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Article

The effect of lead oxide concentrations on the bioluminescence intensity of *Panellus stipticus*

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SUMMARY

Lead is a heavy metal that pollutes soil and water, causing a variety of health issues. As fungi are crucial decomposers and colonize various environments, including lead-contaminated soils, they can serve as bioindicators for heavy metal toxicity. *Panellus stipticus*, a bioluminescent fungus commonly found on decaying wood, is an especially promising organism because changes in its cellular health due to a stressor like lead can be quantified by measuring the intensity of mycelial bioluminescence. The objective was to examine if *P. stipticus* could be used as an effective and sustainable method for detecting the presence of lead in an environment's soil. In this study, we examined how different lead oxide concentrations affect the bioluminescence intensity of a bioluminescent fungus, *P. stipticus*, over time. We hypothesized that when the concentration of lead present increases, the bioluminescence intensity of *P. stipticus* would exhibit a statistically significant decrease. We subcultured 20 *P. stipticus* samples on malt extract agar, then divided the plates into four groups exposed to different lead oxide concentrations. We quantified bioluminescence intensities via mean pixel brightness analysis through digital imaging. We found that higher lead oxide concentrations cause bioluminescence intensity to decrease over time, yet increasing concentrations of lead oxide did not necessarily cause a significant increase in the rate at which bioluminescence intensity was reduced. The results imply that *P. stipticus* could be used as a sustainable, effective method for detecting the presence of lead in an environment, helping to reduce the cases of lead poisoning through early detection.

INTRODUCTION

Heavy metal pollution is a global issue that affects a multitude of environments, often negatively impacting the health of individuals who interact with and live in these areas (1). Heavy metals refer to metals and metalloids which have toxic effects on biological systems at extremely low concentrations (1). As heavy metals pollute the ecosystem, they often accumulate in the human body as individuals are exposed to the contaminated environment (1). Lead is one such devastating and relatively common heavy metal that pollutes the environment. Lead is a prevalent pollutant in soil, water, and air, known for its toxicity and ability to be easily absorbed into organisms, including humans living in polluted areas (2). For instance, lead poisoning of refugee children in the United States commonly occurs due to contaminated water, lead dust, and industrial pollution (3).

Lead pollution and poisoning stem from several anthropogenic activities. For example, lead often enters the ecosystem through paint wear-and-tear, smelting, manufacturing, vehicle emissions, sewage runoff, and landfill runoff (e.g., from mobile phone batteries in landfills) (2). Lead that leaches into the environment often combines readily with soil, water, and air, forming lead compounds such as lead sulfate, lead carbonate, and lead oxide, which do not degrade naturally (1). Due to this, communities living in lead-polluted areas unintentionally ingest lead by interacting with the environment around them. For example, food crops contaminated with lead are absorbed into the body through consumption, and livestock raised for meat can also bioaccumulate lead via the environment in which they graze (2, 4).

When absorbed into the human body, lead accumulates rapidly and is not easily broken down. Lead in the human body is known to cause significant damage to numerous bodily processes, such as nerve function, skeletal formation, reproduction, blood cell formation, kidney function, and cardiovascular circulation (5). As lead is absorbed into these systems, it causes a range of devastating consequences, including paralysis, comas, loss of brain functions, osteoporosis, fetal damage, damage to the DNA of reproductive cells (e.g., sperm), anemia, renal failure, cancer, heart disease, and even death (1). For this reason, the United States Environmental Protection Agency (USEPA) has set a safe soil threshold limit of 400 parts per million total lead and an action limit of 4,000 parts per billion (6, 7).

Given the serious risks associated with lead pollution, detecting the presence of lead pollution is an extremely important process that informs both citizens and researchers about contaminated areas requiring remediation. Currently, the three most common methods for detecting lead pollution are spectrometry, colorimetric assays, and electrochemical procedures (7, 8). However, these methods have several disadvantages, making them impractical for everyday individuals. Spectrometry requires specialized equipment to separate a sample into its ions and then to measure the frequency of each ion's appearance in the sample (9). For this reason, spectrometry equipment is highly expensive, and testing can only be performed in a laboratory setting (8). Colorimetric tests require chemicals that change color at relatively high concentrations of a certain substance and equipment that measures this color change. Although less expensive than spectrometry, the procedure often involves toxic chemicals, produces excessive liquid waste, or lacks high sensitivity (8, 10). Lastly, electrochemical tests measure the changes in current when various concentrations of voltage are applied to an electrode in contact with a sample to determine if a substance is present within a sample (7).

