of engineering technology between male and female students, overrepresented majority or underrepresented minority students, and rising high school seniors (11^{th}) and incoming first-year college (12^{th}) grade students. Additionally, participants were asked to rate their pre- and post-understanding of various ET industry and college success skills. Independent sample t-tests were used to examine the differences in students' understanding across the whole sample and for specific demographic groups. P-values were determined using two-tailed hypotheses to ensure no prior assumptions of the directionality of the summer bridge program's effect were made. Additionally, equal variances were not assumed across the samples due to the low sample size. Paired sample t-tests were used to examine pre/post scores of students' understanding of industry-related skills and college success skills.

Prior Knowledge of Engineering Technologies

To examine the efficacy of the Introduction to Engineering Technology and Data Centers component of the summer bridge program, students were asked four questions about their understanding of various aspects of Engineering Technology. Namely, students were asked how the bridge program developed their understanding of the types of available ET careers, the types of available ET degrees and certifications, and the skills required for ET careers. Students were also asked how the program increased their interest in the program. Responses from across all four questions scored an average of approximately 4 (Agree), with standard deviations ranging from 0.729-0.8, which is depicted in full detail in Table 3. Additionally, independent sample t-tests were conducted to examine if certain subgroups of participants gained more from the summer bridge program when compared to their counterparts.

Table 3: Student Understanding of Engineering Technology Careers (n=34)

Participating in the summer bridge program increased my understanding of ...	Mean	SD
Types of ET Careers	4.11	0.8
Interest in ET Careers	4.14	0.729
Types of ET degrees and Certifications	4.18	0.81
Skills for ET Careers	4.04	0.85

Note: SD Standard Deviation. Scale: 1=Strongly Disagree; 2=Disagree; 3=Neither Agree nor Disagree; 4=Agree; 5=Strongly Agree.

The second research question is concerned with examining the efficacy of various aspects of the summer bridge program for all students, especially those who have been historically underserved in STEM and ET fields. To answer this research question, Table 4 highlights the differences in scores across student demographic groups through independent sample t-tests. There were no statistically significant differences in examining scores across demographic groups of the four areas pertaining to ET. The high p values indicate that the hands-on instruction and industry tours effectively informed students of various backgrounds and identities about engineering technology.

Table 4. Difference in Student Understanding of Engineering Technology Across Demographic Groups

Participating in the Summer Bridge Program has increased my...	Male/Female p-value	ORM/URM p-value	11th/12th Grade p-value
Understanding of Types of ET Careers	0.237	1.00	0.286
Understanding of Types of ET degrees and Certifications	0.254	0.141	0.833
Understanding of Skills for ET Careers	0.428	0.348	0.171
Interest in ET Careers	0.460	0.801	0.238

*Note: *p<0.05; **p<0.01; ***p<0.001. ORM=Overrepresented Majorities, URM=Underrepresented Minorities*

Student Perceptions of ET Industry Skills

Another pivotal aspect of the summer bridge program was the industrial safety course, which is a requirement for all ET credentials, which focuses on safety standards and OSHA 10. As a part of the post-participation survey, students were asked to rate their understanding of various ET industry-related skills on a 5-point Likert Scale, both at the current time and prior to the bridge program. Using paired sample t-tests, we examined the significance of the differences between student perceptions of various ET industry-related knowledge and skills. Table 5 reports the t-values for pre/post differences for each demographic group and the full sample. Results highlight statistically significant differences between pre and post-scores across all survey items for both the full sample and each demographic subgroup. The results indicate that students perceived the SAF 130 course to be effective in educating them on some ET industry-related skills, specifically regarding OSHA regulations and workplace safety.

Table 5: T-values for Pre/Post Student Understanding of Industry-Related Skills

Participating in the Summer Bridge Program has increased my understanding of…	Male df = 20	Female df = 12	11th df = 21	12th df = 11	ORM df = 16	URM df = 16	All Sample df = 33
OSHA Safe Workplaces	3.98***	2.69*	4.16***	2.42*	3.52**	3.23**	4.84***
Personal Protective Equipment Use	3.87***	2.14	3.13**	3.07*	3.27**	2.75*	4.31***
Common Workplace Safety and Health Hazards	4.16***	3.25**	4.42***	2.89*	4.04**	3.45**	5.33***
Maintenance of Walking and Working Surfaces	3.76**	3.05*	4.11***	2.59*	3.38**	3.46**	4.88***
Identifying and Resolving Fall Hazards	4.94***	3.45**	4.55***	3.98**	3.93**	4.59***	6.09***
Identifying and Resolving Fire Hazards	4.11***	2.38*	3.18**	3.75**	3.41**	3.14**	4.67***
Emergency Action Plans	4.79***	4.22**	5.26***	3.63**	4.24***	4.67***	6.38***
Preventing Workplace Violence	4.93***	4.22**	5.11***	3.92**	4.07***	5.1***	6.49***

*Note: *p<0.05; **p<0.01; ***p<0.001. df= degrees of freedom, ORM=Overrepresented Majorities, URM=Underrepresented Minorities*

Independent sample t-tests were utilized to examine the differences in prior understanding of industry-related skills across demographic groups. Comparisons were drawn between male and female students, historically represented and underrepresented in STEM, and rising high school seniors and incoming college first-years. Results found no statistically significant differences across different demographic groups for any of the industry skills, whether in their preconceptions of the industry skills or their perceived competency after the bridge program. This indicates that students were entering the bridge program with similar levels of knowledge regarding ET industry-related skills. Through the SAF 130, all students left the bridge program with similar levels of perceived competence after the course.

Student Perceptions of College Success Skills

In addition to the course on ET industrial safety, bridge program participants also took part in a College Success Skills CDV 101 course. In alignment with the course outcomes, students answered seven survey items on their post-participation survey after the bridge program concerning the prior knowledge and perceived competence of college success skills. Paired sample t-tests were conducted across all seven survey items to examine the differences between student perceptions of their understanding of pre and post-bridge programs. Results indicated statistically significant differences between pre/post perceptions across all seven skills for the entire sample: underrepresented minority students, overrepresented majority students, rising high school seniors (11th grade), and male students. These results indicate that these subsets of students perceived the course to improve their understanding of the skill listed in Table 6. Of the 49 t-values calculated, 4 of them were not statistically significant, three from incoming first-year college students and one from female students. Incoming first-year college students indicated no significant difference in their understanding of learning styles, ability to identify their preferred learning style, and strategies for effective studying. Similarly, female participants did not statistically significantly improve their understanding of strategies for effective studying.

Table 6: T-values for Pre/Post Student Understanding of College Success Skills

Participating in the Summer Bridge Program has increased my understanding of…	Male df = 20	Female df = 12	11th df = 21	12th df = 11	ORM df = 16	URM df = 16	All Sample df = 32
Different Types of Learning Styles	3.08**	2.27*	3.36**	1.81	3.09**	2.21*	3.75***
Identifying My Own Learning Styles	2.94**	2.25*	3.17**	1.79	2.66*	2.75*	3.60***
Strategies for Managing College Work	4.36***	2.65*	3.92***	3.19*	4.04**	3.17**	5.05***
Strategies for Effective Studying	3.29**	2.00	3.60**	1.46	2.67*	2.79**	3.81***
Money Management	3.94***	2.38*	3.40**	3.07*	2.93*	3.43**	4.49***
Effective Communication Strategies	3.9***	2.42*	3.60**	2.63*	3.22**	3.09**	4.40***
Strategies to Manage Anxiety and Stress	3.24**	2.80*	3.17**	3.13*	3.34**	2.7**	4.21***

**p<0.05; **p<0.01; ***p<0.001. df = degrees of freedom, ORM=Overrepresented Majorities, URM=Underrepresented Minorities*

Discussion

The results presented above support the bridge programs' goal of improving student awareness and understanding of the engineering technology field (ET). First and foremost, results indicated that the bridge program was successfully able to recruit students that are historically underrepresented in STEM and ET fields, which was a primary goal of the program. Regardless of gender, ethnicity, race, or academic level, students entered the program with no statistically significantly different preconceptions of engineering technology and its associated skills, corroborating a general lack of student awareness of the ET field [13-14]. Additionally, across all demographic groups, results highlighted a statistically significant difference in student perceptions of their understanding of ET and ET-related skills. These results indicate that the bridge program was successful in improving student awareness of the ET field through hands-on instruction, labs, and industry tours.

Also, results showed that the students perceived their understanding of their ET industry and college success skills to improve over the bridge program, with a few notable exceptions. Specifically, incoming first-year college students indicated a lack of learning around studying strategies and learning styles. While there was no statistically significant data differentiating the pre-program understanding of learning styles between rising first-year college students (M = 3.70, SD = 0.675) and rising high school seniors (M = 3.32, SD = 1.129), there is a difference in the scores, which corroborates prior research highlighting that study habits develop with age [15]. One potential reason for the lack of significant difference is the high variance of the rising high school seniors cohort. While common in instructional practice, most research evidence suggests there

is little to no benefit of learning styles on student learning. However, research suggests it possibly indicates student preference [16]. Students who are further along in their careers may have had more time to develop a preference for a learning style, or students may have already been exposed to learning styles in their high school courses prior to the bridge program.

Additionally, results indicated that female students did not significantly improve their understanding of studying habits as opposed to their male counterparts. While the pre-test for studying habits was not statistically different between male and female students, female students (M = 3.54, SD = 1.05) scores were higher than their male counterparts (M = 3.35, SD = 0.933), potentially highlighting a discrepancy in studying habits. However, prior literature is split on gender's impact on studying habits, with some literature highlighting that there is no difference across genders [15,17], with literature also highlighting that female students have better study habits [18].

Conclusion

To meet the needs of emerging technologies for an educated workforce in those technologies, high-tech manufacturing, and technician workforces require investment from higher education institutions and industry. Through intentional partnerships, we hope to broaden and diversify the STEM pipeline through programs that increase awareness of engineering technology education programs that can increase the pool of qualified candidates [1]. This research highlights several successful mechanisms within a bridge program out of NOVA to increase student awareness and understanding of ET careers to serve the surrounding area.

Due to the funding and program limitations, the bridge program was limited in the breadth of its student outreach, which we hope to increase in future iterations of the bridge program. Additionally, due to the smaller sample size, we were not able to use more robust methods of statistical analysis, such as MANOVA or multiple regression, to examine how the interactions of demographic groups influenced participants' development throughout the program. Future extensions of this research will examine student and parent preconceptions of the differences between engineering and engineering technology. Additionally, outreach to parents and partnerships with secondary institutions may further develop the pipeline for providing students with a more comprehensive view on the engineering technology careers open to them.

Acknowledgements. This work was supported in part by the National Science Foundation (NSF) under award 2055717. Additional support comes from the Growth and Opportunity Virginia grant program, the NOVA Foundation, and private grant programs to cover participant transportation costs.

Disclosures. The authors declare no conflicts of interest.

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WBL in Two-Year Colleges: What's in a Name?

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Abstract: Workplace-based learning (WBL) provides participants with a valuable experiential learning opportunity to apply knowledge from the classroom to a real-world business or industry location. Yet despite calls to invest in or expand WBL opportunities in two-year institutions, no standard language or definitions appear to exist. Literature and research on WBL in two-year institutions is scant, and what is available suggests a lack of a common lexicon but does not address why it persists. This mixed-method study, using the Advanced Technological Education (ATE) program as its sample, addresses this gap and provides further insight into WBL language. Study results confirm that the language used to define and describe different types of WBL lacks standardization; ATE projects use various terms for WBL opportunities, with no clear pattern of characteristics distinguishing among types of WBL. The choice of terms for particular types of WBL opportunities is driven not by the opportunities' goals and characteristics but by external factors. The response to whether language in WBL matters also varied across the study population. This article concludes by reviewing the potential implications of these findings for research and practice and suggesting what can be done now to capture the impacts of workplace-based learning.

Keywords: internship, apprenticeship, externship, co-op learning, workplace-based learning

Introduction

As part of their commitment to growing the nation's skilled technological workforce, many two-year colleges offer students valuable opportunities to engage in workplace-based learning (WBL) while completing career and technical education programs, associate degrees, and other industry-approved skills- and credential-based pathways to gainful employment. Connecting classroom instruction with the workplace in such a way is particularly beneficial for science, technology, engineering, and mathematics (STEM) fields, where jobs are in high demand, require technical expertise, and feature a knowledge base that is constantly evolving and advancing [1]. As recognition of the potential benefits of WBL grows, federal policy initiatives, industry leaders, and educators have called for sustaining and expanding funding for WBL at two-year institutions and for more research on the impacts of WBL on students' professional and educational outcomes [2-4]. Yet, despite these calls, no standardized definition of WBL exists. Yet, despite these calls, no standardized definition of WBL exists.

