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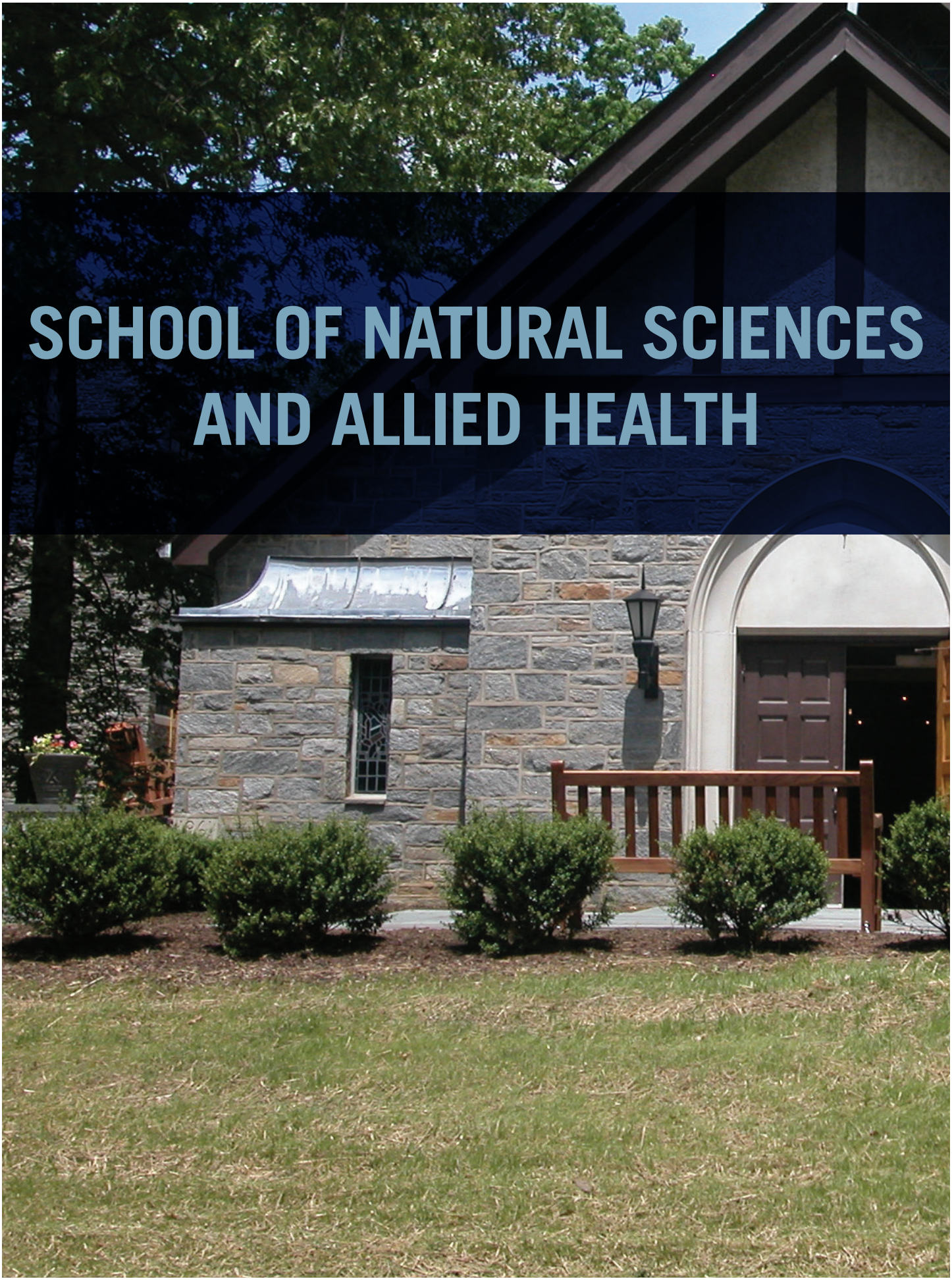

# SCHOOL OF NATURAL SCIENCES AND ALLIED HEALTH

# Use of a modified tube dilution method to determine bacterial susceptibility to antimicrobial proteins from *Eisenia hortensis*

Anna Bauer*, Alyssa Rothman, Sophia Scarpone & Sheryl Fuller-Espie
Research Mentor: Dr. Sheryl Fuller-Espie
*Primary author of this work

Science Department, Cabrini University, 610 King of Prussia Rd, Radnor, PA 19087-3698