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Compared to the previous two methods, electrochemical tests are cheaper and easier to use. However, the equipment used for these tests must be readily mass-produced and thus is not entirely sustainable (8). Consequently, there is an urgent need for a sustainable and practical alternative method for detecting lead that is accessible to everyday individuals.

Fungi can absorb lead when grown in lead-contaminated soil and thus remediate the soil (11). Lead can potentially inhibit the growth of certain fungi species (12). Additionally, bioluminescent fungi could possibly be used effectively as bioassays for detecting environmental pollutants (13). *Panellus stipticus*, also known as the bitter oyster, is a species of fungus found in the forests of North America, Europe, Asia, and Australia (14). It is capable of bioluminescence through the organic compound luciferin, which facilitates a light-producing biochemical reaction (13). The mycelia of *P. stipticus* produce a green glow observable to the naked human eye in low-light environments (14). However, there has not been detailed research on how increasing lead levels in the environment would affect the bioluminescence intensity of *P. stipticus* fungi. If the light output intensity of *P. stipticus* is proven to change with increasing levels of lead, then *P. stipticus* would have possible future applications as a practical and sustainable method of detecting harmful levels of lead in the environment. The everyday individual wishing to detect lead in the environment would simply have to grow *P. stipticus* in a nutrient medium combined with a soil or water sample, a process that can be done at home without requiring complicated laboratory equipment. In addition, *P. stipticus*, as a living organism, could be subcultured and constantly grown without the need for factory manufacturing or excessive materials for mass production, unlike chemicals or specialized equipment. Thus, using *P. stipticus* would be a more sustainable method of detecting lead pollution than common methods used today.

Regarding the light output intensity, previous studies have validated the use of Adobe Photoshop (a graphics editing software) as an effective method for quantifying bioluminescence intensity (15, 16). Specifically, we can calculate the relative bioluminescence light intensity within a selected area of an image with the "Histogram" tool in Adobe Photoshop, using the following function.

$$\text{Relative bioluminescence} = \frac{\sum_{i=0}^{255} n_i(i)}{n_{\text{tot}}}$$

In this formula, n_i represents the count of pixels at each level of brightness i (0 to 255) within the selected area of mycelial growth. Also, n_{tot} indicates the total count of pixels within the selected area of mycelial growth (16).

The purpose of this study was to examine how different lead oxide concentrations affect the bioluminescence intensity of a bioluminescent fungus, *P. stipticus*, over time. Based on previous research indicating that lead can potentially inhibit the growth of fungi, we hypothesized that when the concentration of lead present increases, the bioluminescence intensity of *P. stipticus* would exhibit a statistically significant decrease. We found our hypothesis to be true, and there was a statistically significant decrease in *P. stipticus* bioluminescence intensity as lead concentrations increased. These results demonstrate that measuring the bioluminescence intensity of *P. stipticus* could be used to detect lead pollution in the environment in a cost-effective, simple, and sustainable manner. Thus, *P. stipticus* could be used as an effective bioassay tool to detect lead pollution and prevent lead poisoning in lead-contaminated areas.

RESULTS

In this study, we aim to examine how different lead oxide concentrations affect the bioluminescence intensity of a bioluminescent fungus, *P. stipticus*. To test our hypothesis, we cultivated 20 subcultures of *P. stipticus* until bioluminescence was visibly evident. Then, we randomly assigned these 20 subcultures into 4 groups. To each of these 4 groups, we applied 0 mg (Group 1, 0 ppm), 8 mg (Group 2, 125.75 ppm), 17 mg (Group 3, 267.22 ppm), and 25 mg (Group 4, 392.98 ppm) of lead oxide, respectively. We calculated these concentrations of lead oxide by scaling the 400ppm limit of lead pollution to the volume of the agar in the petri dish to obtain a maximum concentration of 25mg (392.98 ppm). Then, we observed the fungi over a period of 17 hours. We measured bioluminescence intensity through the Adobe Photoshop "Histogram" tool, which measures the average pixel brightness within a selected area of an image (**Figure 1**). (15,16) We initially measured the bioluminescence intensity of each specimen before applying the lead oxide. Then we applied the lead oxide to each specimen according to the concentrations for each group. Afterwards, we measured the bioluminescence intensity every hour for a further 17 hours. We hypothesized that increasing concentrations of lead oxide would inhibit the bioluminescence intensity of *P. stipticus,* as prior research has demonstrated that lead exposure can inhibit the overall growth and metabolic processes of fungi, even in fungi used for bioremediation that accumulate and absorb high concentrations of lead (12,13).