Nascent research examining WBL language has identified potential implications arising from this lack of standardized lexicon in community colleges. This work has identified issues such as the challenges for measuring and reporting student outcomes, impediments to advancing WBL research, and barriers to developing shared standards and expectations among industry partners, students, and educational institutions [5]. While this research sheds light on an important issue, especially given calls to diversify and expand WBL opportunities, it does not provide insight into why non-standardized language persists, nor into the perspectives of WBL practitioners on the need for a common lexicon in two-year institutions [5-7]. We conducted a mixed-methods study to address this gap and facilitate conversations about its potential impacts on STEM education. Our study collected and analyzed engagement in WBL activities and nomenclature amongst principal investigators in the National Science Foundation's (NSF's) Advanced Technological Education (ATE) program.

Background

In the existing literature, WBL definitions tend to converge around WBL, serving as an opportunity for students or workers to transfer classroom knowledge to a workplace setting. Examples of WBL definitions from various sources are shown in Table 1. However, the types of models that constitute WBL and how they are defined vary widely in the literature and among practitioners. For example, Cahill [8] reports that different types of WBL include internships, apprenticeships, on-the-job training, co-ops, and transitional jobs tied to classroom instruction and may or may not provide academic credit and payment. Rodriguez et al. [9] likewise identify apprenticeships and internships as WBL, but they also include clinical placements, school-based enterprises, service learning, and community-based learning as types of WBL.

Table 1. Definitions of WBL Across Institutions of Higher Education and National Organizations

Source	Definition
The Academic Senate for California Community Colleges [10]	"an education strategy used to connect classroom instruction to careers by providing students with opportunities to reinforce and make relevant their classroom experiences." (p.1)
Western Piedmont Community College (WPCC) [11]	"an opportunity to receive college credit for on-the-job experience. The work experience, conducted under the direction of WPCC, must be significantly related to the student's program of study."
Community College of Denver [12]	"a teaching methodology that blurs the lines between school and work" and includes activities that "provide a learner with hands-on experience that allows them to apply the knowledge of the classroom in a simulated work environment."
The Federal Partners in Transition [13], a group of federal agencies that formed the National Collaborative on Workforce and Disability for Youth	"a supervised program sponsored by an education or training program that links knowledge gained at the work site with a planned program of study." (p.1)
Jobs for the Future [14], a non-profit examining and promoting workforce development	"an approach to training in which a student or worker completes meaningful tasks in a workplace."

WBL definitions and types of opportunities considered WBL also vary across educational institutions and federal and state government levels. For example, while some institutions characterize an internship as a short-term, unpaid opportunity tied to a specific course, others characterize an internship as a long-term, potentially paid opportunity that does not have to be associated with academic coursework [5, 8, 9]. A review by Giffin et al. [15] at the College and Career Readiness and Success Center at the American Institutes for Research found variations in how WBL activities are identified and described. Their review found that only two state labor or workforce departments (Illinois's and New Hampshire's) had formal definitions for WBL, and 19 out of 23 national organizations that identified WBL as a priority had publicly available definitions for WBL [15]. The closest that any type of WBL comes to having uniform characteristics and using standardized language is the U.S. Department of Labor's (DOL) Registered Apprenticeships [16]. Overseen and recognized by the DOL's Office of Apprenticeships in conjunction with state apprenticeship agencies, DOL Registered Apprenticeships are designed to meet strict guidelines and specific industry standards [16].

Research on WBL has provided little guidance to inform the development of WBL frameworks or definitional criteria, but the potential consequences of unstandardized language have surfaced. One consequence is the inability to measure, understand, or translate WBL outcomes, especially across different contexts and types of WBL. Lucero et al. [4] conducted a literature review examining internship characteristics and program outcomes at community and tribal colleges. Their findings showed that programs utilized a broad range of student and employer outcomes to gauge WBL experiences and found little overlap among the outcomes used by these institutions [4]. Other reviews of WBL outcomes were limited or showed mixed results [5].

In recognition of the lack of clarity for WBL outcomes and few empirical research studies on WBL benefits, some WBL researchers and advocates have made calls to standardize WBL language [5, 8, 10]. Given these calls and limited research on WBL, an opportunity exists to examine perceptions among those responsible for administering or otherwise supporting WBL activities in two-year institutional contexts.

Methods

Research Questions

To better understand WBL in two-year institutions, our study was guided by four research questions:

1. What types of workplace-based learning (WBL) opportunities are offered by Advanced Technological Education (ATE) projects?
2. What characteristics differentiate the types of WBL offered?
3. Why do ATE projects use certain terminology for their WBL opportunities?
4. Does WBL language matter? Why or why not?

Design

We utilized an explanatory sequential mixed-methods design. This involved collecting and analyzing quantitative data, then collecting and analyzing qualitative data to help explain the quantitative results and answer the complete set of research questions [17]. In the first phase of this study, we surveyed NSF-funded ATE projects regarding their WBL practices. In the second phase, we selected a purposeful sample of survey respondents for structured interviews to better understand survey responses.

Population

This study was conducted within the context of the NSF ATE program. The ATE program is particularly well-suited to explore the topic of WBL language, given its reach through funding projects across the United States and U.S. territories and its focus on STEM and skilled technological workforce development in primarily two-year institutions, which frequently offer WBL opportunities as part of their workforce education pathways.

Data Collection

Quantitative data was collected from ATE principal investigators (PIs) through the annual ATE Survey. First launched in 2000, the ATE Survey collects and disseminates data on ATE projects' activities and accomplishments during the previous calendar year. This long-standing data collection mechanism was used to collect data for this study because of its generalizability to the ATE population, given historically high response rates of over 90%.

Data used for this study is from a survey section entitled "Workplace-Based Learning (WBL)," which first appeared in the 2019 ATE Survey. WBL is defined in the survey as a situation in which a student gains experience at a work site. Working Partners Research Project, an ATE grantee, conducted a survey and focus groups of ATE projects in 2016-17, which indicated five different types of WBL. These five types were then permanently integrated into the ATE Survey and included job shadowing, externships, internships, co-op learning, and apprenticeships. Definitions for WBL options in the ATE Survey were intentionally not provided to allow respondents to provide details of their specific activities rather than their labels. For each type of WBL opportunity that they reported offering students, survey respondents were asked to report the number of hours per week and the number of weeks per year that students were engaged, the total number of students who participated, if academic credit or payment was provided, if participation was required by an academic program, and if it was coupled with a specific course(s).

Survey data for this analysis were drawn from the 2020 ATE Survey and represent activities conducted from January 1, 2019, through December 31, 2019. Survey respondents completed the survey between February 2020 and May 2020 online via the Qualtrics survey software. To ensure that the data used in this study was not an anomaly or outdated, we also analyzed data from the WBL section of the 2021 and 2022 ATE Surveys. We found no meaningful differences in the types of WBL opportunities offered, who offered them, or how these opportunities were characterized by respondents (i.e., students received academic credit or payment, participation required, coupled with a course). This indicates that findings from the 2020 ATE Survey align with more recent surveys and are not an outlier.

Given the study's sequential design, the qualitative phase of the study was informed by and conducted after the quantitative phase. We developed a structured interview to clarify survey results and better understand the respondents' applied definitions of WBL opportunities and activities. Interviewees were asked to confirm their survey responses (i.e., type of WBL offered, number of students participating, payment or academic credit provided, participation required or not), describe the origins of the name of the type of WBL offered (e.g., why an internship is called an internship) and how it differs from other types of WBL that they offer. Interviewees were also asked to define each type of WBL opportunity, regardless of whether they offered them or not, talk about their experiences with inconsistent WBL language, if any, and discuss the potential implications of unstandardized WBL language on students, institutions, or the STEM field.

Sample

The survey was a census of the 325 ATE-funded projects identified by NSF in 2020, of which 294 projects responded. Out of 294 projects, 76 indicated that they offered WBL to students, while 218 did not offer WBL. As Table 2 shows, these 76 ATE projects were primarily at two-year institutions distributed across the United States, and they provided programming in a variety of STEM disciplines. Over one-third of ATE projects that offered WBL were located at minority-serving institutions, with most representing Hispanic-serving institutions (n=26) and none representing historically black colleges and universities (HBCUs).

Table 2. Quantitative Sample Characteristics, by Survey Respondent

Region (n = 76)	
South	29
West	25
Midwest	13
Northeast	9
Institution Type (n = 76)	
Two-year college	65
Four-year college	10
Non-profit organization	1
Minority-Serving Institution (n = 76)	
Yes	29
No	47
Minority-Serving Institution Type (n = 29) [a]	
Hispanic-serving institution	26
Native Hawaiian-serving institution	2
Tribal college or university	1
STEM Field (n = 76)	
Information and securities technology	20
Engineering technologies	20
Advanced manufacturing technologies	13
Agricultural and environmental technologies	9
Bio and chemical technologies	7
General or interdisciplinary	6
Micro and nanotechnologies	1

The remaining 47 ATE projects were not minority-serving institutions.

A purposeful saturation sampling strategy utilized six inclusion criteria to identify interviewees from the 76 ATE projects who reported offering WBL on the 2020 ATE Survey. Criteria included number and types of WBL opportunities offered; institution type; ATE project type (e.g., funded as a project or as a regional

center); apprenticeship type (i.e., DOL Registered Apprenticeship or not); number of students served; and type of NSF ATE grant (i.e., project, center, small new-to-ATE). ATE projects were selected based on their ability to meet these criteria. We oversampled ATE projects that offered multiple types of WBL to understand how WBL types were differentiated from one another at the same institution. These criteria and the study design, which emphasizes the ability of qualitative data to provide insight into quantitative findings over a large sample, led to an initial sample of 10 PIs and co-PIs from 10 different projects. Figure 1 provides an overview of the sampling process. They were invited to participate in the study via email. Two PIs did not respond; one PI was no longer associated with the grant, and one PI's email address was no longer active. The final sample consisted of 10 individuals across the six ATE projects. One project included five different institutions that served as sub-awardees, and it was decided to interview one person at each institution because of their diverse geographic locations and autonomous operating structures. Please note that subawardees do not complete the annual ATE Survey, so these five institutions are not represented in Table 2. After interviewing all ten individuals and analyzing the resulting data, no additional information was revealed, indicating that our sampling strategy was achieved. As a result, no additional ATE projects were interviewed [18].

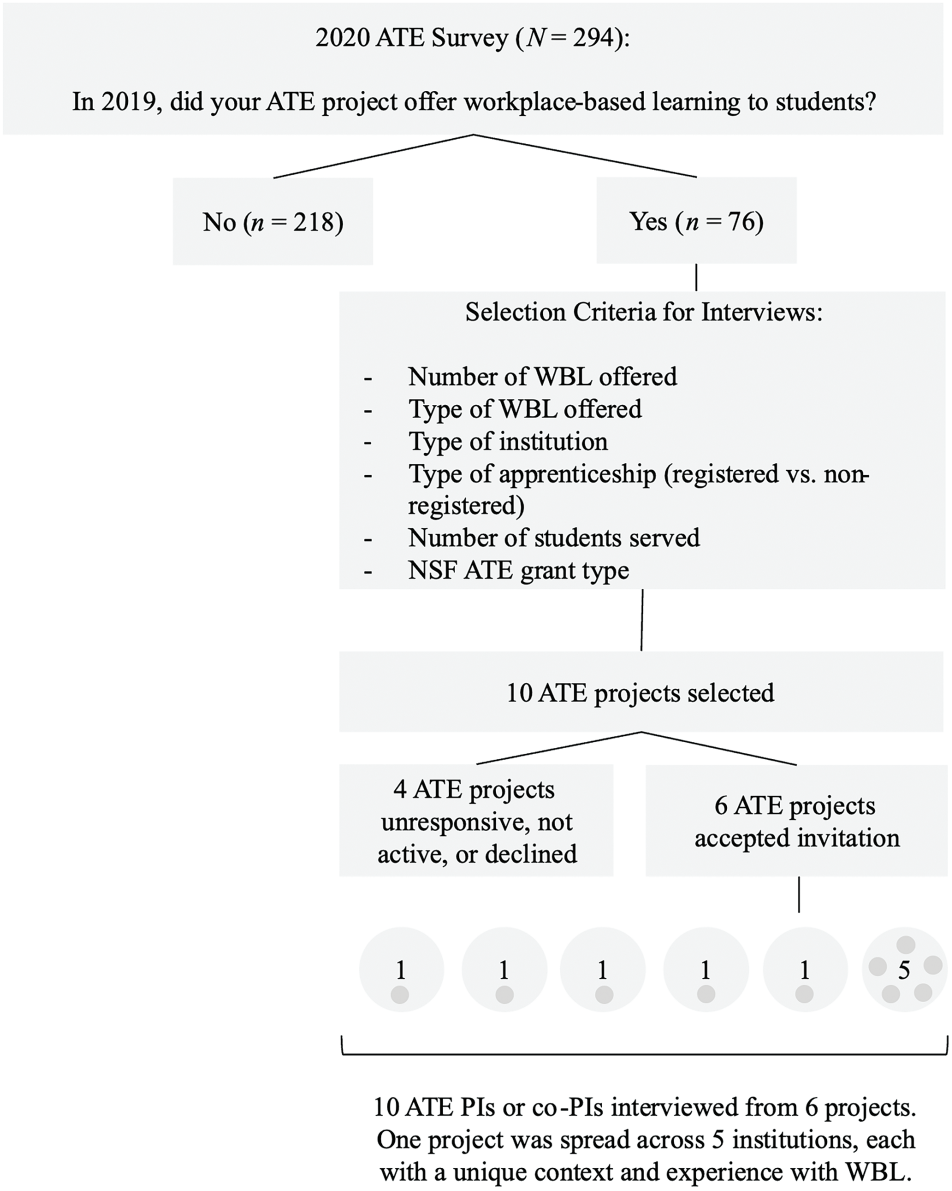

Fig. 1. Final sample selection for qualitative interviews with PIs using the 2020 ATE Survey

Table 3 displays the characteristics of the final sample of 10 interviewees and shows that they represented a range of STEM disciplines and were located in diverse areas. Most offered one type of WBL, primarily internships.