**Abstract**

The objective of this investigation was to determine if a protein extract purified from the the earthworm *Eisenia* hortensis is inhibitory to the growth of selected Gram positive bacteria using a broth-based method, and if so, at what concentration. Protein extracts were prepared using 85% ammonium sulfate precipitation, dialysis, filtration and concentration techniques. Previous results in the lab using a disk diffusion method revealed zones of inhibition for two bacteria, *Micrococcus luteus* and Bac*illus megaterium*, using disks impregnated with 500 or 2,000 g and placed on agar plates streaked with each of the bacteria. A tube dilution method was modified to determine the concentration of extract required to inhibit growth in liquid culture. Overnight tryptic soy broth cultures of bacteria were diluted to OD600 ~0.1 and incubated with three concentrations of extract (high, medium, and low) for 16-24 hours. In two separate trials conducted in duplicate, the number of colony forming units (CFUs)/ml for treated and untreated control samples was determined by preparing ten-fold serial dilutions made in 0.85% saline and plating on tryptic soy agar in the absence of protein extract to permit recovery of bacteria. Statistically significant ($p < 0.05$) decreases in CFUs/ml were observed for *M. luteus* treated with 0.82 (high) and 0.082 (medium) mg/ml, but not with 0.0082 (low) mg/ml compared to untreated controls. Results show that *B. megaterium* exhibited less sensitivity to the extract than *M. luteus*, with significant inhibition observed at 2.5 but not 0.25 or 0.025 mg/ml. Future studies will focus on the concentration of protein extract required to inhibit the growth of *M. roseus*.

## Introduction

Antibiotics have helped reduced the risk of bacterial infections for decades, but bacteria are becoming increasingly more resistant to antibiotics and more challenging to control during infection. In recent years, antibiotics used in agriculture to increase livestock growth rate have caused concern for resistant bacteria that could affect human health (Cheng et al., 2014). To combat this problem, there are alternative microbial agents that can be used. Bacteriophages and antimicrobial peptides (AMPs) are alternatives that can help combat multidrug resistance in harmful bacteria. A recent review by Lin, Koskella, and Lin (2017) emphasizes the importance of pursuing phage therapy as an alternative to microbial drug regiments to combat clinically significant multi-drug resistant pathogens. Antimicrobial peptides have the capacity to destroy the integrity of the cell wall and cell membrane of both Gram-positive and Gram-negative bacteria making them attractive candidates for therapeutic use. AMPs were shown to be effective in humans in *in vitro* studies. Alternative antimicrobial agents can come from invertebrates, such as earthworms.

In common with other invertebrates, earthworms do not possess adaptive immunity providing the ability to produce antibodies and must compensate with other innate immune defenses. Earthworms, an oligochaete annelid, have been used for both traditional medicine for centuries in the Far East, and medical research in recent years to more fully understand the therapeutic effect of active factors (Bilej, De Baetselier, & Beschin, 2000). Earthworms, including *Eisenia hortensis* (also known as the European nightcrawler - the model organism used in this study), have multiple layers of defense mechanisms for protection against the myriad of pathogens encountered in their natural habitat. The skin has a cuticle layer with antimicrobial mucopolysaccharides and has mucus that also acts as an antibacterial barrier (Bilej et al., 2000). Internally, the coelomic fluid and coelomocytes residing in the coelomic cavity provide multiple defense mechanisms. A number of humoral products have been identified in *Eisenia fetida*, a closely related species of *E. hortensis*, including lysozyme, hemolysins, antimicrobial peptides, lysenin, fetidin, eiseniapore, and Lumbricin I (reviewed in Bilej et al., 2010). Coelomic cytolytic factor 1 (CCF-1) has also been isolated from *E. fetida* which binds to O-antigen of lipopolysaccharide, □-1,3, glucan of zymosan, and muranmic acid and muramyl dipeptide of peptidoglycan, activating the prophenoloxidase cascade (Beschin et al., 1998). Earthworm coelomocytes make up three distinct subpopulations derived from a common progenitor (prohemocyte) including hyaline amoebocytes (large coelomocytes), granular amoebocytes (small coelomocytes), and chloragocytes (eleocytes) (Hartenstein, 2006). The hyaline amoebocytes are phagocytic, the granular amoebocytes exhibit natural killer-like activity, and the chlorogocytes contain chloragosomes that enable secretion of lytic substances (Cossarizza et al., 1995; Fuller-Espie et al., 2010).

There are other invertebrates that have been used in antimicrobial research. Two species of horseshoe crab have a cationic protein, known as tachyplesin, that has antimicrobial effects on Gram-negative and Gram-positive bacteria (Tincu & Taylor, 2004). The crustacean *Penaeus vannamei*, a shrimp specie, has an antimicrobial peptide known as penaeidin that is effective against Gram-positive bacteria (Tincu & Taylor, 2004). Antimicrobial peptides have also been identified in the fruit fly *Drosophila* (Dimarcq, Bulet, Hetru, & Hoffmann, 1998). The use of therapeutic alternatives to traditional antibiotics are attractive candidate drugs from invertebrate species thanks to these previous studies.

The research described in this paper focuses on the antimicrobial properties of protein extracts purified from the earthworm species *E. hortensis*. These investigations have not been previously described in the literature for this particular organism. A crude protein extract was tested at various concentrations to examine inhibitory effects on growth of selected bacteria. It was hypothesized that *M. luteus* and *B. megaterium* will show growth inhibition to the protein extract using a modified tube-dilution broth method, and that the concentration of extract required to inhibit growth could be ascertained through serial dilution techniques.