Our experiment showed that the average bioluminescence intensity for Group 1 (0 mg) was 62.28 by the end of the 17-hour period, while the average light outputs for Group 2 (8 mg), Group 3 (17 mg), and Group 4 (25 mg) were 42.29, 29.30, and 33.87, respectively **(Figure 2)**. When analyzing a specimen, these light output values represent the average pixel brightness on a scale of 0–255 within the area of mycelial growth visible in the image of the specimen. Thus, these values report the bioluminescence intensity of the specimen. We compared the initial bioluminescence intensity (Hour 0) before any lead oxide was added and the final bioluminescence intensity (Hour 17) for each group to examine whether the bioluminescence intensity remained the same, increased, or decreased after the lead oxide was applied. We conducted a series of paired sample *t*-tests **(Figure 3)**. The results showed that, in Group 1 (0 mg), the mean intensity increased from 53.08 (SD = 7.52) to 62.28 (SD = 4.94), with a statistically significant difference ($t(4) = -2.329$, $p < 0.05$). In Group 2 (8 mg), the mean intensity decreased from 75.47 (SD = 18.60) to 42.49 (SD = 10.16), with a statistically significant difference ($t(4) = 5.079$, $p < 0.05$). In Group 3 (17 mg), the mean intensity decreased from 73.65 (SD = 13.93) to 29.30 (SD = 12.10), with a statistically significant difference ($t(4) = 7.324$, $p < 0.05$). In Group 4 (25 mg), the mean intensity decreased from 57.01 (SD = 9.68) to 33.87 (SD = 8.29), with a statistically significant difference ($t(4) = 14.350$, $p < 0.05$). Bioluminescence intensity

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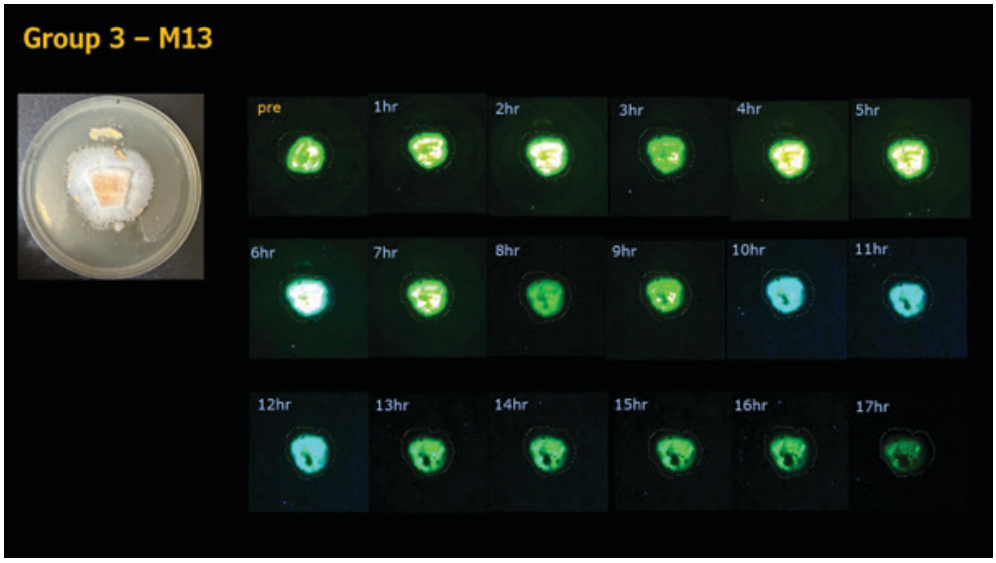

Figure 1: Example of photos from Group 3, subculture number 13, displaying the level of bioluminescence intensity by hour. *Panellus stipticus* subcultures in Group 3 were grown on malt extract agar under 17 mg of lead oxide.