Table 3. Qualitative Sample Characteristics, by Interviewee

Region (n = 10)		
South		3
West		1
Midwest		1
Outside of continental USA		
American Samoa		1
Mariana Islands	1	
Marshall Islands	1	
Micronesia	1	
Palau	1	
Institution Type (n = 10)		
Two-year college	9	
Four-year college	1	
Offered Multiple WBL Types (n = 10)		
Yes		2
No		8
Types of WBL Offered by Project (n = 10)		
Internship		8
Externship		1
Co-op learning		1
STEM Field (n = 10)		
Information and securities technology	1	
Engineering technologies	1	
Advanced manufacturing technologies	1	
Agricultural and environmental technologies	5	
Bio and chemical technologies	1	
General or interdisciplinary	1	

Data Analysis

We analyzed survey data using SPSS. Analyses were primarily descriptive, including frequencies and cross-tabulations. Interview data were analyzed using MAXQDA 2020 software. One author served as the primary coder and utilized a two-cycle inductive coding process that employed both concept coding and descriptive coding before identifying patterns in the codes and aggregating them into larger thematic categories. Descriptive

coding uses short, descriptive words or phrases to label themes within qualitative data to provide an overview of topics and ideas discussed [19]. Concept coding allows analysts to identify underlying constructs, or concepts, that are at play in the phenomenon being studied to form a broader understanding [19]. The second and third authors reviewed all codes, categories, and patterns that emerged for accuracy and reliability.

Results

In this section, we provide a summary of results by research question.

What Types of WBL Are Offered by ATE Projects?

Survey data indicated that the majority of WBL offered by ATE projects fell into four groups: internships (82%), co-op learning (18%), job shadowing (17%), and apprenticeships (16%) (Figure 2). Only 3% of ATE projects offered externships to students. Except for externships, a mix of STEM fields was represented across WBL types.

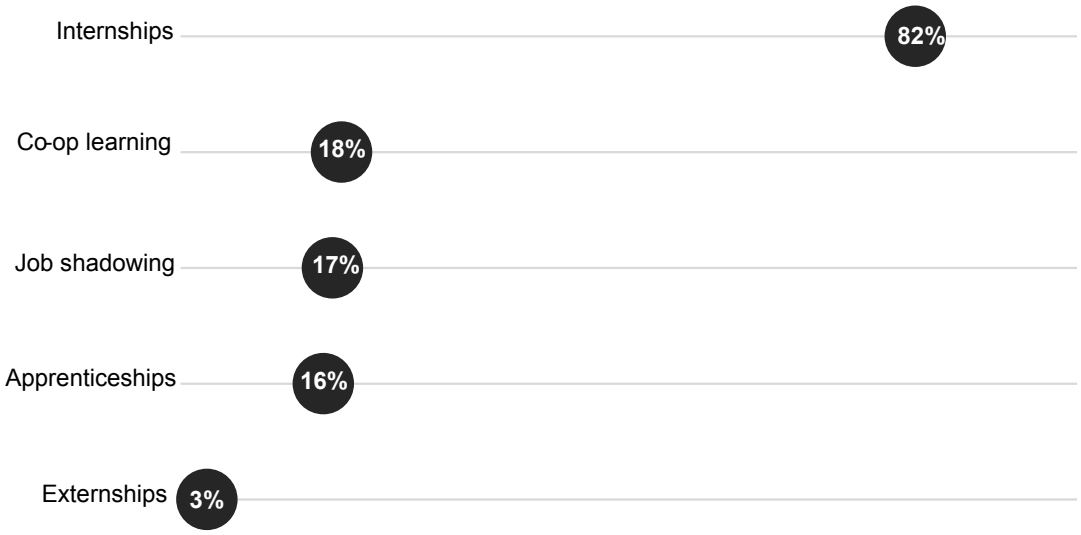

Fig. 2. Percent of Survey Respondents Offering WBL by Type (n=76)

What Characteristics Differentiate the Types of WBL Offered?

Survey results indicated that DOL Registered Apprenticeships and job shadowing came closest to having the same characteristics identified for them across different institutions. As shown in Table 4, a majority of ATE projects offering DOL Registered Apprenticeships coupled these opportunities with specific courses, academic credit, and payment. A majority of ATE projects participating in job shadowing did not offer students payment or academic credit, required participation, or couple the WBL opportunity with specific courses. Co-op learning tended to provide students with academic credit for participation, but no other common characteristics emerged. No clear pattern of characteristics emerged for internships, as 50% to 60% of ATE projects indicated each characteristic. With only two ATE projects reporting on externships, larger implications about characteristics for this WBL type cannot be meaningfully drawn.

Table 4. Characteristics of ATE Projects' Workplace-Based Learning Opportunities (n=72)

WBL Type	Total number of respondents engaged in this type	Students received payment	Students received academic credit	Coupled with specific course(s)	Participation required by program
Internships	62	65%	66%	55%	53%
Co-op learning	14	50%	71%	57%	64%
Job shadowing	13	15%	23%	23%	38%
Apprenticeships					
Non-registered	4	25%	25%	50%	25%
Registered	8	75%	100%	88%	50%
Externships	2	100%	100%	100%	100%

While some loose patterns may emerge from the characteristics held by ATE WBL opportunities, there is enough variation to throw into doubt whether the experiences that students receive are similar from one internship to another, or one co-op learning opportunity to another.

Similar to WBL characteristics, the number of hours and number of weeks per year that students engaged in WBL was not a reliable differentiator among different types of WBL opportunities. The box and whisker plots in Figure 3 show that the number of hours students engaged in different types of WBL opportunities varied, with apprenticeships, internships, and job shadowing having the largest range. The average time commitment was highest for apprenticeships (964 hours per year, approximately 18.5 hours per week). Apprenticeship time commitment also varied the most widely among programs, with a minimum of 320 hours per year (6 hours per week) and a maximum of 2,080 hours per year (40 hours per week). In comparison, internships required an average of 237 hours per year (4.5 hours per week), co-ops an average of 123 hours per year (2.3 hours per week), and job shadowing an average of 108 hours per year (2 hours per week).

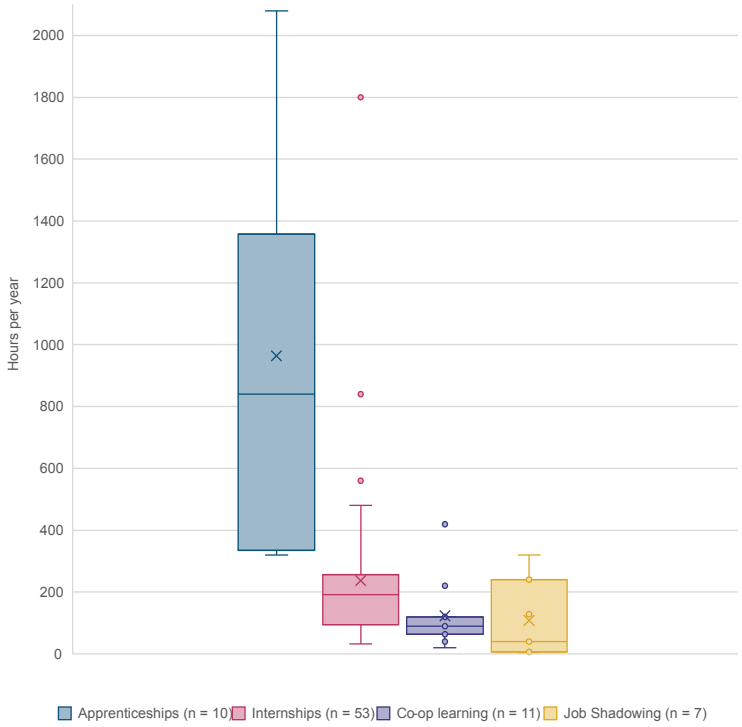

Fig. 3. Number of Hours per Year Students Engaged WBL Opportunities by Type

Interviews largely mimicked survey results and showed a lack of differentiation among the different WBL types. When asked to define the terms "internship," "co-op learning," "job shadowing," "apprenticeship," and "externship," regardless of the type(s) of WBL their own project offered, interviewees struggled to identify definitions, especially for externships and co-op learning. For example, when asked to define externship, one interviewee responded, “I've never heard that term before. I don't know what to think,” while another interviewee noted, “I've never personally used that, and I've never run across it.” Out of all WBL types, apprenticeships came closest to being defined consistently across different institutions compared to other WBL types. A majority of interviewees defined apprenticeships as closely aligned with a particular industry and identified them as longer-term, structured opportunities that may be connected to certification or credentialing programs.

In contrast with the ATE Survey, which used characteristics such as receipt of payment or academic credit to define and describe different types of WBL, interviewees stressed the purpose or goal of the opportunity. For example, four interviewees reported that internships were opportunities to gain hands-on experience. As one interviewee said, internships are a "hands-on activity to upgrade their skills" that helps ensure that "their skills aren't stuck" when they get into the real world. Two interviewees noted that an apprenticeship led to a specific credential. As one interviewee noted about apprenticeships, "I think about a structured long-term program leading to a state or other government-endorsed credential." Interviewees reported the same purpose or goal for various types of WBL, which was to enhance or develop participants' skills.

Interviewees did not view WBL opportunities as activities that targeted students enrolled in academic programs. For example, one interviewee characterized an apprenticeship as representing “a specific rank and level” (more specifically, as an apprentice, “you are a beginner at this job, and you are in that job”) rather than an opportunity directed toward degree-seeking students. One interviewee defined externships as activities to enhance educators’ skills rather than students’: “Teachers will go out and do an externship in the summer to enhance their abilities.” Thus, as in the survey findings, no clear pattern of characteristics that were cited by interviewees, such as purposes or goals and target audience, emerged that would help differentiate WBL types from one another.

Why Do ATE Projects Use Certain Terminology for Their WBL?

To explore how naming conventions of WBL types arise, each interviewee was asked to identify the origins of nomenclature used for the WBL types supported by their project. More specifically, why is an internship, for example, called an internship, and has it always been called an internship? Two patterns emerged from this analysis.

First, interviewees reported that what a WBL opportunity is called in their institution is driven by its ability to facilitate understanding and communication among students, faculty, or external entities. "Internship is something that's kind of standard across the board," one interviewee reported, "I think a lot of people understand what that is, and so it makes sense that they would use that." The understanding facilitated by terminology may also be rooted in the institutional history of a school or program, reinforcing and normalizing its usage. As one interviewee stated, "We've only ever referred to it as an internship." Another remarked that the WBL terminology was "definitely what it was called before I came in; I believe that it's written into the grant as an internship."

A second pattern revealed that what a particular type of WBL opportunity is called is dictated by terminology used by external organizations, specifically employers. Academic programs' language matched the various lexicons used in the industry settings they worked. As one interviewee succinctly stated, “I use the business term ‘internship’ because they like that.” Thus, the nomenclature used to differentiate one WBL type from another is partially dictated by its ability to easily translate into the familiar language of the workplace or employer.

Does WBL Language Matter? Why or Why Not?

Interviewees offered arguments both supporting and negating the idea that the names and terms used to describe and identify types of WBL matter. The most frequently cited reason WBL nomenclature matters, identified by four interviewees, was that it provides standardization that facilitates shared understanding and application across contexts. One interviewee suggested that common terms help to facilitate the assessment of students’ experiences and the transfer of credit when changing academic programs, stating, “If you don't have

a clear outline and definition of what something is, I, as chair of assessment, cannot approve that as a transfer from that institution to this institution, unless I know exactly what it means." Another interviewee noted that standardized language "could help clarify what students are doing" in a WBL opportunity and thus facilitate shared expectations with industry partners; this interviewee explained, "If we can't communicate correctly to our community partners what we want our students to do, how are they - how are we - expecting them to actually do it?"