## Methods

### *Preparation of TSB, TSA, PBS, and glass tubes*

500 mL of tryptic soy broth (TSB) was prepared and 50 mL was aliquotted into ten 100 mL flasks. 2.5 L of 0.85% NaCl was prepared and aliquotted into five 1 L flasks each filled with

500 mL of PBS. Tryptic soy agar (TSA) plates were prepared according to manufacturer's directions. All solutions were sterilized by autoclaving.

A large quantity of glass test tubes was sterilized by autoclaving prior to the start of the experiments. Once autoclaved, 9 mL of the sterile NaCl solution was pipetted into each tube, sealed tightly, and placed into a test tube holder for serial dilutions (saline blanks).

### *Preparation of earthworm protein extract*

**Figure 1** depicts the method used to prepare the protein extract from the earthworm *E. hortensis* (Rothman, 2018). Briefly, after precipitation with 85% ammonium sulfate, dialysis, and filtration, the final concentration was determined to be 247.4 mg/mL by Nanodrop. Confirmation of protein heterogeneity and integrity is shown in **Figure 2** where an SDS polyacrylamide gel was employed to separate proteins according to mass and charge (Rothman, 2018).

### *Treatment of M. luteus and B. megaterium with earthworm protein extract*

An overnight culture was prepared by inoculating 20 mL of TSB with isolated colonies obtained from a TSA Petri dish culture. After incubation at 30OC with aeration, the culture was diluted with TSB to OD600 ~ 0.1. Duplicate tubes containing diluted bacteria included the negative control (no earthworm extract), and high, medium, and low concentrations of earthworm extract as specified using ten-fold serial dilutions. The initial goal was to use the earthworm extract at a range of concentrations using 1 mg/mL as the upper limit. After factoring in dilutions in growth media, however, the working concentrations used were less than originally planned for *M. luteus*; 0.82, 0.082, and 0.0082 mg/mL were actually used. *B. megaterium* exhibited less sensitivity to the earthworm extract than *M. luteus* necessitating a three-fold increase in the amount of extract used, i.e. 2.5, 0.25, and 0.025 mg/mL were used. After overnight incubation with aeration, ten-fold serial dilutions of 10-1 – 10-6 were made in 9 mL saline blanks before plating 0.1 mL of the 10-4 – 10-6 dilutions onto TSA Petri dishes. Following

overnight incubation, colony forming units (CFU's) were counted and subjected to statistical analyses. **Figure 3** summarizes the protocol setup and dilution schemes.

## Results

### *Growth inhibition of M. luteus with earthworm extract*

The two trials for *M. luteus* showed that the earthworm extract inhibited the growth of the bacteria. **Figure 4** shows two separate trials conducted with *M. luteus* at 10-4 dilution, with trial 1 on the left, and trial 2 on the right. The plates are arranged with the negative control (no extract) in row 1, high concentration (0.82 mg/mL) in row 2, medium concentration (0.082 mg/mL) in row 3, and low concentration (0.0082 mg/mL) in row 4. The graphs underneath each trial show the respective statistical analyses. For both trials, the high and medium concentrations showed statistical significance (* $p < 0.05$). These concentrations of extract had few to no colonies, while the low concentration showed growth of the bacteria at levels similar to the untreated control.

### *Growth inhibition of B. megaterium with earthworm extract*

*B. megaterium* also showed inhibition of growth. **Figure 5** shows the two trials done, with trial 1 using the 10-4 dilution, and trial 2 using the 10-5 dilution. The plates are arranged with the negative control (no extract) in row 1, high concentration (2.5 mg/mL) in row 2, medium concentration (0.25 mg/mL) in row 3, and low concentration (0.025 mg/mL) in row 4. The two trials for *B. megaterium* also have their respective graphs below the plates. The graphs show the statistical analysis done for both trials, and showed statistical significance (* $p < 0.05$, ** $p < 0.005$) for the high concentrations in both trials. *B. megaterium* showed less sensitivity to the inhibitory properties of the earthworm extract compared to *M. luteus*. These results corroborated with previous results in the lab with the extract (Rothman, 2018), where a higher concentration of earthworm extract was employed when using *B. megaterium*. The extract needed to be 3X more concentrated to exert negative effects on growth than what was required to inhibit *M. luteus*.

## Discussion

Ideally these experiments would have benefited from another round of testing providing data for three replicate trials. Given the high degree of consistency and reproducibility of the data acquired in the duplicate trials reported in this study, it is believed that the results are an accurate demonstration of the antimicrobial effects of the earthworm extracts. Overall, *M. luteus* was more sensitive to the earthworm extract than *B. megaterium*, since *B. megaterium* required 3X the amount of extract in order to show any inhibitory effects. It would be interesting to explore the basis for the difference in sensitivity between the two Gram-positive bacteria used in this study; this information would provide a better understanding of the mechanism of action of the antimicrobial component of the extract.