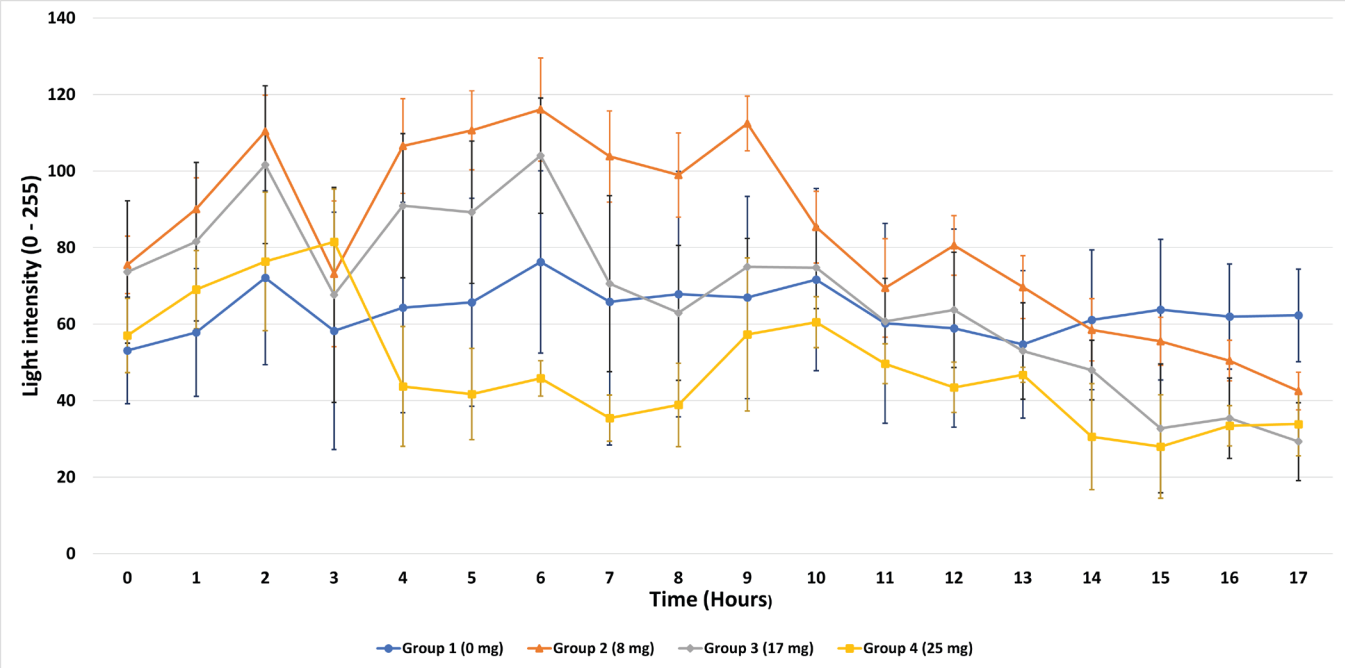

Figure 2: Mean bioluminescence intensity over time for *Panellus stipticus* subcultures exposed to increasing concentrations of lead oxide. The lines represent Group 1 with no lead oxide (control group), Group 2 with 8 mg of lead oxide, Group 3 with 17 mg of lead oxide, and Group 4 with 25 mg of lead oxide that were incorporated into malt extract agar. Each group contains *n*=5 samples. Bioluminescence intensity was measured hourly over a 17-hour period and is reported in light units (0-225). Error bars represent one standard deviation of bioluminescence intensity at each hour.

changed across all groups from 0 to 17 hours. In the control group (0 mg lead oxide), the intensity increased significantly, while in all treatment groups (8 mg, 17 mg, and 25 mg), the intensity decreased considerably.

We further conducted a linear regression analysis to determine how the bioluminescence intensity changed over time for each group. The slope of each line showed how quickly the light level decreased **(Figure 4)**. We interpreted a more negative slope as the light fading faster. Across all groups, the average slope was -2.10 (SD = 1.47), showing a steady decrease in bioluminescence intensity over time. The slope value for each group was -0.06 (SD = 0.55) for Group 1, -2.94 (SD = 0.96) for Group 2, -3.38 (SD = 0.40) for Group 3, and -2.07 (SD=0.90) for Group 4. To compare how the slopes differed across the four lead oxide concentration groups, we performed a one-way Analysis of Variance (ANOVA). The

