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The Tolerance of Shewanella woodyi for Electric Potentials and Heavy Metals as Biofilms

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Abstract:

Shewanella woodyi is a bioluminescent marine organism that is known to be metal tolerant and modulate the intensity of its luminescence with electrochemical potential. The viability of *S. woodyi* as a bioreporter for the toxic heavy metal zinc, copper, and silver was analyzed. Biofilms of *S. woodyi* was grown on marine broth agar plates and then exposed to various concentrations of each metal ion to evaluate biofilm response to the metal ions that were generated from an operating short circuited electrode containing either Zn, Cu, or Ag metal. The ability of the bacteria to tolerate the heavy metals and continue to luminesce was evaluated at designated distances from the electrode by ICP-OES. The possibility of an electricidal effect was determined to be insignificant near the electrodes. So, even though *S. woodyi* showed unprecedented tolerance for Zn(II), it would ultimately be a marginal living bioreporter without genetic modification.

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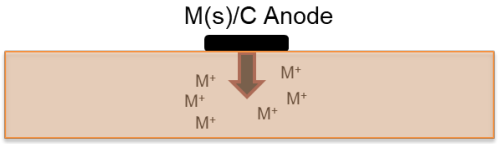
Introduction:

The ability of a living organism to act as a bioreporter for heavy metals is reliant on the organism's ability to tolerate the heavy metal present. Other members of the *Shewanella* family have been shown to have this ability such as *Shewanella putrefaciens* (Stone, Burgos, Royer, & Dempsey, 2006) (Lu, et al., 2015). Heavy metals such as Zn and Ag have been established to be toxic to microorganisms (Dhas, Shiny, Khan, Mukherjee, & Chandrasekaran, 2014). The ability of an organism to tolerate such toxins would be a valuable bioreporter for knowing if a body of water has been contaminated with toxic metals from dumping sites or leech fields. To act as an efficient bioreporter we need a way to measure the presence of the heavy metals reliably. Bioluminescent microorganisms that can tolerate heavy metals could be excellent bioreporters if the bioluminescence output could be modulated along with a high rate of survival.

The heavy metal tolerance by the *Shewanella* family varies from species to species. Though it is not the only organism that can resist toxic heavy metals (example *Geobacter sp* and *Desulfuromonas sp*.) . There was a study done in 2011 with *Vibrio harveyi, V. fischeri, Photobacterium phosphoreum,* and *P. leiognathi* that suggested that the ability of organisms to be resistant to the toxic effects of heavy metals was due to a specific type of plasmid (Ranjitha & Karthy, 2012). *Vibrio* species are marine bacteria and a significant portion of this family showed resistance to zinc, copper, mercury, barium, silver, and cobalt. It was also noted that most of the organisms tested contained multiple plasmids which imparted some of the adaptability of this organism to these toxic metals. It is believed that some of the plasmids that might have been involved in the

toxic heavy metal resistance were being mutated to evolve in a polluted environment which required the specific plasmid, or plasmids, to survive in that environment.

Other living bioreporters have also been documented for the metals we are testing (Zn, Ag, and Cu). The organisms that are used as bioreporters primarily use bioluminescence as an indicator. Some organisms exhibit bioluminescence in the presence of certain metals while others will shut off their bioluminescence in the presence of the metals. We recently published an article showing that S. woody (the strain used in the study) also shuts off bioluminescence but shows a high metal tolerance (Theberge, et al., 2019). Another example would be *Pseudomonas putida* which has an efflux pump regulating the amount of metal inside the cells. When the efflux pump is removed and a certain amount of metal builds up in the cells, the cells begin to produce bioluminescent proteins which would be the exact opposite effect that we would expect from *Shewanella woodyi.* This organism, with the efflux pump removed, can also be used as a bioreporter for the presence of metals that would cause bioluminescence to be emitted. We present here the first example of a bioluminescent bacterium as a bioreporter for toxic metal ions as a biofilm. These toxic metal ions were generated from the short circuiting of carbon-based electrodes using either Ag(s), Zn(s) Cu(s) into a semi-conductive agar support. Figure 1 shows a pictorial representation of the expected effect of these electrodes along with some rational behind the overall electrochemical experimental design.