Standardization also fosters an understanding of what WBL participants have achieved upon completion of the activity. One interviewee likened the need for WBL standardization and the clarity it provides to the benefits provided by other standardization efforts occurring across academia. They compared it to "the whole micro-credentialing concept, where things are sort of standardized, and I know this person has 'x' credential, so I know exactly what they've done, I can look at the outcomes that they've achieved."

Standardizing WBL language was also seen as potentially beneficial for educators who pursue professional development related to WBL. One interviewee noted that if the terms and characteristics for various WBL types were consistent, newly acquired information could more easily be shared across one's home institution and used to support the building of WBL effectiveness.

Five interviewees noted that specific language is not always demanded in certain contexts or environments, and as such, language concerning WBL language is not of concern. In fact, using precise or specific language for WBL that would distinguish WBL types was seen as potentially leading to administrative burdens and other challenges. As one interviewee, in speaking of a multi-institutional collaboration, noted, "Because of the different challenges each college has ... and the way that it still works ... is to leave as much flexibility in the wording so that each school can use that allotment of internship funding for what works for their school in their situation."

Two interviewees felt that language matters less than the opportunities that WBL offers students, with one stating, "As long as there's something going on, and something [students] can benefit from and learn from, I don't care what you call it." Thus, while interviewees identified reasons why WBL language mattered, they also identified reasons why uniform language was not needed, indicating a lack of consensus on the topic.

Discussion

Similar to WBL definitions in the literature and those used in practice by educational institutions and government bodies, findings from this study indicate that WBL opportunity types are not clearly or consistently differentiated from one another. As evidenced by survey results, while some loose patterns emerged for the characteristics of WBL types reported by the ATE community, there was enough variation to prevent clear, defining characteristics that would distinguish one WBL type from another. For example, at least half of all internships and co-op learning opportunities offered students payment and academic credit, were coupled with a specific course, and were required by the academic program. The lack of clearly defined types of WBL opportunities contributes to the lack of standardized language that characterizes WBL. The only type of WBL to demonstrate consistency in survey responses was DOL Registered Apprenticeships, a consistency that may be tied to the guidelines and regulations set forth by the government for these opportunities.

Interviews with a subset of survey respondents confirmed the absence of any unifying theme in definitions of WBL types across different institutions. When asked to define five types of WBL, interviewees' answers described the purpose of the opportunity or whom it targeted (e.g., students, displaced workers) rather than features such as payment or academic credit received as asked about in the survey. Like survey results, no clear pattern emerged in interviews that would distinguish WBL types, even when interviewees used their own framework to define WBL types.

The lack of standardization of definitions for types of WBL reflects the varying institutions, policies, and industrial environments that support them. Interviews revealed that WBL nomenclature is often a function of external conditions, such as historical norms or administrative restrictions, or mimics terminology that is easily translated to industry partners rather than being descriptive of the WBL activity itself. As a result, WBL terminology is context-dependent and fails to detail common, standardized features of an activity that would help distinguish the goals of one WBL type from another. Internship characteristics, for example, were defined differently by survey respondents, and their purpose, as articulated by interviewees, also varied. Thus, the purpose of internships, whom they engage and why, the skills developed, or the outcomes targeted may

vary widely from school to school and even program to program. This reiterates previous research that found a lack of shared WBL definitions at state, national, and organizational levels while providing new insight into why and how this is occurring [15]. Rather than being grounded in a well-defined body of literature that has reached a basic level of consensus among scholars and practitioners about what does and does not constitute a particular activity, WBL language emanates from the needs and requirements within a specific two-year institution or program.

When we asked interviewees whether variation in WBL nomenclature mattered, as with definitions of WBL types, no clear picture emerged. Some interviewees noted that students and faculty benefit from standardized language as this facilitates a shared understanding of the purposes of different opportunities clarifies expectations for both students and industry partners, and also allows school administrators, students, and faculty to translate their experiences to transfer programs and the marketplace. Thus, standardized language is viewed as benefiting multiple groups and individuals engaged in WBL while helping participants achieve shorter-term outcomes, such as attaining academic credit, or longer-term outcomes, such as earning transfer credit or recognized industry experience. This suggests that language is connected in meaningful ways to aspects of the opportunity itself. Conversely, other interviewees noted that WBL nomenclature did not pose barriers for participants, and standardization may create adverse effects for administrative processes that are bounded by specific rules and terminology. Interestingly, none of the interviewees cited assessing WBL outcomes for students, faculty, or industry partners on an individual or broader level, such as across their department, institution, or region, as a reason to standardize WBL language or not. Instead, answers were focused on the individual level.

Given the variation in definitions and naming conventions of WBL opportunities, differences in respondents' opinions concerning whether these differences mattered are not surprising. Standardization has never occurred in WBL across two-year institution landscapes in the United States, and given the scarcity and recency of literature examining WBL nomenclature and its various implications, a culture of standardization in this arena is absent. Policies funding WBL have also failed to set an expectation or highlight the need for standardized language, although some school administrators are calling for change [3, 20]. Further, failing to address the potential implications of WBL language on students' employment and educational trajectories impacts many individuals, especially female, first-generation, low-income, and racial and ethnic minority students, whom community colleges disproportionately serve [6].

Conclusion

Results from this study have important implications for school administrators, educators, and WBL researchers within and outside of the ATE community. Advocates of WBL have called for more research examining the outcomes of student and program participation in WBL [6, 15, 21]. Understanding WBL outcomes in two-year institutions is particularly important because programs offered in these settings focus on preparing a diverse student body to transition directly into middle- or high-skilled jobs, such as those in STEM. However, a lack of consensus on WBL definitions and nomenclature hampers research efforts to identify and scale best practices across diverse contexts and support the development and achievement of a range of WBL outcomes [5, 21]. For example, different educational settings and programs may identify and track the same outcomes, but if differing definitions of WBL are utilized, aggregating these outcomes to draw meaningful conclusions or lessons learned is not plausible. Previous research has already surfaced this issue by finding either no outcomes being reported or a lack of uniformity among them [5, 17].

The ability to demonstrate WBL outcomes, such as benefits to students, industry employers, and local economies, is particularly important given recent calls from both industry and educators to sustain and expand funding for WBL in two-year institutions [6, 9, 21]. Federal policy initiatives such as the Workforce Innovation and Opportunity Act of 2014 and Perkins V have renewed past efforts to expand the school-to-work pipeline, while government programs, such as NSF's ATE program, have helped strengthen technician education and student opportunities such as WBL [1-3, 22]. These calls for expanded or continued funding for WBL will inevitably be accompanied by requirements to collect evidence of program impact and outcomes. Meaningful WBL outcomes necessitate standardized WBL definitions and language, and without them, the ability to build an evidence base for WBL benefits is limited.

Although formal, uniform terminology for various types of WBL is unlikely to occur in the immediate future, current work in this area can begin now. School administrators and faculty can begin by clarifying the purposes and characteristics of WBL opportunities offered at their institutions and then look more broadly. Are expectations aligned across schools, students, and industry? How do these experiences and the language used to describe them map onto industry credentials and requirements? Addressing these questions sooner rather than later is advantageous, given the demand for STEM jobs, quickly evolving industry needs, and growing support and interest in WBL opportunities in two-year institutions. Given the experiential nature of WBL, business and industry leaders should also be involved in answering these questions and discussing WBL terminology and any efforts to standardize it.

Lastly, school administrators, industry, and others involved in overseeing or engaging in WBL could also explore developing a classification system for WBL types that would allow students to meaningfully communicate their experiences and researchers to study them without creating or standardizing language. A classification system could include categories such as payment or academic credit received, placement types (e.g., early or advanced program internships), duration (i.e., short or long-term), and rotation (i.e., stays at same employer or engages with multiple employers). This type of system could be instituted within a department or program, institution, or even on a broader level such as a region. Doing so would allow for a meaningful comparison of outcomes (e.g., student, employer) and, in doing so, a way to demonstrate WBL's impact.

Limitations

This study is not representative of all two-year institutions or STEM disciplines in the United States. While our work confirmed previous findings about WBL language and uncovered potential reasons why inconsistent terminology may be occurring, it does not capture the extent of ambiguity around naming conventions of WBL types nationwide. We also recognize that five interviewees were drawn from the same ATE project. Despite differences among them, such as the different locations and institutions, there may be similarities that were not accounted for.

Acknowledgments. The authors would like to thank the ATE community for their participation in the ATE Survey and the interviewees who volunteered their time and insight into making this study possible. This work was supported by the National Science Foundation (NSF) under award 1841783 and award 1931215 . Any opinions, findings, conclusions, or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

Disclosures. The authors declare no conflicts of interest.

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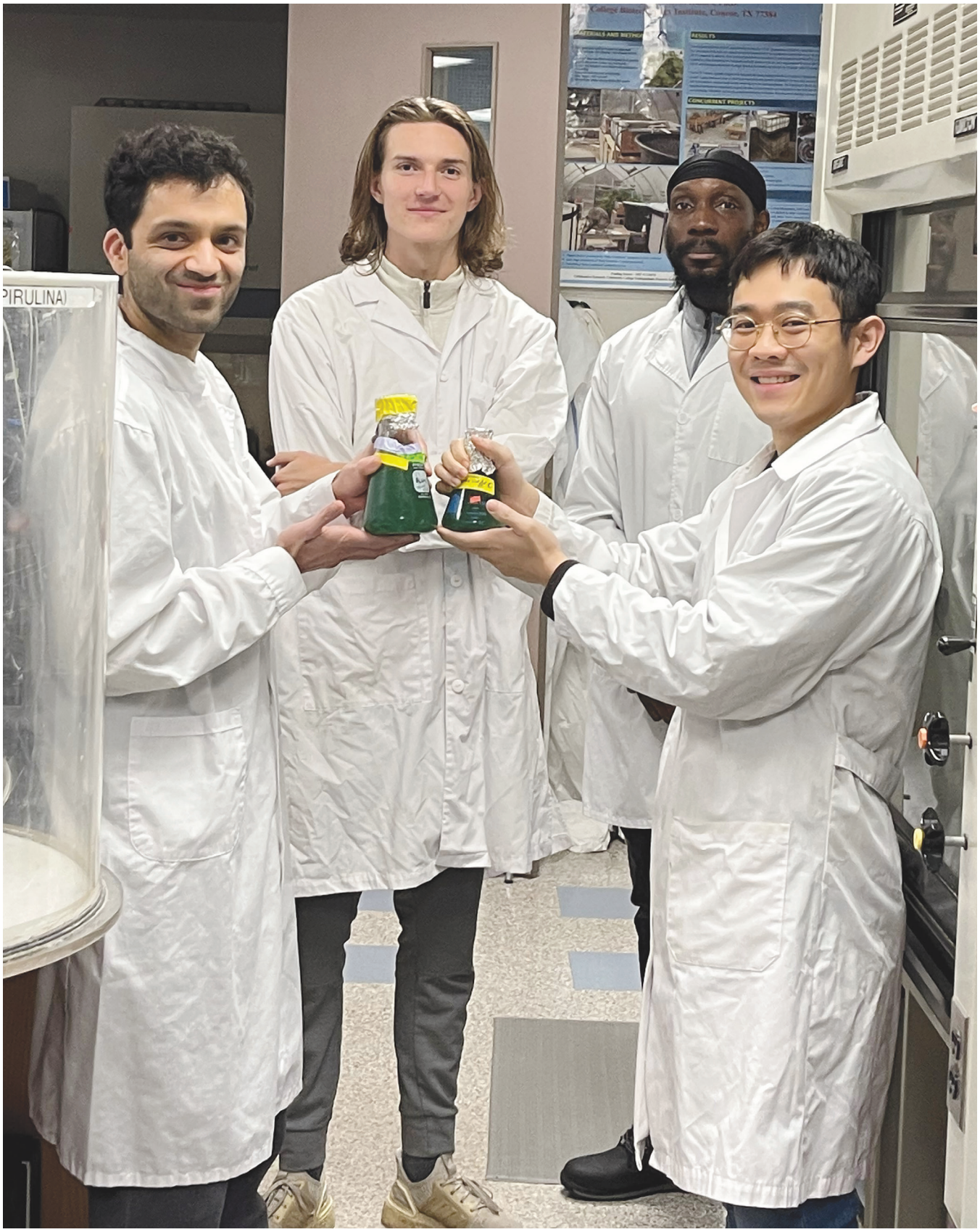

Lone Star College students prepare reagents in support of insect-mediated plastic degradation research. From left to right, they are Ranjit Inamdar, Landon Sanz, Nathanael Salako, and Thien Tran.