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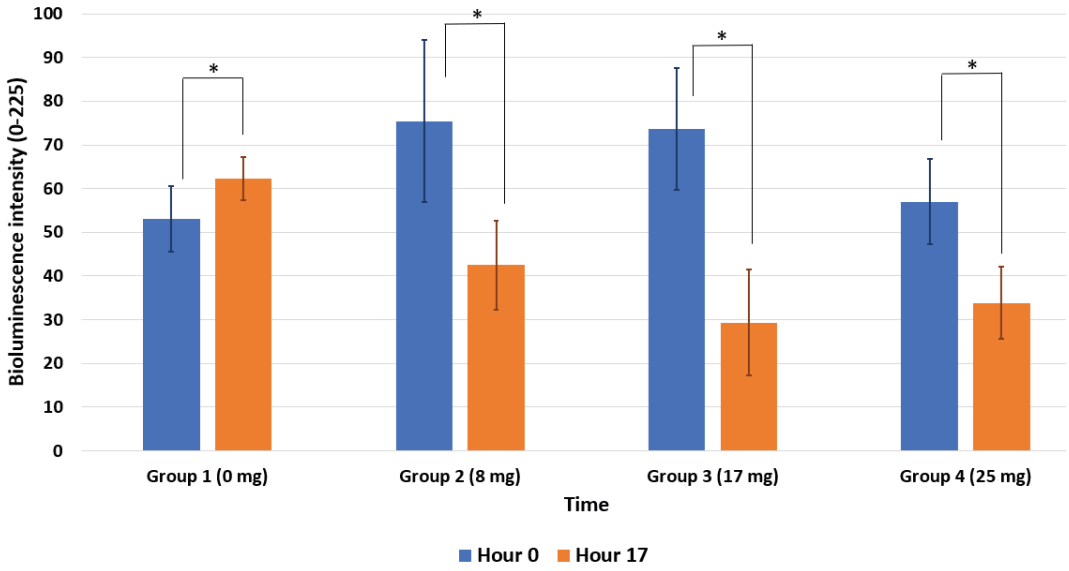

Figure 3: Change in mean bioluminescence intensity of *Panellus stipticus* in each group from Hour 0 to Hour 17. Bar graph showing mean ± SD bioluminescence intensity for four groups comparing Hour 0 and Hour 17 (n=5). Fungal subcultures were grown under either control conditions, 8 mg lead oxide, 17 mg lead oxide, or 25 mg lead oxide for 17 hours. Paired samples *t*-tests, *:$p < 0.05$. Error bars represent one standard deviation of bioluminescence intensity at Hour 0 and Hour 17.

findings showed a significant difference between the groups ($F(3, 16) = 20.00$, $p < 0.001$). A follow-up Tukey post-hoc test found that Group 1 with 0 mg of lead oxide had a significantly slower rate of decrease in light intensity compared to Groups 2, 3, and 4 ($p < 0.01$). However, there were no major differences between Groups 2, 3, and 4. This suggests that when the concentration of lead oxide is higher than a certain value, adding more lead does not make the light fade significantly faster.

DISCUSSION

The results of this study supported our hypothesis that increased concentrations of lead oxide caused a statistically significant decrease in the bioluminescence intensity of the *P. stipticus* specimens over time. However, we found that increasing concentrations of lead oxide did not necessarily cause a statistically significant increase in the rate at which bioluminescence intensity was reduced. These results show that *P. stipticus* specimens have the potential to be used as a bioassay tool for detecting lead pollution levels in an environment's soil, even at lead concentrations lower than 400 ppm (the USEPA's safety threshold limit for lead in soil). Using *P. stipticus* specimens would provide an alternative method for detecting lead pollution that is simpler than using complicated technology or equipment in a laboratory. *P. stipticus* could also easily be subcultured and cultivated on a large scale, providing a sustainable and accessible method of lead detection. By providing an easier way to detect lead in polluted environments, this method could possibly be used to warn of the presence of lead before lead poisoning occurs.

We noticed that the initial bioluminescence intensities (Hour 0) in Groups 2 and 3 (75.47 and 73.65, respectively) were higher than those in Groups 1 and 4 (53.08 and 57.01, respectively). These differences may have been due to subtle variations during the subculturing process, such as slight differences in the initial subculture size, fungal growth phase, or microenvironmental conditions in the petri dishes. To further examine these variations, the differences between the mean bioluminescence intensities for each group at Hour 0 and Hour 17 were calculated. While Group 1 showed an increase (+9.20), Groups 2, 3, and 4 exhibited notable decreases in bioluminescence (−32.98, −44.35, and −23.14, respectively). These absolute changes further supported our hypothesis. Although applying increasing concentrations of lead oxide did not necessarily cause a greater decrease in bioluminescence intensity, application of lead oxide did consistently cause decreases in the bioluminescence intensity of the *P. stipticus* specimens over time.

There are several possible directions for future research based on the findings of this study. One direction may involve exploring additional species of bioluminescent fungi, such as *Armillaria borealis*, to evaluate their sensitivity and effectiveness as bioassay tools for detecting lead pollution (14). Another area of research could be investigating whether bioluminescent fungi can be used to detect other heavy metals or environmental pollutants, such as zinc or copper (5). Such studies would enable researchers to analyze the versatility of using bioluminescent fungi as bioassays for pollutants in general. Finally, further studies may examine the potential of bioluminescent fungi to aid bioremediation by testing their effectiveness not only as detection tools but also as a method of reducing pollutants from contaminated environments.