The crude extract was a heterogeneous mixture of proteins and showed antimicrobial activity, however, the specific component responsible for this effect is currently unknown. The isolation of this specific component should be explored in more detail. Several ways to accomplish this would include gel filtration, ion exchange, or hydrophobic interaction chromatography methods. These methods would help separate and purify the antimicrobial component in question. The next step would be to isolate the gene encoding the protein responsible for antimicrobial activity and then mass produce the protein. One way to do this would involve the use of reverse genetics where the amino acid sequence of part of the purified protein is first determined, then a synthetic degenerate nucleic acid probe based on codons would be generated, and finally the genome would be screened by hybridization of either a copy DNA (cDNA, based on messenger RNA sources) or genomic DNA (gDNA) library. It would be useful to expand the scope of the present study to include a more comprehensive panel of pathogenic bacteria to further elucidate the clinical usefulness of antimicrobial proteins isolated from *E. hortenis*.


## Acknowledgements

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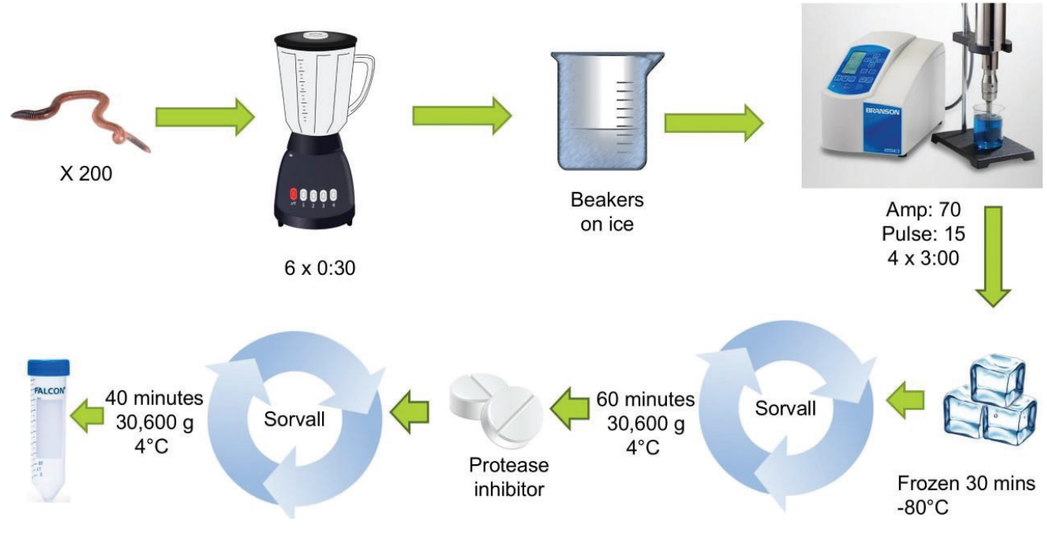



**Figure 1: Preparation of the earthworm protein extract.** Two hundred earthworms were liquefied in 6 pulses of 30 seconds at 4°C. The samples were sonicated and frozen to disrupt cellular membranes. The samples were centrifuged, then a protease inhibitor was added. Once the samples were centrifuged again, the samples' pellets were placed into PBS. Then ammonium sulfate was added (85%) to precipitate proteins. Following centrifugation and resuspension of pellet in PBS, dialysis was conducted followed by filtration. Nanodrop was performed to determine protein concentration (247.4 mg/ml) (Rothman, 2018).

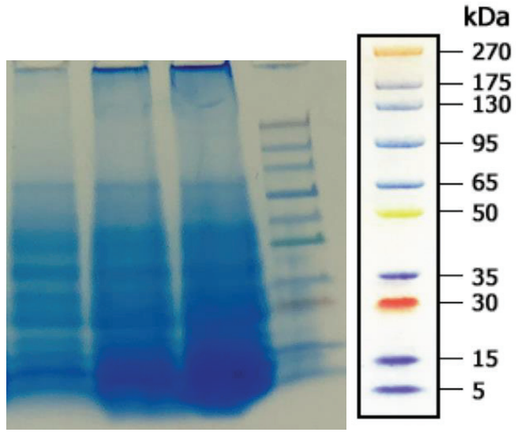



**Figure 2: Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).** Earthworm protein extracts at three different amounts (0.309, 0.618, and 1.236 mg) were electrophoresed as shown in lanes 1-3 using a BIO-RAD Mini-PROTEAN TGX™ precast gel. Molecular weight standard is shown in lane 4 (Precision Plus Protein dual Xtra, prestained protein standard). The banding pattern reflects the heterogeneity of the protein sample (Rothman, 2018).