Potential of *Tenebrio molitor* and *Zophobas morio* in Plastic Degradation: Mechanisms, Microorganisms, and Enzymes

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Abstract: Plastics have become a central part of society, yet their benefits are short-lived compared to the enduring environmental impact caused by their resistance to biodegradation. Their persistence endangers natural ecosystems and all living creatures, infiltrating every part of the human food chain. Hydrolyzable plastics have functional groups that make them more susceptible to degradation, and as such, much progress has been made in understanding the factors and mechanisms that ultimately lead to their degradation. On the other hand, non-hydrolyzable polymers are devoid of functional groups, which has made elucidating their mechanism significantly more challenging, and consensus in literature can be sparse. Degradation by microorganisms has grown in popularity as a potential solution, but the rate of degradation is extremely slow in the environment. Interestingly, the larvae of *Tenebrio molitor* and *Zophobas morio* have been found to be able to degrade various resistant polymers at much higher rates than microorganisms alone. Although their ability is closely tied to their gut microbiome, their high rates of degradation are ultimately dependent upon the synergistic relationship between the host insect and gut microbiome.

Keywords: plastic, degradation, *Zophobas morio, Tenebrio molitor,* mealworm, superworm, enzymes, microorganism, mechanisms, biodegradation mechanisms, biodegradation enzymes, biodegradation, biodegradation microorganism, plastic biodegradation

Introduction

Since their widespread adoption in the mid-20th century, plastics have become integral to economic development and societal progress. They have been ubiquitous across various industries: packaging, construction, healthcare, and electronics [1]. To date, humanity has produced approximately 6,300 million metric tons of plastics [2]. This proliferation of plastics has been driven by their durability, lightweight nature, and low production costs, making them indispensable in modern life [3]. However, those characteristics make them environmentally catastrophic [4]. Plastics decompose in anywhere between a few decades to thousands of years, depending on the type of polymer, size, and environment in which they accumulate. Even then, they do not disappear; they physically break into smaller pieces known as microplastics [5–7].

To mitigate the ecological impact of plastic waste, effective recycling strategies are essential. Recycling methods are categorized into four main processes: primary, secondary, tertiary, and quaternary. They range from mechanical to energy recovery [8]. Primary recycling, or closed-loop recycling, repurposes plastic waste into equivalent products while maintaining original quality. Although this method is low-cost, it is limited by diminishing quality after several reuse cycles and is only applicable to industrial materials that have minimal contamination [9]. Secondary recycling involves cleaning, melting, and remolding plastics into lower-quality products with limited applications [10]. Tertiary recycling breaks down long hydrocarbon chains of polymers into monomers and oligomers, which can be used to synthesize new plastics, chemical feedstock, and fuels. Methods like pyrolysis, methanolysis, and glycolysis are employed in tertiary recycling but are often resource-intensive, costly, and environmentally challenging [11–14]. Quaternary recycling converts plastic waste into energy through processes like incineration. This method is primarily used to generate electricity or heat from the combustion of municipal solid waste. While it reduces landfill volumes and recovers energy, it faces criticism for its environmental impact due to emissions and ash residue requiring proper disposal [15–17].

Despite these varied recycling strategies, 79% of plastic waste ends up in landfills or the environment, 12% is incinerated, and only 9% is recycled. This low recycling rate is due to several factors, including contamination of recycling streams, economic challenges, and the technical limitations of current recycling methods. Consequently, innovative strategies are urgently needed to enhance plastic recycling rates. The production of plastics continues to grow at an alarming rate of 8.4% annually [2]. In 2022 alone, an estimated 400 million metric tons of plastic were added to the global total [18].

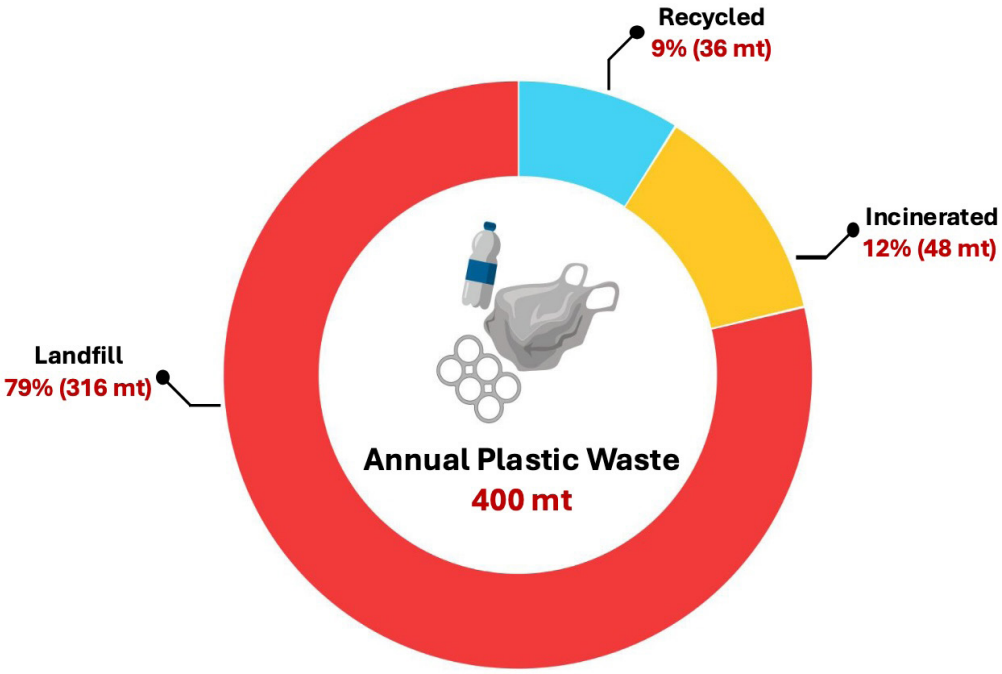

Fig. 1. Fate of Annual Plastic Waste

In this quest, the potential of biological degradation by organisms has shown promise. Various species, ranging from microbes to larger organisms, have demonstrated the ability to break down plastic materials [5,10,19,20]. For example, *Ideonella sakaiensis* can break down polyethylene terephthalate (PET) into components for new polymer synthesis [21]. Other studies have found similar results in two highly active enzymes isolated from the bacterium *Thermobifida fusca*, and LC-cutinase. Slight enzyme modifications resulted in a weight loss of 19.2 mg (42% of the original total) in PET films over 50 hours [22].

Despite the promising biodegradation avenues being explored, there are still concerns that current microbial degradation approaches are too slow. Additionally, most studies that have close to elucidating mechanisms behind biodegradation have been done on hydrolyzable plastics, namely PET. Non-hydrolyzable plastics are much less susceptible to more typical biodegradation pathways because they lack functional groups. This makes them comparatively much more difficult to degrade, and as such, very few enzymes have been identified. Given that non-hydrolyzable plastics constitute the majority of global plastic production, ongoing research into their biodegradation is essential [1,2].

Interestingly, a few organisms capable of degrading plastics have come to light and shown much higher rates of degradation. *Tenebrio molitor* and *Zophobas morio (=Zophobas atratus)* are commonly known as the mealworm and superworm, respectively. Their degradation capabilities ultimately depend on their gut microbiome, as the use of antibiotics halts degradation abilities in most cases. However, isolates of the gut microbiome alone show slower rates of degradation, suggesting that the gut itself is an important contributor.

The process of plastic degradation in the two species includes physical chewing, microbe colonization, enzyme secretions, bioemulsifying agents, assimilation into biomass, and subsequent metabolism. This complex system has been termed as a "bioreactor" [23]. The co-dependent synergistic effects of the gut microbiome and the host itself offer an interesting perspective on the possible mechanisms behind the biodegradation of more recalcitrant polymers. This review aims to provide comprehensive yet accessible information on all relevant aspects, enabling independent researchers to explore their hypotheses regardless of their prior experience in this field. It does so by offering an overview of plastic degradation, *T. molitor, Z. morio*, mechanisms, key potential enzymes and microorganisms, techniques to evaluate biodegradation, factors affecting consumption, and responses of *T. molitor and Z. morio* to plastic. By synthesizing current research and identifying gaps, this review advances the field while also serving as a valuable resource for researchers across various disciplines.

Methods

To gather relevant literature for this review, a comprehensive search was conducted using ScienceDirect, Google Scholar, and Scopus. No date restrictions were applied to capture the full development of the literature, but experimental data on degradation were primarily drawn from articles published between 2013 and 2024. Articles were included regardless of cost of access. Only articles originally in English were included. Abstracts were screened for relevance, prioritizing studies with experimental data on *T. molitor and Z. morio* related to plastic degradation. Reviews and theoretical papers were excluded unless they provided significant insights into degradation mechanisms, plastic pollution, alternative uses, techniques, or ecology. Limitations include inconsistent experimental conditions, language bias, and potential publication bias. The following keywords were employed either individually or in combination with "*Tenebrio molitor*" and/or "*Zophobas morio*": "plastic degradation," "biodegradation," "microbial degradation," "polymer biodegradation," "plastic waste," "plastic waste management," "polystyrene," "PS," "polyethylene," "PE," "non-hydrolyzable plastics," "hydrolyzable plastics," "polymer," "microplastic," "microplastic pollution," "plastic consumption," "plastic degradation rates," "depolymerization," "depolymerization mechanisms," "enzymatic degradation," "oxidative degradation," "biofilm formation," "biodeterioration," "functional groups," "molecular weight," "carbon mass balance," "mineralization," "biodegradation pathways," "bioremediation," "circular economy," "mealworm," "superworm," "insect degradation," "gut microbiome," "gut bacteria," "microbial communities," "microbial colonization," "microbial enzymes," "enzymes," "hydrolase," "cutinase," "lipase," "alkane hydroxylase," "monooxygenase," "cytochrome P450," "enzyme activity," "reactive oxygen species," "oxidative cleavage," "bioemulsification," "emulsifying agents," "microbial adherence," "microbial isolation," "plastics in the gut microbiome," "nitrogen fixation," "bioreactor," "chewing plastic," "carbon sources," "isotopic analysis," "biomass assimilation," "hydrocarbon chains," "synthetic microorganisms," "biodegradation efficiency," "temperature," "nutritional supplements," "review," "biodegrading organisms."

Results and Discussion

Understanding the Plastics: PS and PE

Of the vast different types of plastics, polystyrene (PS) and polyethylene (PE) are some of the most resistant to degradation and will be the focus of this review. PS consists of a linear chain of carbon atoms with a phenyl group attached to every second carbon atom. PE consists of a linear chain of carbon atoms with two hydrogen atoms attached to each carbon. These polymers are less susceptible to degradation for several reasons. Most importantly, they lack hydrolyzable groups such as carbonyl, amide, and carbon-alkene bonds. Hydrolysable bonds are common targets for enzymatic attack by the likes of hydrolase, cutinase, lipase, etc [24, 25]. Without the ability to attack said functional groups, initial degradation is largely limited to terminal depolymerization. The ends of the hydrocarbon chains are oxidatively cleaved into shorter chains (oligomers, dimers, and monomers) via enzymes that have high redox potential, resulting in broad or limited-extent depolymerization, depending on the molecular weight [25–29]. Additionally, the absence of hydrolyzable groups also increases hydrophobicity, making it difficult for enzymes and microorganisms to interact with the plastic surfaces effectively [30, 31].

High molecular weights (MW) also contribute to resistance, as they are often too large to penetrate microbial cell walls where they would be further degraded by intracellular enzymes [24, 32]. Additionally, polymers consist of regions with tightly packed molecular chains, known as crystalline regions, and regions with loosely and randomly packed molecular chains, known as amorphous regions. Amorphous regions are more prone to enzymatic attack [33]. Therefore, polymers with high crystallinity, which have more tightly packed chains, are more resistant to biodegradation, also known as the chain-flexibility hypothesis [34].

Finally, additives are often incorporated into polymers during manufacturing to enhance their properties and tailor their uses, frequently increasing stability. One of the most common types of additives are stabilizers [35], including antioxidants, which inhibit or delay oxidative degradation [36]. Additives have also faced scrutiny due to their documented human health risks [37] and the generally apparent lackluster control of their usage [38]. Compounding this issue is the fact that additives are not covalently bonded to the polymers, making them prone to leaching into the environment over time [39, 40]. The spread of harmful additives is exacerbated by the increasing prevalence of microplastics (MPs), defined as plastic particles under 5mm [38–40]. MPs can be categorized into primary MPs, directly released into the environment through industrial activities, and secondary MPs, formed from the gradual degradation of larger plastic pieces [41, 42]. Secondary MPs are the most abundant, and the effect on marine life is well-documented. Some studies on commercially sold marine life documented that up to 100% of the analyzed fish contained MPs, most notably in Southeast Asia [43–48]. The widespread presence of MPs thus facilitates the broader distribution of toxic additives, heightening environmental and health concerns and warranting more research into how plastic pollution can be controlled.

Mechanism of Biodegradation

A distinction must be made between the terms "consumption" and "biodegradation." Consumption, in the context of these studies, refers to the act of an insect ingesting a feed, the feed traveling through the digestive system and then being egested. It is measured by the difference in the mass of the feed before and after insect inoculation. Consumption does not indicate whether the feed's chemical structure has been modified and broken down by biological means; this is part of biodegradation. Biodegradation encompasses many complex processes that are necessary to understand. The general mechanisms of biodegradation are becoming increasingly understood, with new insights consistently emerging through the study of host insects.