Despite the promising findings of this study, there are several limitations. First, the application of lead oxide to the fungi through a wooden stirring stick may have caused some uneven distribution of the lead oxide. A more uniform

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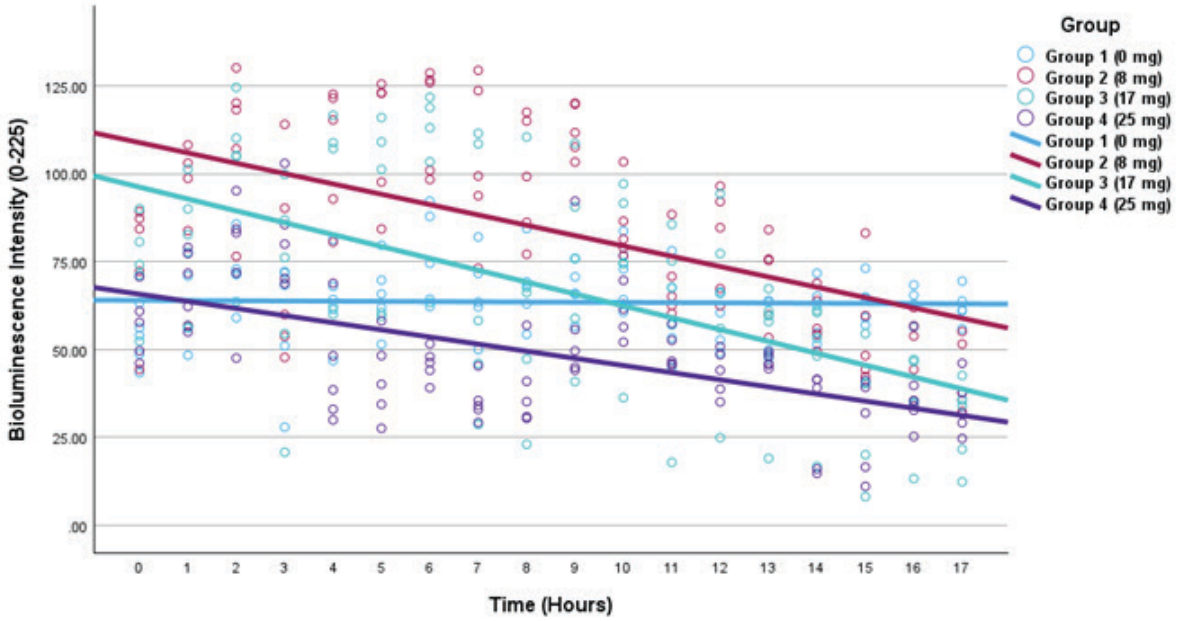

Figure 4: Linear regression analysis of bioluminescence intensity. Bioluminescence intensity (0–225 scale) measured over 17 hours for four groups receiving 0 mg, 8 mg, 17 mg, or 25 mg of lead oxide, respectively. Individual data points represent recorded intensities at each hour, and solid lines depict the linear regression trend for each group. Higher-dose groups (17 mg and 25 mg) show steeper negative slopes, indicating faster declines in bioluminescence over time compared to the 0 mg and 8 mg groups.

application method, such as dissolving the lead oxide in solution and spraying it onto the fungal subcultures, could reduce this variability. However, the consistent decrease in bioluminescence intensity observed across all treated samples in multiple trials suggests that even with some variability in the application of the lead oxide, the inhibition effect was clear and reproducible. Thus, any potential unevenness in the application of the lead oxide would have introduced some variability but would not have invalidated the overall trend of the data. Second, while this study used a single strain of *P. stipticus*, the statistically significant decrease in bioluminescence intensity suggests a genuine response to lead exposure in this species of fungus. Although different strains may vary in the strength of their inhibitory responses, it is unlikely that other strains of this species would show no or little reaction to lead oxide. Thus, the usage of a single strain is not a significant limitation of this study. The results of this study are robust for *P. stipticus* and provide a foundation for future strain comparison studies.