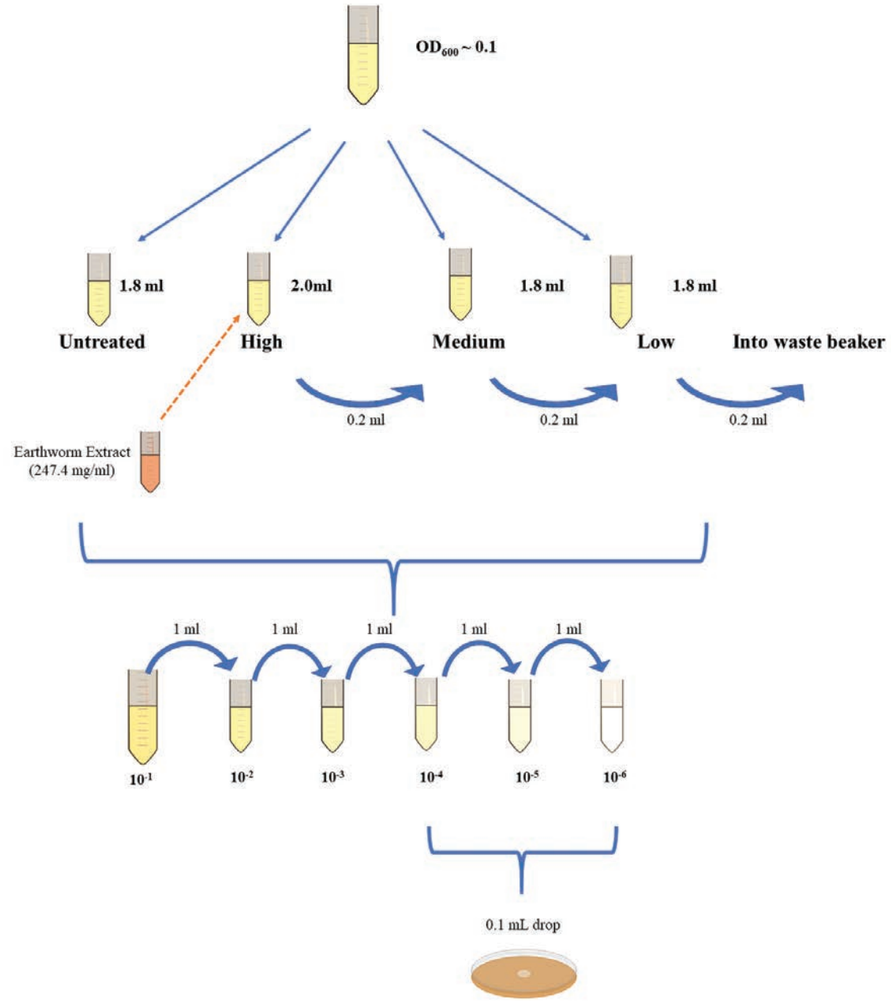
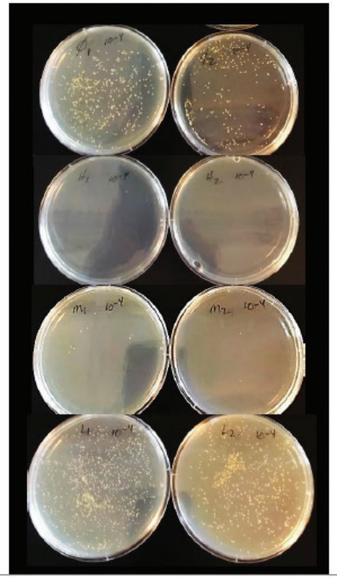
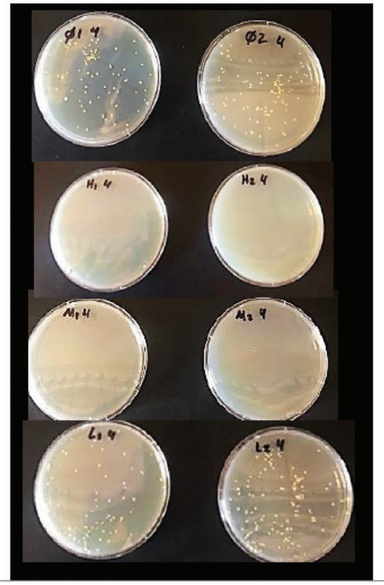
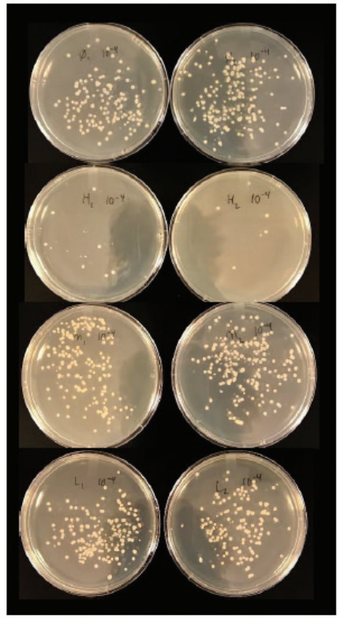
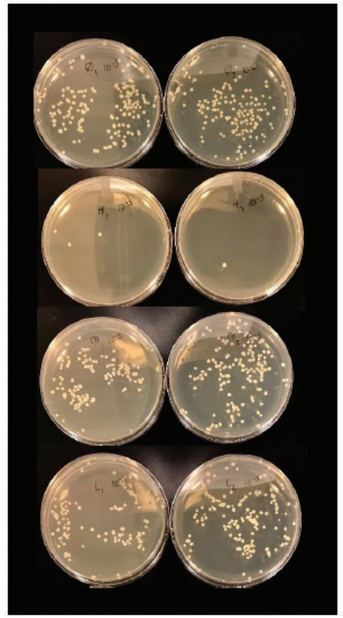