Biodegradation through insects can be broken down into a few key steps: (1) Biodeterioration of the surface and structure of the polymer, increasing its susceptibility to enzyme attack and facilitating microbial attachment and colonization. First, environmental factors such as UV radiation, heat, moisture, pH, salinity, and atmospheric pressure modify the crystallinity, molecular masses, hydrophobicity, and functional groups of the polymer [25, 49]. Next, physical chewing by insects increases surface area, roughness, porosity, and pore size, facilitating microbial colonization [49, 50]. (2) Microbes adhere to the polymer surface and create biofilms, which are effective strategies for supporting growth on hydrophobic surfaces [50–52]. (3) The host secretes emulsifying agents that are able to coat and separate, thus creating more surface area on hydrophobic plastic particles for microbes to colonize further. These secretions are independent of the gut microbiome [53]. (4) The surrounding colonizing microbes and host secrete extracellular enzymes such as alkane hydroxylase, alkane monooxygenase, cytochrome P450, monooxygenase, flavin-binding monooxygenase, aromatic ring hydroxylase, lipases, depolymerases, esterases, proteinase K, cutinase, urease, and dehydrase, which depolymerize polymers into small fragments such as oligomers, dimers, and monomers [26, 54–58]. (5) The newly formed cleaved fragments then permeate through the microbial membrane into the cell. (6) Intracellular enzymes further oxidize and break down these fragments into fatty acids. (7) Fatty acids are then assimilated into microbial biomass or oxidized to go through metabolic pathways, forming mineralized end products. Mineralization typically results in H_2O under aerobic conditions, CH_4 under anaerobic, and CO_2 under either [57–59].

Techniques to Measure and Confirm Degradation of Plastics

Fourier-transformed Infrared Spectroscopy (FTIR) tracks chemical changes on the polymer surface and the formation of functional groups that result from biodegradation, thus characterizing modifications in the polymer structure. Typically, any peaks associated with a digested plastic but not with the undigested plastic feedstock will be evidence of biodegradation. However, FTIR results can be skewed by additives that may have been released during degradation, so special care must be taken during analysis to control for this, typically

with pre-treatment. Researchers have found that PS digested by *T. molitor* exhibits weaker peaks in regions characteristic of benzene rings, suggesting ring cleavage. Additionally, studies have found a broadening of peaks associated with hydrogen bonds of carboxylic acid and hydroxyl groups, which are not typically present in PS [60–62]. This indicates oxidation and chemical changes consistent with biodegradation.

Proton nuclear magnetic resonance (^{1}H NMR) determines molecular structure with respect to hydrogen atoms within molecules. It is used to characterize degradation by identifying new peaks in digested PE associated with alkene bonds and new peaks in digested PS associated with oxygen incorporation [62]. Similarly, new alkene bonds have also been identified in digested polyvinyl chloride (PVC) [63]. None of these peaks are found in their respective natural chemical structures, indicating a chemical change and, thus, degradation.

^{13}C Cross-polarization/magic angle nuclear magnetic resonance (CP/MAS NMR) enhances the signals of low-sensitivity nuclei in solid-state samples. It has identified phenyl derivatives and possible indicators of fragments produced during the depolymerization of PS [23]. Another study used CP/MAS NMR to detect weaker intensities for the resonance signals assigned to the PS carbons in the frass spectrum, indicating a decline in the PS content in the frass [64].

Thermogravimetric analysis (TGA) can provide evidence of degradation through composition analysis. The composition changes are measured by changes in the mass loss of plastic over a range of temperatures. Studies using this technique find plastics that were digested by *T. molitor* or *Z. morio* have different weight loss rates than undigested plastic, specifically, the thermal stability decreases. For example, in one study using both insects, feedstock PS had a 95.5% weight loss rate at a range of 366.7- 431.4°C, while digested PS had weight loss rates of 71.4% and 87.4% at the same range for *Z. morio* and *T. molitor*, respectively [65]. This technique has also been used with polyurethane (PU) [66], PVC [63], and low-density polyethylene (LDPE) [27].

Gel permeation chromatography (GPC) is one of the most widely used measures of biodegradation. It separates components of a mixture by size and allows for the number-average molecular weight (Mn), weight-average molecular weight (Mw), and size-average molecular weight (Mz), which describe the distribution of the size of polymers. Typically, a reduction in molecular weights (Mn, Mw, Mz) in digested polymers compared to pre-digestion will be evidence of degradation via broad depolymerization (BD). However, after digestion, polymers that naturally have very high MW will express a lesser reduction or even an increase in Mn, Mw, and Mz; this classifies limited-extent depolymerization (LD). LD most likely occurs because low MW chains are depolymerized at a faster rate than high MW chains, leaving a greater proportion of high MW chains post-digestion, overall increasing the Mn, Mw, and Mz. However, depolymerization still occurs; thus, an LD pattern is still evidence of degradation when the overall molecular weight distribution (MWD) shifts towards a low-MW direction [28]. A study observed this pattern when PS with varying MWs was fed to *T. molitor*. PS with low to high MW (<~600kDa) showed consistent BD patterns, while the ultrahigh MW PS (1346 kDa) showed LD patterns [29].

Mass balance tests are a strong estimative way to quantify degradation rates by measuring the difference between inputs and outputs. Inputs include the feed, while output is the weight of the frass prior to extraction, the weight of the residual polymers in the frass recovered through solvent extraction, and the change in weight of the surviving insects. If the output no longer matches the input, biodegradation is evident. Capturing and quantifying gasses during the process allows for the estimation of mineralization, i.e., the amount of plastic (organic material) that was converted into gasses, presumably CO_2 (inorganic material) [25]. This test has commonly shown increased plastic degradation efficiency over time. For example, just 18.84% of PU fed to *T. molitor* was converted to CO_2 in the first five days of incubation; this number rose to 29.80% by day 20 [67]. Another study saw the amount of PS being converted to CO_2 rise from 20.7% to 47.7% over 15 days [23].

Carbon-13 (^{13}C) isotopic tests are used to quantify biodegradation by feeding ^{13}C-enriched plastic is fed to insects, who incorporate it into their biomass and form metabolic products. As plastic is degraded, the insect releases $^{13}CO_2$. By trapping and measuring the amount of $^{13}CO_2$ released, biodegradation and mineralization can be quantified. Isotopic tests can also be used to see if plastic was assimilated into the insect biomass by measuring the ^{13}C values in fatty acids [23]. One study measured an increase in $^{13}CO_2$production from *T. molitor* fed ^{13}C-enriched PS compared to those fed non-^{13}C-enriched PS, indicating mineralization. They also found higher ^{13}C values in the fatty acids of *T. molitor* fed ^{13}C-enriched plastic, indicating that a limited fraction of the ^{13}C was assimilated into the mealworm biomass [26]. These increases in ^{13}C indicated biodegradation of the PS.

Scanning electron microscopy (SEM) and atomic force microscopy (AFM) are additional techniques used to evaluate the surfaces of polymers before and after inoculation. These methods are often applied in experiments that involve isolating bacteria and incubating them with a polymer. Initial signs of degradation observed include changes in color, holes, surface roughening, cracks, and biofilm formation. However, these techniques should be used in tandem with other strategies, as changes in the surface could only be an indicator of biodeterioration rather than biodegradation. Nonetheless, studies that have detected surface changes using these techniques often confirm biodegradation through additional methods, establishing a strong correlation between surface alterations and actual degradation [50, 52, 68, 69].

Finally, differential scanning calorimetry (DSC), X-ray photoelectron spectroscopy (XPS), X-ray diffusion diffraction (XRD), the "clear-zone" test, and mass spectrometry (MS)-based detection methods are additional techniques that can provide evidence of biodegradation. They were less commonly used in the reviewed studies, so a description will not be provided. However, they have been reviewed in excellent depth elsewhere [25, 58, 70].

Natural Diet, Environment, Life Cycle, and Behaviour: *Zophobas morio*

Z. morio belongs to the beetle family of Tenebrionidae, with origins tracing back to tropical regions of Central and South America [71]. Rearing *Z. morio* efficiently depends on humidity and temperature, requiring 22-27°C [72] and 50-60% humidity [73]. While wheat bran with raw vegetables as supplements for nutrition and water is the most commonly used diet for rearing *Z. morio* [74, 75], the exact nutritional requirements are not agreed upon. Studies have successfully used various organic wastes such as vegetable waste, garden waste, chicken feed, horse manure, and cattle manure [76]. Avian feed and high-calcium cricket feed have also seen success [48]. By-products from beer brewing, potato processing, and bioethanol production have also been somewhat viable feeds [77, 78]. However, less-optimal substrates increase larval periods and mortality [79]. For example, high-starch diets that contained cookie remains had a survival rate of 0% [78]. A notably interesting behavior of *Z. morio* is that pupation is dependent on 4-6 days of isolation; crowded larvae will completely fail to begin metamorphosis [74]. Additionally, crowding increases rates of cannibalism and dehydration [80, 81].

Natural Diet, Environment, Life Cycle, and Behaviour: *Tenebrio molitor*

T. molitor, like *Z. morio*, belongs to the beetle family of Tenebrionidae and is said to originate in Europe [82]. For both adult and young *T. molitor* larvae, a humidity of 50%-75% and a temperature of 25°C are optimal parameters for maximal survival rate [83]. Notably, extreme humidity conditions (12% and 98%) at normal temperatures (25°C) showed no detrimental effect on *T. molitor* survival, indicating that temperature is a more influential factor [84, 85]. One study reported the greatest growth rate at 31°C but did not control humidity nor mention ending survival rates [64].

Substantial interest in using *T. molitor* as an alternate protein source has resulted in numerous studies on the most effective type of substrate. The most common and reliable substrate used is wheat bran [86–90], often combined with a source of moisture such as vegetables [91, 92]. A source of moisture maximized growth [93] by lowering developmental time and increasing survival rate [91]. Adding an additional protein source, such as casein, lactalbumin, or yeast (at concentrations of 5-10%), further enhances growth, survival rates, and weight gain [86, 94, 95]. Crucially, a range between 50% [96] and 80% [95] of carbohydrates has yielded the best biomass growth, longevity, and reproductive capacity results. Similarly to *Z. morio, T. molitor* has been successfully reared on food by-products and waste, such as brewery spent grains [97], orange albedo [98], watermelon rind [99], malt residual pellets [100], and a host of other vegetables, cereals, oats, legume, and beverage by-products that have been reviewed more in-depth elsewhere [101].

Alternate Uses: *Tenebrio molitor* and *Zophobas morio*

Z. morio and *T. molitor* have been studied extensively, particularly in the context of circular economies, due to their flexible substrate-rearing requirements and rich nutritional profiles [102-104]. While exact measurements of nutritional value tend to vary depending on study conditions, both species are extremely dense in nutrition. An extensive review [105] found protein concentrations to be 47% and 54% for *Z. morio* and *T. molitor*, respectively, and 18:1n9 (a common fatty acid present within all species) concentrations of 39% and 41% for *Z. morio* and *T. molitor*, respectively. In addition to fat and protein, both species are rich in calcium, zinc, copper, magnesium, iron, aluminum, and manganese [106, 107]. This nutrition is why both species have been

considered a possible protein source for animal feed and human consumption. One study [108] found that a small supplementation of *Z. morio* (2%) for fishmeal in the diet of young pigs had no negative effect on growth and increased fat digestibility, which is likely because *Z. morio* has high concentrations of monounsaturated and low polyunsaturated fatty acids [109]. Beyond nutrition, both *Z. morio* and *T. molitor* are a viable source of chitin, which can be extracted and used in various applications, including medical and pharmaceutical products [110–112]. Additionally, both *Z. morio* and *T. molitor* are suspected to be sources of antimicrobial peptides, as studies have found improved immunity in animal livestock after supplementing minor amounts of feed with *Z. morio* and *T. molitor* [108, 113]. Despite limited research on this, it is documented that insects can synthesize antimicrobial peptides [114]; thus, further research into these two species is warranted.

Polystyrene and Polyethylene Biodegradation by *Tenebrio molitor* and *Zophobas morio*

While publications on insects and their ability to degrade plastic date back to the 1950s [115, 116], research on the subject was relatively quiet until it was propelled into the spotlight of bioremediation when Yang et al. published their team's findings on the surprising effectiveness of *T. molitor* in degrading PS [23]. *T. molitor* was able to degrade PS within hours, with a consumption rate of 12 mg/100 worms/day, resulting in a 31% total mass loss over 30 days. Egested frass from *T. molitor* was analyzed, revealing that the long-chain structure of PS molecules had been depolymerized, producing lower molecular weight fragments. Carbon mass balance tests were conducted to test the efficiency of carbon usage, which indicated that the carbon content of the egested frass decreased from 73.6% to 49.2%. This surprising finding suggested that *T. molitor* adapted to utilize the carbon source better, resulting in increased degradation efficiency over time. Another study found the same increase in degradation efficiency and hypothesized that it is likely due to an increase in microbial abilities [60]. Furthermore, ^{13}C-labeled PS was used to confirm that the carbon from PS was being mineralized to $^{13}CO_2$, with significant ^{13}C enrichment observed in the CO_2 released by the mealworms compared to the control. These tests demonstrated that the carbon was being broken down and utilized by *T. molitor* rather than simply passing through their digestive systems.