Standard deviations, shown by error bars in the graph, indicated the variation in bioluminescence intensity among the five samples in each group **(Figure 2)**. In Group 1 (0 mg), the standard deviations were small and stayed consistent over time. This means the five samples in Group 1 have low variability. But Groups 2 (8 mg), 3 (17 mg), and 4 (25 mg) showed larger standard deviations between Hours 2 and 10, indicating greater variability in their bioluminescence response. Group 4, with the highest concentration of lead oxide, showed the largest variations overall. However, the standard deviations in all groups treated with lead oxide became smaller by the final hour, Hour 17, compared to the standard deviations prior to Hour 17. This trend in standard deviations suggests that *P. stipticus* would be a reliable bioindicator for lead contamination when measured over longer periods (i.e., 17 hours or more), as the results of this study demonstrated that the variability in bioluminescence intensity between samples decreased with time.

Moreover, before *P. stipticus* can be reliably deployed as a bioindicator, additional research is needed to assess the accuracy and practicality of this method. Future studies should focus on testing whether *P. stipticus* consistently shows reduced bioluminescence intensity when grown in different environmental conditions containing lead, such as in different types of soil or water. In addition, it is necessary to test *P. stipticus* in these different environmental conditions to ensure that abiotic (e.g., pH, temperature) and biotic factors (e.g., soil microorganisms) do not elicit similar responses in bioluminescence intensity, potentially leading to false positives.

Analyzing such parameters would improve the robustness of this bioassay method, ensuring that *P. stipticus* provides an accurate and reliable indication of lead contamination in diverse ecological settings.

Overall, the findings of the study supported our hypothesis that if the concentration of lead present increased, the bioluminescence intensity of *P. stipticus* would exhibit a statistically significant decrease. Thus, through further analysis, *P. stipticus* could be used as an effective and sustainable method for detecting the presence of lead in an environment's soil and water due to its lead sensitivity. *P. stipticus* could provide an alternative to current methods of detecting lead pollution, which are expensive and unsustainable, and help to reduce the cases of lead poisoning through early detection of lead in polluted areas.

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Figure 5: Culturing process. (Step 1) Gather materials. (Step 2)Mix 38 g of malt well and extract agar into 750 ml of water. (Step 3) Transfer the mixture into a microwave-safe bowl and microwave for 4 minutes. (Step 4) Take the bowl out and let the mixture cool for 1 minute. Pour the mixture into each petri dish at a depth of 1cm for each plate. Let the agar set. (Steps 5&6) Use the scalpel to cut 20 one-centimeter cubes from the edge of the growing *Panellus stipticus* culture. (Step 7) Invert and place one cube in the middle of each of the 20 dishes. Avoid touching the scalpel to the inside of the dish to avoid contamination. (Step 8) Incubate all dishes at room temperature in the dark for 7 days.

MATERIALS AND METHODS

Subculture Preparation

For our experiment, one *P. stipticus* culture was purchased online from the Carolina Biological Supply Company (17). To prepare the agar plates for the fungal cultures, the work area was first sterilized with antibacterial wipes. Then, 38 g of malt extract agar, obtained from Seaweed Solution Laboratories, was thoroughly mixed into 750 mL of water. After this, the mixture was transferred to a microwave-safe bowl and microwaved for 4 minutes. Then, the bowl was taken out, and the agar mixture was allowed to cool for 1 minute. The mixture was poured into each of the 20 petri dishes obtained online (Amazon.com) to a depth of 1 cm for each plate. After the agar mixture was solidified, the scalpel obtained online (Amazon.com) was used to cut 20 one-centimeter cubes from the edge of the growing *P. stipticus* culture. One cube was inverted and placed in the middle of each of the 20 petri dishes **(Figure 5)**.

Lead Oxide Treatment

All dishes were incubated at room temperature in the dark for seven days. After rhizomorphs began to form in all the dishes and bioluminescence was present, the 20 dishes were randomly assigned into 4 groups, with 5 dishes in each group. Different concentrations of lead (II) oxide powder (in a 30 g container), obtained from the Home Science Tools company (SKU: CH-PBO), were applied to the 20 dishes. Specifically, 8 mg of lead oxide was applied evenly across each dish in Group 2, 17 mg evenly across each dish in Group 3, and 25 mg evenly across each dish in Group 4. No lead oxide was applied to the remaining control group, which was Group 1. The lead oxide powder was carefully spread across the specimens using a wooden stirring stick (**Figure 6**).