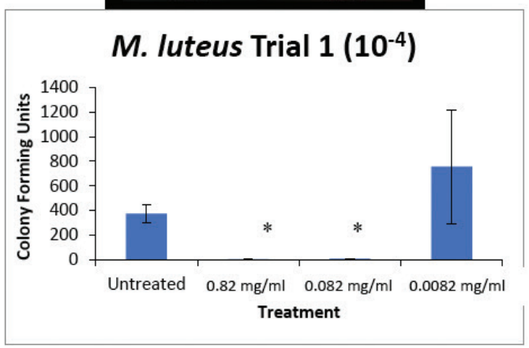

**Figure 3: Testing antimicrobial activity of earthworm extracts using a modified tube dilution method.** First an overnight culture of the bacteria was grown in TSB and subsequently diluted to achieve an OD600 ~ 0.1. Then the dilution was aliquoted in duplicate into tubes labeled untreated, high, medium, and low. The earthworm extract was added to the desired concentration in the high tube, and then medium and low were prepared by ten–fold serial dilutions. The samples were incubated overnight at 30°C before preparing ten-fold serial dilutions in saline blanks to create 10-1 to 10-6 dilutions. Aliquots of 0.1 ml of 10-4 – 10-6 were added to each TSA plate, spread thoroughly using sterile technique and inverted plates were incubated overnight. The CFUs were counted after 1 day (*B. megaterium*) or 2 days (*M. luteus*) of incubation.











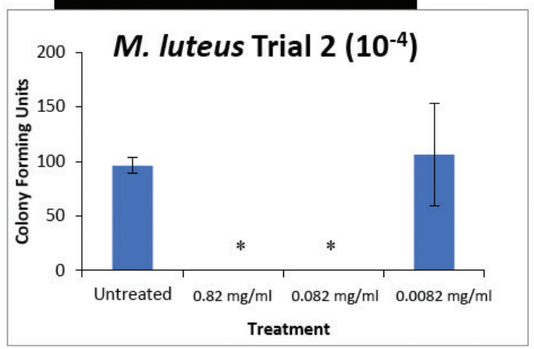
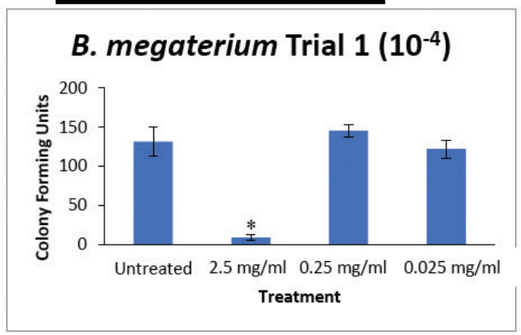




**Figure 4: Earthworm protein extract possesses antimicrobial activity on *M. luteus*.** Petri dishes are shown in duplicate: row 1=untreated (control), row 2=high (0.82 mg/ml), row 3= medium (0.082 mg/mL), and row 4=low (0.0082 mg/mL). Colony forming units on plates of the 10-4 dilution were graphed and statistical significance (* $p<0.05$) is indicated.



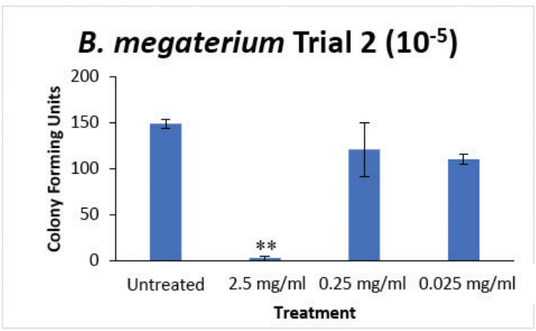




**Figure 5: Earthworm protein extract possesses antimicrobial activity on *B. megaterium*.** Petri dishes are shown in duplicate: row 1=untreated (control), row 2=high (2.5 mg/mL), row 3=medium (0.25 mg/mL), and row 4=low (0.025 mg/mL). Colony forming units on plates of the 10-4 dilution (Trial 1) or 10-5 dilution (Trial 2) were graphed and statistical significance (* $p<0.05$; ** $p<0.005$) is indicated.