This ability is not restricted to specific strains or the environments of which they are from. A study found that *T. molitor* from 12 different sources from around the world displayed similar results of consumption and degradation and were confirmed by the same techniques as used before [117]. Yang et al. then tested the effect of antibiotics on *T. molitor*. The result was a loss of ability to degrade PS, indicating that PS biodegradation depended on the gut bacteria. This was validated through an insignificant change in average molecular weights compared to undigested PS and a lack of $^{13}CO_2$ production after being fed ^{13}C-labeled PS [56].

To test the viability of bacteria outside the gut, gut suspensions from *T. molitor* fed PS for two weeks were prepared and incubated with PS pieces. The resulting culture was spread on LB agar, and isolated colonies were obtained. These colonies were then spread on a CFBAM plate with added PS films. After 28 days of incubation, the film's surface exhibited deterioration with pits and cavities, while the uninoculated control film remained smooth. After 60 days of incubation, decreases in the average molecular weight of the PS films inoculated by the bacteria confirmed PS degradation. Yet, the weight loss was just 3.89 mg (7.4% of the original total), indicating that factors inherent to the mealworm itself significantly impact degradation rates. Yang et al. theorized the physicochemical treatments by *T. molitor*, such as chewing, ingesting, and host secretion of enzymes, are likely critical for the effective depolymerization of PS.

A later study further differentiated the role of the host body and gut microbiome in degradation [53]. When incubated with PS, respiration activity in the gut microbiome cultures of *T. molitor* increased when supplemented with gut supernatant from PS or bran-fed *T. molitor*, but not with supernatant from antibiotic-treated *T. molitor*. This suggests the gut microbiome secretes factors that enhance PS degradation. The supernatant also exhibited emulsification activity that effectively coated and separated hydrophobic particles, creating more surface area on the hydrophobic plastic particles for microbes to adhere to, which is one of the initial steps in microbial degradation [22, 110, 111]. Emulsification was observed in all diets, including antibiotics, which suggested that it is a factor of *T. molitor* itself, independent of the gut microbiome, and could be a reason why the isolated gut microbiome is less effective at degradation. The supernatant was fractioned by molecular weights and assessed for two qualities: increased respiration activity when used as a supplement and increased surface coating. The two groups most efficient at both (<30 kDa and 30-100 kDa, respectively)

were combined and used as supplements. This combination led to the greatest increase in respiration activity seen. The author noted that because unfractionated supernatant had lower respiration activity, supernatant >100 kDa may contain inhibitory agents [53]. The independence of microbial secretions and host secretions was further proven when the only antibiotic-treated supernatant that had increased activity was 30-100 kDa. These findings indicate that although isolated gut microbiomes have lower degradation rates, there is potential to enhance them.

Once PS susceptibility to *T. molitor* was established, Brandon et al. investigated the potential biodegradability of PE [62]. They found that PE and PS consumption rates (mg/100 worms/day)/(32-day PS consumption %) were comparable at 23.1 mg/48.3% and 16.9 mg/31.6%, respectively. It was also evident that *T. molitor* could adapt and more efficiently utilize PE throughout the experiment, as previously shown with PS [23]. Biodegradation of PE was proven through the same techniques used previously with PS, showing decreased average molecular weights and new functional groups. Specifically, ^{1}H NMR showed new peaks associated with alkene bonds in PE groups, and FTIR provided evidence of oxygen incorporation through the presence of alcohol groups and C-O bonds. These new functional groups align well with the theory that oxidation is the first step in PE depolymerization [118-120]. Mass balance tests showed that a higher percentage of carbon from PE was assimilated into the biomass compared to PS.

The success of *T. molitor* in degrading PS and PE prompted researchers to explore whether other insect species might possess similar or even greater capabilities. This led to the discovery that *Z. atratus (=Z. morio)* can also degrade and mineralize PS [64]. *Z. atratus* showed a consumption rate of 58 mg/100 worms/day, a rate over 3 times greater than that of *T. molitor*, and consumed 65% of the total PS over 28 days. This difference could be due to physical differences between the two species. *Z. atratus* are larger and have a mandibulate mouthpart that enables them to chew plastics better, which is the first step in insect biodegradation mechanisms. Analysis of the egested frass revealed lower weight-average molecular weights than that of undigested PS, once again suggesting the depolymerization of long chains of PS. Thermal characterization through TG-FTIR showed the egested frass had weaker styrene peaks than undigested PS, indicating a reduced presence of styrene. New peaks attributed to aromatic carbons of phenyl derivatives were also observed. *Z. atratus* was able to mineralize PS at rates similar to *T. molitor*. Finally, antibiotic treatment suppressed PS-degradation, once again suggesting the role of the gut bacteria.

Changes in the Gut Microbiome of *Tenebrio molitor* and *Zophobas morio*

For the sake of brevity, it should be assumed that all discussed changes in the gut microbiome of *T. molitor* and *Z. morio*, as well as the microorganisms involved, have been demonstrated to be associated with the depolymerization and/or mineralization of plastic. Each referenced study has adequately proven these processes through one or more established techniques, of which most were mentioned above, and controlled for relevant variables.

Although the specifics of each step in the proposed mechanism are not well known, the importance of the gut microbiome has been well established. When *T. molitor* and *Z. morio* are fed with plastic, massive shifts in their microbiota occur. The diversity of the microbial community exposed to a specific plastic depends on many factors and does not always respond the same way to the same plastic. For example, when *T. molitor* was fed PS with low MW, the phylum Firmicutes had a relative abundance of 83.15%. However, in groups fed PS with medium and high MW, the abundance of Firmicutes dropped to 20.13% and 21.84%, respectively. At the family level, Streptococcaceae was the most abundant in the low MW PS group at 63.33%, while Enterobacteriaceae dominated in the medium MW PS group at 79.84% [29].

Diversity in the microbiome can also be influenced by the structural complexity of a polymer. For instance, the gut microbiome of *T. molitor* on a natural bran-fed diet had 180 operational taxonomic units (OTUs). When fed polypropene (PP), which has a relatively simple chemical structure, the number of OTUs dropped to 102. In contrast, in the PU and ethylene vinyl acetate groups, which are more structurally complex plastics, the OTUs increased to 186 and 188, respectively. Despite the survival rate decreasing as plastic complexity increased, all three groups consumed nearly the same amount of plastic [121].

Availability of nutrition also alters the microbial communities and typically increases degradation rates by providing an energy source for microorganism growth and synthesis of enzymes [60, 61, 122]. Recent interest in nitrogen fixation has emerged because plastics are completely nitrogen-deficient. Despite this, both *T. molitor* and *Z. morio* have been able to survive surprisingly long on plastic alone. Some of the bacteria found in these insects have nitrogen-fixation potential, which could provide the necessary nitrogen for their survival. This is relevant because many bacteria associated with plastic degradation, such as *Klebsiella* sp., *Mixta* sp., *Kluyvera* sp., and *Citrobacter* sp., are also known to have nitrogen-fixation potential and may actually be less significant in the degradation process [123, 124]. Instead, they could be microorganisms that grow under nutritional stress, creating a more competitive environment that inhibits the growth of bacteria actually capable of degrading plastics [122–124]. However, nitrogen fixation and the ability to biodegrade polymers are not mutually exclusive. For instance, *Klebsiella grimontii* MA76 was recently isolated and showed plastic-degrading potential despite the species being diazotrophic. The same study found that three different strains of *Acinetobacter septicus* had different growth rates on PS plates, highlighting the variability of microbial behavior even among strains of the same species [69]. These observations illustrate the necessity of researching microorganisms responsible for degradation at the strain level and that isolation is a powerful and essential technique.

While the functions of microbial communities are complex, and the effectiveness of biodegradation can vary among strains of the same species, many studies consistently arrive at similar conclusions across various taxonomic ranks and testing settings [13, 119, 125, 126]. One study that highlighted this was when *T. molitor* from 12 different sources worldwide were analyzed. Despite the original bacterial communities being diverse among all sources, feeding them PS resulted in similar patterns of bacterial abundance across all sources, suggesting that the ability to digest and degrade PS is genetic. Notably, the Enterobacteriaceae family abundances nearly doubled when fed PS only compared to a normal diet [117].

Among the many microorganisms that are associated with plastic degradation, the abundance and growth of the Enterobacteriaceae family when exposed to plastic has been quite consistent, regardless of the type of plastic or the species of the host. Brandon et al. [62] found *Citrobacter* sp. and *Kosakonia* sp. in *T. molitor* to be strongly associated with both PE and PS diets and are part of the Enterobacteriaceae family. Wang et al. [127] found that *Cronobacter, Lactococcus,* unclassified *Enterobacteriaceae, Lactobacillus*, and *Citrobacter* were significantly increased in abundance in PE-fed *Z. morio*. Luo et al. again found Citrobacter sp. to be associated with PE-fed *Z. morio*, along with *Dysgonomonas* sp. and *Sphingobacterium* sp. in the PS-fed group, and *Mangrovibacter* sp. in the PU-fed group. Tang et al. [128] isolated *Klebsiella pneumoniae* (part of the Enterobacteriaceae family) and *Aeromonas* sp. from PS-fed *Z. morio* and *T. molitor*, respectively, by growing them on PS plates.

Shan Jiang et al. [126] found that the relative abundance of Enterobacteriaceae increased in all *T. molitor*, *Z. morio*, and *Galleria mellonella* (another plastic-degrading insect) when fed PS. When Bo-Yu Peng et al. [63] fed PVC to *T. molitor*, the gut microbiome shifted from a diverse one to one dominated by families Streptococcaceae, Spiroplasmataceae, Clostridiaceae, and Enterobacteriaceae. In a subsequent study, Bo-Yu Peng et al. [129] tested polylactic acid with *T. molitor* and found similar shifts in the microbiome to families of Streptococcaceae, Spiroplasmataceae, Clostridiaceae, Lactobacillaceae, and Enterobacteriaceae. This trend continued in a study by Yumeng Wang et al. [65], when the relative abundance of unclassified Enterobacteriaceae sharply rose in *Z. morio* when fed PS and PU. While the abundance of *Klebsiella* did not significantly change across different diets, it remained relatively high. In another study, *Klebsiella* sp. and *Sierra marcescens* from *T. molitor* were associated with PS degradation [53], but these bacteria failed to degrade PS when isolated, indicating a dependence on the host [53].

Interestingly, Woo et al. [130] isolated *Serratia* sp. WSW from *Plesiophthalmus davidis* (another plastic-degrading insect) onto a PS plate. Biofilms formed, resulting in a six-fold increase in bacteria, and C-O bonds covered the newly formed cavities, indicating biodegradation. The molecular weights were unchanged, but the MWD shifted toward a low-MW direction, indicating an LD pattern more attributed to *Z. morio*. *Serratia marcescens, Klebsiella oxytoca*, and *Pseudomonas aeruginosa* were isolated and found to be able to degrade PS films [131]. When fed PS, *Pseudomonas* sp. and *Lactococcus* were dominant in *Z. morio*, while *Spiroplasma* was dominant in PE-fed *T. molitor* [127]. *Pseudomonas* sp. is able to degrade PVC, PU, and PE

[132], so it was studied more in-depth, and the strain *Pseudomonas aeruginosa* DSM 50071 was isolated. The strain degraded PS, diminished much of the polymer's hydrophobicity, and formed carbonyl groups. The gene expression of serine hydrolase was upregulated during PS degradation, and when the strain was treated with a serine hydrolase inhibitor, bacterial growth on the PS was diminished, weight reduction of the PS was halved, and no carbonyl groups formed in comparison to the control group [133]. This study showed that PS degradation depended on a well-known hydrolase to be active, suggesting that it likely has a role in the depolymerization of PS into monomers.