Bioluminescence Imaging and Quantification

A digital smartphone camera with adjustable exposure settings was used to capture long-exposure photos of the *P. stipticus* through the Slow Shutter App, purchased from the App Store. First, a photo of each specimen in normal lighting using the default camera on the smartphone with original exposure settings was taken. Next, the Slow Shutter app's settings were appropriately modified to "Low Light," "High Noise Reduction," a shutter speed of eight seconds, and an ISO of "Auto." Using these settings, digital images of each specimen were captured in complete darkness every hour. This process continued for 17 hours, as minimal changes in the bioluminescence intensity of the fungi were observed beyond this time frame.

To find the bioluminescence intensity of one specimen in one image, the digital image editing software Adobe Photoshop was used. First, a specimen was chosen for analysis. Within Photoshop, the Magic Wand tool was used to trace the outline of the chosen specimen in the first image taken of the specimen in normal lighting. This selection was then saved and superimposed onto the second image taken in the dark of the chosen specimen. The histogram tool was applied to this selected area in Photoshop to obtain the mean bioluminescence intensity value of the specimen on a scale of 0–255 in the photo as well as the standard deviation value (16). These values, in arbitrary units, represent pixel brightness on an 8-bit RGB scale, where 0 corresponds to black (no intensity) and 255 to white (maximum intensity). This procedure was repeated for all remaining photos of the chosen specimen in the dark until the end of the 17-hour period

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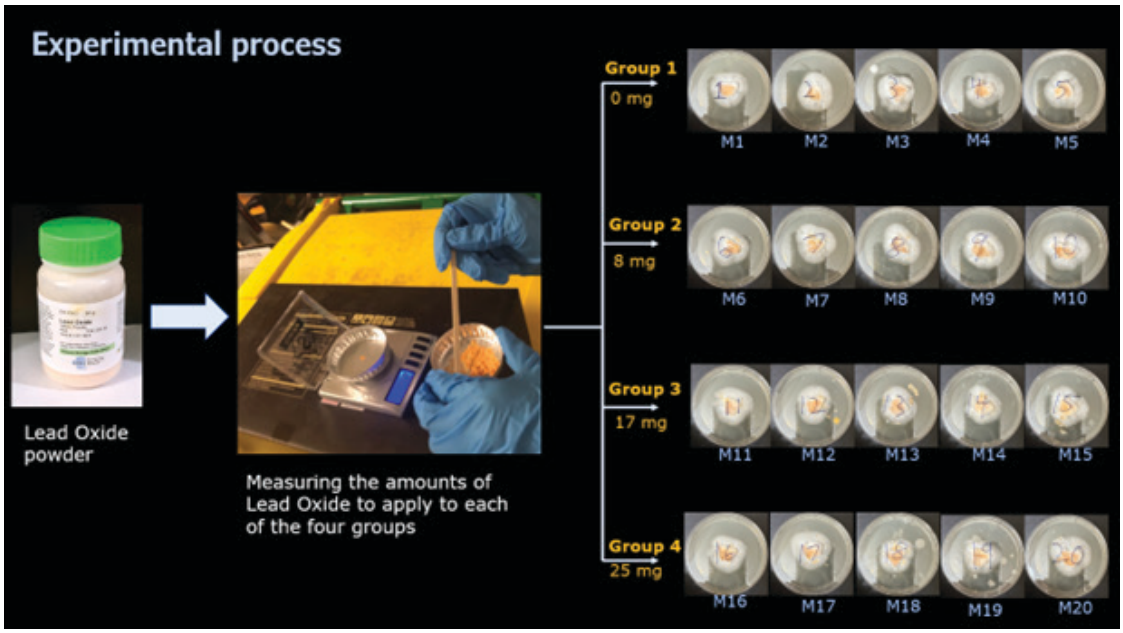

Figure 6: Experimental process. Lead (II) oxide powder was applied in varying concentrations to 20 dishes: 8 mg, 17 mg, and 25 mg for Groups 2, 3, and 4, respectively, and none for Group 1 (control group).

to examine the trends in the specimens' bioluminescence intensity over time. This entire procedure for measuring bioluminescence intensity over time from a specimen's digital image was repeated for all specimens across all groups.

Statistical Analysis

All statistical analyses were conducted using SPSS (version 29.0). A significance level of 0.05 was used throughout the analysis. To examine changes in bioluminescence intensity from Hour 0 to Hour 17 within each group, paired samples t-tests were conducted. To analyze the rate of change in bioluminescence intensity over time, linear regression was performed for each group to compute slope values. The slope values were then compared across the four groups using a one-way ANOVA. Since the ANOVA showed a significant effect, follow-up Tukey post-hoc comparisons were conducted to identify which groups differed significantly from one another.

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