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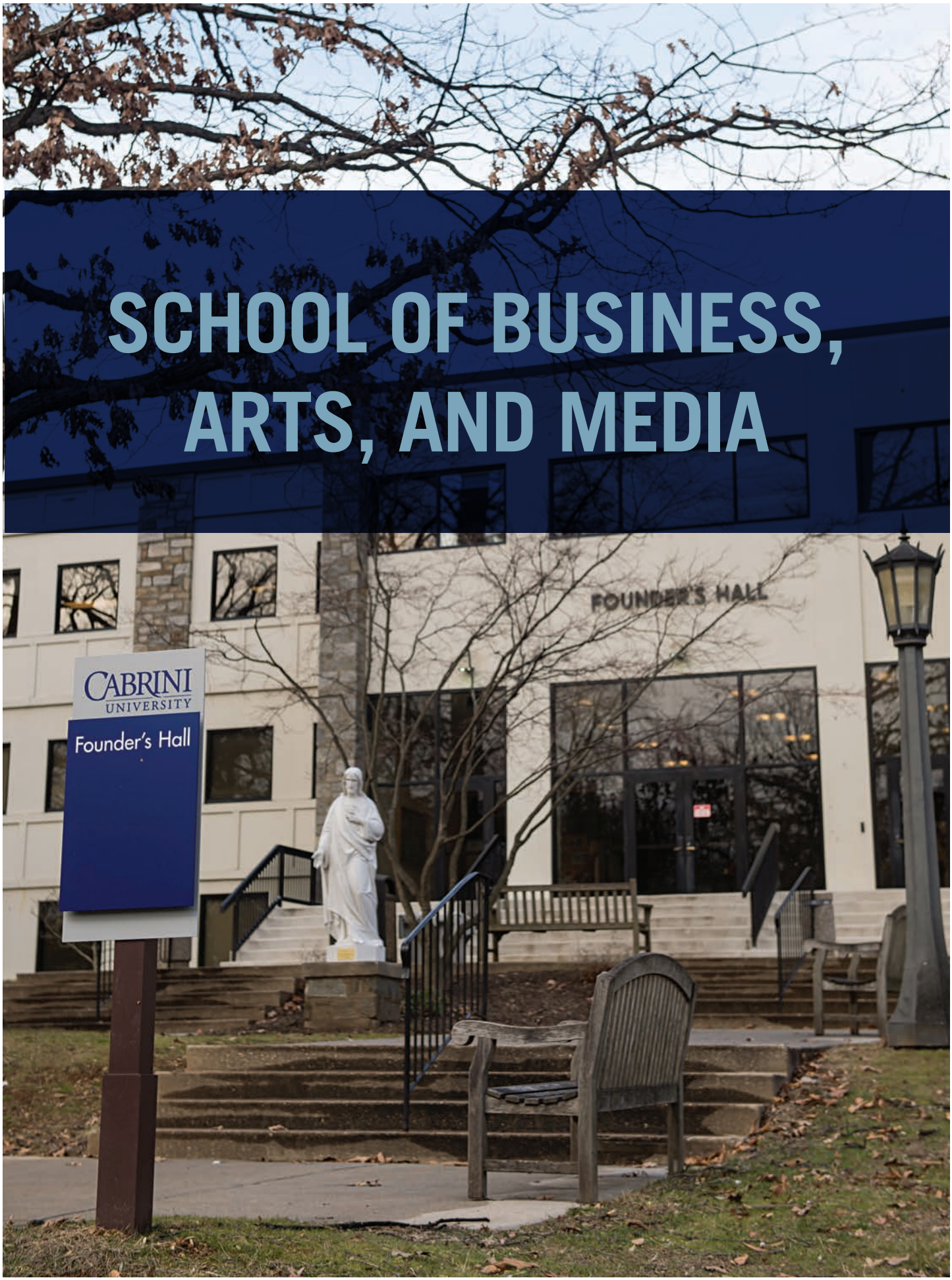