Enzymatic Activities and Roles

With a strong foundation of knowledge regarding which microorganisms are most associated with plastic degradation, studies have shifted focus toward identifying enzymes involved in the biodegradation mechanism. Przemieniecki et al. conducted one of the earlier studies in this area, using a combination of metagenomic analysis and enzymatic activity tests with *T. molitor* [122]. The study first measured microbial abundances, and the results were consistent with previous studies [62, 117, 134]. Planctomycetes and Nitrospirae increases were specific to PS diets, and *Pantoea* was specific to PE diets. Enzymatic testing revealed that while the types of enzymes were similar, the digestive tract produced them much more actively than the microorganisms. This finding could be one explanation as to why isolated microorganisms have such slower degradation rates. Alkaline phosphatase and acid phosphatase activity had a strong correlation with PS, while C8 ester lipase activity was elevated in PE diets [122]. Luo et al. theorized that the increased lipase and proteinase activities in PE-fed *Z. morio* were a result of nutritional deficiencies [135]. Przemieniecki et al. mentioned that high levels of depolymerizing enzymes tend to be correlated with high levels of phosphatase activity; this idea merges the correlations of elevated degradation rates, lipases, and phosphatases. A transcriptomic analysis on *T. molitor* showed that hydrolases were the most upregulated in both PE and PS groups. In LDPE, the upregulated hydrolases acted on ester bonds, sugars, and C-N bonds, overall expanding fatty acid metabolic processes. This aligns with another study that saw significant changes in fatty acid profiles and metabolic activities, supporting the theory that assimilation into the biomass and, ultimately, mineralization depends on the decomposition of fatty acids [119, 136]. Furthermore, the consumption of both expanded PS and LDPE in *T. molitor* enriched 42 KEGG pathways that indicated fatty acid degradation is involved in the breakdown of PS and PE [137].

After biodeterioration, microbial adherence, and the secretion of bioemulsifying agents, the depolymerization of hydrocarbon chains begins. As established, the method for depolymerization depends on the type of plastic. Non-hydrolyzable plastics, such as PS and PE, are more difficult to degrade, and their exact mechanisms are still unknown. PS depolymerization most likely starts with either an attack by functional enzymes at the β-carbon (main chain cleavage) or the aromatic ring (side-chain cleavage). The main chain is more likely to be cleaved since the alkane chain is weaker than the aromatic alkene bonds. Enzymes possibly responsible for this include alkane hydroxylase, alkane monooxygenase, cytochrome P450, monooxygenase, flavin-binding monooxygenase, or aromatic ring hydroxylase [54, 125, 138]. Cytochrome P450 is a likely candidate because of its ability to participate in monooxygenase, peroxidase, and peroxygenase reactions [54]. Additionally, cytochrome P450 contributes to the production of reactive oxygen species (ROS) [139]. ROS generation increases in the gut when *Z. morio* is fed PS and degraded. However, if ROS generation is prevented, there is a significant decrease in PS depolymerization, proving the significance of ROS [140].

The initial oxidation of PS and PE is most likely by cytochrome P450 on an alkane, forming a primary alcohol. The primary alcohol is further oxidized into an aldehyde and subsequently converted into a fatty acid [120]. Cytochrome P450 can regioselectively oxidize subterminal carbons, forming secondary alcohols, which are then again oxidized into ketones. Subsequent ketones can be converted to esters by the addition of an oxygen atom by Baeyer-Villiger monooxygenase, which are then finally cleaved by esterase to form an alkanol and fatty acid [54, 120, 141]. Finally, these fatty acids are subsequently stored in the host or undergo β-oxidation for the citric acid cycle to produce metabolic products [142]. A metatranscriptomic analysis ties all the aforementioned concepts together [143]. It revealed similar findings as previous studies [122, 144]; xenobiotics, aromatic compounds, and fatty acid degradation pathways were enriched in *T. molitor*-fed PS or corn straw. Monooxygenase, superoxide, dehydrogenase, and cytochrome P450 were all shown to be involved in PS degradation. Additionally, the analysis revealed an upregulated gene, *lac640*, in both PS and

corn straw groups. When overexpressed in *E. coli*, this gene exhibited PS and lignin degradation abilities. These comprehensive findings that involve many of the suspected mechanisms behind PS degradation show that research is on the correct path. The successful PS degradation abilities in the overexpressed gene are a step towards harnessing and manipulating these biochemical mechanisms for bioremediation strategies.

Factors that Affect PS Consumption Rates

While each species exhibits varying degrading capabilities on a host of different types of polymers, such as PS, PE, PVC, PU, PLA, and PP [56, 62, 63, 129, 135, 145], PS has seen the most attention. This focus is likely due to the early preliminary successes and consistency in results. In the following years, Yang et al. investigated factors that may affect PS consumption rates, such as added nutrition, temperature, and multiple common types of PS waste [61]. *T. molitor*-fed PS with added bran or soy protein had consumption rates (mg/100 worms/day)/(32-day PS consumption %) of 44.1 mg/67.6% and 49.1 mg/76.8%, respectively, significantly higher than the 22.2 mg/39.1% rate observed when fed PS alone. These results were further amplified with a higher bran-to-PS ratio (16:1) and higher temperatures (25°C and 30°C), resulting in 84% and 78.5% PS consumption rates, respectively. Another study [60] found that the consumption rate of PS alone was nearly identical at 24.3 mg/41.5%. Yet, there was a lower rate in the 1:1 bran:PS co-diet group at 33.23 mg/56.8%, compared to the previous study [61] that reported ~64% consumption in their 1.3:1 bran:PS ratio diet. The difference can likely be attributed to the group sizes tested (130 vs. 410 worms), as crowding increases stress and lowers consumption rates [146]. Further studies consistently reveal the same positive correlation between the addition of nutrition and increased PS consumption rates; increasing the nutrition:PS ratios and adding protein amplify this even more [117, 147]. However, this has mainly been done with standard diets such as bran and soybeans.

One interesting study [148] focused on three different factors: bedding, pre-treatment of PS, and supplemental nutrition. The bedding was made from either inedible beads or oats. Pre-treatment involved soaking expanded PS cups in either lemon-lime soda, lemon juice, or tomato paste. Supplemental nutrition was either spinach, protein powder paste, cucumber, or lemon slices. PS consumption was consistently higher on beaded bedding, which aligns well with previous observations that *T. molitor* strongly prefers nutritious, particularly protein-dense, substrates. Oat bedding is a high protein substrate, suggesting that *T. molitor* consumed it first rather than the PS. This assumption is consistent with a previous study [61] that found extremely high nutrition:PS ratios having lower consumption rates, as the insects interact less with PS and more with the nutritious substrate. The ratio between the number of *T. molitor*/total mass of insects and the masses of nutrition and PS will be an important factor to consider for future optimization of consumption rates. Pre-treatment PS foam also consistently resulted in greater consumption. The author theorized that the acidity of the pre-treatment weakened the cup's harder shell [148]. Other studies [61, 131] also report higher rates of PS consumption among softer, less dense plastics, as species' success is heavily attributed to the mechanical action of chewing [122, 149, 150]. Finally, outside of cucumber, which had no effect on consumption rates in just the oats bed, all supplemental nutrients increased the consumption rate [148]. This is likely due to the added moisture, of which the benefits in both species are well documented [62, 64]. One study proved the benefits of moisture applied to PS consumption too, where adding water to diets increased PS consumption and survival rate; the author suggested that the water facilitates the growth of gut microbiota, leading to the increased degradation [147].

How *Tenebrio molitor* and *Zophobas morio* Are Affected by Plastic Consumption

Plastic consumption and degradation of the worms have been documented heavily in reports from other studies. However, for future applications, it is vital to understand the effect plastic consumption has on insects. Studies vary, but generally, insects' survival rates greatly depend on incubation times. Thirty-day experiments are the most common among studies, and survival rates of *T. molitor*-fed PS stay consistent at around ~85-90%. This tends to be higher than in starvation groups, indicating that the carbon in polymers is of use [56, 61, 151]. However, survival rates during longer incubation times have large drops. One study investigating survival rates found just 4-12% survival rates among *T. molitor*-fed PS after 91 days; another saw a similar 11.5 ± 4.9% survival rate after 98 days [117, 151]. Starvation groups see similar survival rates over longer periods, but groups with supplemented nutrition are much less affected, with survival rates hovering around 80%. These results are relatively consistent with PE-fed groups [152]. In *Z. morio*, survival rates differ among polymers more, with PS-fed groups at a 100% survival rate while PE-fed groups at an 81.67% survival rate [135].

The plastic waste affects the growth of the *T. molitor* because it slows down their metabolism and provides insufficient nutrients like B vitamins, proteins, trace metals, and nitrogen [153]. Furthermore, when mealworms are fed with PS, the protein content of the worms is highest while the fat and carbohydrate content are reduced compared to worms on a conventional diet, which contributes to their weight loss because lipids were consumed due to the low nutrition available [143, 154]. Comparing the two worms, *T. molitor* had some decrease in mass, and they were physically lethargic on a plastic diet. On the other hand, *Z. morio* had an insignificant decrease in weight and similar behavior on a plastic diet versus their normal diet [154]. Indeed, comparing the health outcomes of *T. molitor* and *Z. morio* is challenging due to the variability in study designs, including differences in rearing conditions and the types of polymers tested. The influence of these factors on the results highlights the need for standardization in research methodologies to ensure consistent and comparable outcomes.

Technological Applications and Future Prospects

The studies reviewed highlight the potential of *T. molitor* and *Z. morio* for plastic degradation, but their practical applicability is limited. Generally, these insects use polymers for sustenance rather than growth, leading to mass loss and lower survival rates on polymer-only diets. This makes rearing and subsequently reproducing worms solely on plastic impractical and thereby unlikely for integration into a circular economy focused on plastic degradation. For instance, at a consumption rate of 0.58 mg of PE per *Z. morio* per day found by Yang et al., it would take over 7 tons of *Z. morio* to degrade 1 ton of PE in a month [64]. This scenario is complicated not only by *Z. morio*'s cannibalistic behavior in crowded conditions but also by the significant energy requirements that would pose technological and economic challenges. Although supplementing nutrients might improve survival rates and pre-treatments may improve consumption rates, the added costs and logistical barriers would likely be even more prohibitive for large-scale operations. Such a system may be more practical for small-scale, home-based applications for plastic-conscious individuals and families. While *T. molitor* and *Z. morio* are popular alternative protein sources, non-excreted additives and MPs would likely prevent their integration into human food chains. Their use as feed for non-human food chain animals (e.g., zoo animals) could be more likely considered, but this could raise animal welfare concerns. Nonetheless, if such toxicological concerns could be answered, their use as a feed source would hold economic value.

A promising approach is to understand and apply the degradation mechanisms of these insects to existing waste management technologies. As mentioned, factors of both the host and gut microbiome have shown to be synergistically responsible for more efficient degradation. If researchers can replicate these relationships *in vitro*, microbial-enzymatic solutions could be applied to landfills to potentially reduce their volume. However, isolated microbial communities have shown to be only a fraction as efficient as the insects, highlighting the need for further research. To optimize the comparability and reproducibility of future results, standards need to be established and followed for all aspects of experiments, such as rearing conditions, insect characteristics, co-dieting, polymer pre-treatment, and polymer characterization.

With standards set, studies should consider determining which microbial genera are directly responsible for degradation. While sequencing insect gut tissue alone is possible, the microorganisms that are efficient plastic degraders outside versus inside the gut are likely different. As such, isolating and incubating microbes on polymer films provides a more reliable method for identifying key microorganisms. Once responsible microbes are identified, attention should focus on enzymes, using metagenomics to predict and annotate genes and enzymes based on comparisons with known enzymes. Novel genes, not detectable by metagenomics, can be identified through proteomics techniques such as mass spectrometry, liquid chromatography, and associated tandem systems. Additionally, transcriptomics and metabolomics can provide deeper insights. A combined approach of these techniques has been used to identify a variety of potential PS-degrading enzymes [142]. Often, consortia of microbes are more efficient than isolated ones, likely because the different metabolic pathways and enzymes from various species and strains complement each other; how they do so should be studied. In the future, with this knowledge, synthetic microorganisms could be designed by modifying enzymes and constructing metabolic pathways. Further understanding of mechanisms could support certain modifications of polymer compositions and structures. Finally, there should be an investigation into the differences in enzymatic activity, metabolic pathways, and depolymerization patterns using insect secretions as a supplement versus not. These secretions show promise and warrant further study.

Conclusion

The danger plastic pollution poses to all aspects of life, and the environment should not be understated. Combined with constant growth in plastic production, innovative and cost-effective recycling solutions need to be developed. Current methods have been well-researched yet sparsely implemented due to high costs, technical limitations, and the production of secondary pollution. Microbial degradation, while promising, faces challenges due to slow degradation rates. *T. molitor* and *Z. morio* have shown the ability to not only consume but degrade a variety of polymers. The environment, co-diet, and type of polymers are just some things that affect consumption rates. Their degradation relies on gut microbial communities and is enhanced by host factors, suggesting a complex interaction between mechanical factors, microbes, enzymes, and metabolic pathways. Replicating and utilizing such mechanisms could lead to engineering approaches that optimize degradation rates or establish their use in tandem with established chemical recycling processes.

Acknowledgments. This work was in part supported by the National Science Foundation (NSF) under the award DUE ATE #2325500.

Disclosures. The authors declare no conflicts of interest.

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