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Over the last 30 years, we've gotten pretty good at predicting big swells here at Surfline. Following 'em from when they're just wee blips on the models to full-blown purple blobs in the ocean to actual numbers on the buoys is almost as fun as actual surfing. Seriously. We love this stuff. At risk of sounding overly poetic, it's the ever-changing ocean providing a beautiful, quantifiable narrative arc of wind over water creating waves that gets us excited.
But the best thing about these big swells — sorry, Forecast and Science Teams — is the unpredictability of what happens when they actually hit the beach. Because, like anything, when you introduce the human element, all forecast tools are out the window.
The models couldn't have showed all captains refusing to head out of port at Pillar Point Harbor at 6am this morning, despite about 40 paying customers — Rimers, surfers and spectators — waiting on the dock. LOLA couldn't have predicted Anthony Tashnick and Kai Lenny rolling this morning's low session. Or Lenny's aerial assault. Or Francisco Porcella attempting a floater on a PWC after towing Nic Von Rupp in from very deep. And the spectral buoy readings would've never said in a million years that Lenny was one of the first to paddle and shred at 10am, or that he was joined by a crew of other young Hawaiians, including Nathan Florence, Billy Kemper, Koa Rothman, Danny Fuller and Torrey Meister, who were in the lineup from dawn to dusk, bobbing around on a little boat in between making giant drops and/or taking serious beatings alongside a solid-and-growing local crew.
Even years of firsthand experience covering swells here went out the window today. Normally the parking lot at Pillar Point is full of big trucks and big boards and big-wave dudes walking from the lot down the long trail to paddle out. I usually intercept them on the way out or back and prefer 'em with questions about how it was or what their strategy would be.
Today, I saw only one surfer paddle off the beach all afternoon. Turns out, at this size, everyone opts for a PWC or a boat, which makes sense. Today, the cliff and the trail and the surrounding streets were packed with all manner of Bay Area curiosity seekers — many brought out due to an onslaught of mainstream "Swell of the Century" news coverage, until they realized that wasn't a set, and those things in the water were not birds but jet-skis. Because when sets came, even from a few hundred yards away and looking through spray, glare and rocks, the crowd would utter a collective "Holy crap!"
"The place is beautiful and eerie and scary. The level you could go out there is crazy. Someone's gonna pack a crazy left."
Once I realized that my normal interception on the trail routine wasn't going to work, I improvised. The launch ramp at dusk turned out to be a who's who of big-wave surfers, photographers and filmers as skis made their way in from a long day's work.
First, I cornered longtime Mav's photog/safety person Frank Quirarte. "Kai Lenny won the day for sure," he said. "He was murdering it. It wasn't nearly gigantic. It was hitting on the third reef, but it wasn't focusing on the bowl till this afternoon, which is when the best paddle surfing happened."
Nathan Florence, who snagged one of the day's better waves, loaded up his giant board and said simply, "I had a few fun waves. It wasn't a hundred feet, but still some huge ones. Everyone got fun waves and everyone came back in safe."
Torrey Meister, who popped his Maverick's cherry today, said, "It's like a huge slab but somehow a big wave. The place is beautiful and eerie and scary. The level you could go out there is crazy. Someone's gonna pack a crazy left." (At which point all the godfytoots within earshot said, "Yeah, the left's gonna get crowded this week.")
On the other end of the spectrum, Skindog, who's been surfing Mav's for decades, came onto the launch ramp and says he's going to hang it up. "It was 25-foot out there — and that was my retirement session," he laughed. "Wives don't like these kind of sessions. She's like, 'why don't you go snowboarding instead?'"
After the sun went down, I headed to where everyone else was headed: the GPL. I found Twiggy and Grant Washburn in the corner. "It was very chunky," Twig said. "There was a lot of water in the waves. It wasn't as big as we thought,but it was the perfect size because we got chip-ins on the second reef and roll-throughs. Unfortunately, though, there was a light south wind all day, which made it really bumpy and scary to commit to one. It was similar in size to the 2010 swell, but that one was a lot cleaner."
"We flew in the night before got maybe four hours of sleep and went down the the harbor at first light," Eli Olson said. "It was so big the boats were canceling charters and the boat I planned on going out on canceled last minute, so I was about to just paddle out from the beach and luckily caught a ride on a ski out there. I watched it for a little while, seeing what it was doing, and it was super big and ugly and not inviting at all but I flew over to get amongst [laughs]."
"I sat on the outside ledge, just inside of Twiggy and Pete Mel," said Olson. "And when that set came, Twiggy caught the first one and I was in position for the second one, rolled into it and took my time bottom turning. I saw the wall and I was like, 'If I go straight, I'm fucked, and if I pull in I'm f**ked' — but I just thought it would be so much sicker to pull in. So I went for it. And then thought I might be able to sneak out — and ended up getting completely axed and felt like I was hit by a car."
Said Mav's grandpa Grant Washburn: "There were some huge waves. But the south, lung stayed bad. Gotta say, though, 15-20 years ago, there would've only been one or two people trying for those waves. Now, even though it's taken longer than most of us thought it would, there's dozens of guys charging. Their ability and confidence is super high.
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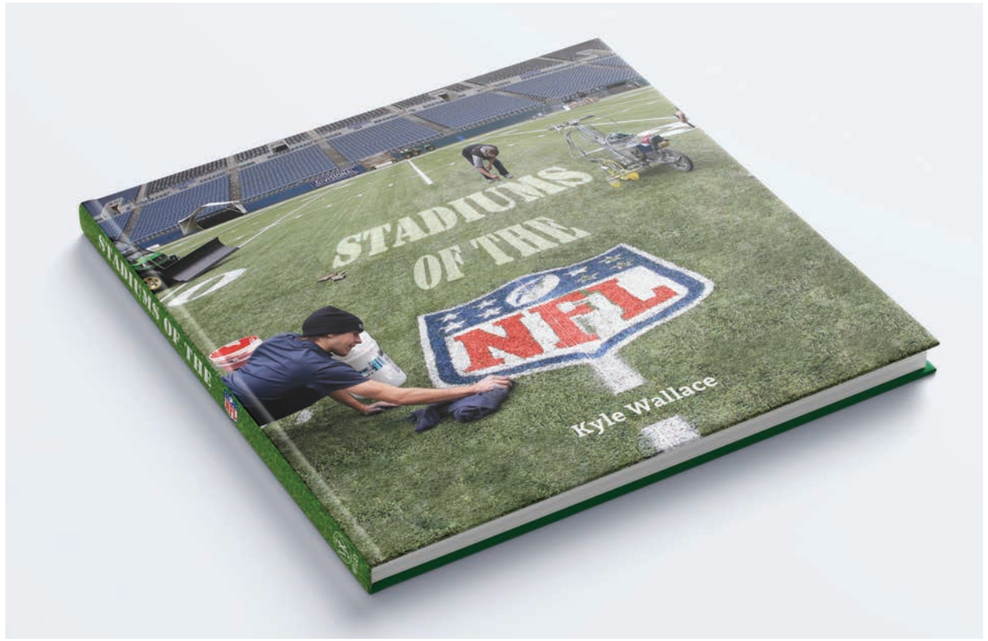



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