Electrode Slowly Discharges into the Agar

Efficiency of the process related to:
1) Ion diffusion rates in agar gels
2) Reaction products from each electrode and the nutrient rich plates
3) Potential of the metal oxidation

Reaction	E^0 (V vs. NHE)	Potential Effect
$Ag(s) \rightleftharpoons Ag^+(aq) + e^-$	+0.76	Oxidizing
$Zn(s) \rightleftharpoons Zn^{2+}(aq) + 2\,e^-$	-0.80	Reducing

Each electrode would react with the agar components differently

Figure 1. Pictorial representation of the overall experimental design with specific electrochemical aspects of the bioassay.

Experimental setup and Methods

Culture Conditions:

We first obtained a small sample of *Shewanella woodyi* from a frozen stock or from a slant that had been grown previously on Marine Broth Agar. We took the sample and put it in a 15mL falcon tube filled with Marine Broth and placed it in an incubator for two days at 20°C (with shaking at 170 rpm). Once the cells reached stationary phase (48 hours), they were removed from the incubator. To wash the cells, we removed 1mL of the grown culture and placed it in an Eppendorf centrifuge tube. The tube was then centrifuged at 12,000 rpm for one minute. The supernatant was removed via micropipette from the cell pellet and replaced with 1mL of ONR7A (Fisher Scientific, 5x) with 10mM glucose solution. The cells were then resuspended in solution by vortex. The tube was then placed back in the centrifuge and pelleted at 12,000 rpm for 1 min. This process was

repeated for a total of three washes with ONR7A. From there 100µL of the washed cells was placed in another Eppendorf tube that contained 900µL of ONR7A with 10mM glucose solution. The solution was then put on a vortex machine for 20 seconds to ensure proper mixing. The process of taking 100µL of one cell suspensions and placing it in 900µL of ONR7A with 10mM glucose solution was repeated 4 times until we had a dilution of 10^{-4} dilution of cells that would eventually be drop cast at pre-designated differences from the electrode.

Composition of Agar Plates for Biofilm Assay:

To make the agar plates we took 500 mL of RO water and added it to a 1L container. We then massed out 18.7 g of Marine Broth mix and added it to the container. We then massed 7.5 g of Agar and added that to the container. The container was then autoclaved for 1 hour. Once done, we pipetted 25 mL of the solution into the culture plates. Once done the plates were left to sit for 2-3 hours and were then placed in the fridge until they were needed.

We also created agar plates with soluble zinc ions to confirm at what concentration zinc ions become toxic to *S. woodyi.* To make the zinc agar plates we took 500 mL of RO water and added it to a 1L container. We then massed out 18.7 g of Marine Broth mix and added it to the container. To make the plates have zinc, we added a volume of 0.2 M $ZnCl_2$ to get the ppm of Zn we needed to test. We then massed 7.5 g of Agar and added that to the container. The container was then autoclaved for 1 hour. Once

done, we pipetted 25 mL of the solution into the culture plates. Once done the plates were left to sit for 2-3 hours and were then placed in the fridge until they were needed.

Carbon/Metal Electrode Inks:

To make the electrode inks used we first massed 5 mg of the heavy metal we were going to use (Cu(s), Zn(s), or Ag(s)) in a glass vial. In general, we then added 20mg of XC-72 Vulcan carbon. After that we added 30µL of a 5% Nafion solution, 150µL if isopropanol, and 450µL of RO water. The vial was then placed on a vortex apparatus for 30 seconds. Once done the vial was placed in a sonicator for 10 min (at room temperature) in order to ensure that the Vulcan carbon was completely broken up and the metal was properly mixed into the ink.

For the 20mg Zn ink that was created, we first massed 20mg of Zn(s) in a vial. We then added 20 mg of XC-72 Vulcan carbon, 60µL of a 5% Nafion solution, 300µL of isopropanol, and 900µL of RO water. The vial was then mixed by vortex for 30 seconds. Once done the vial was placed in a sonicator for 10 min in order to ensure that the Vulcan carbon was completely broken up.

Biofilm Electricidal Agar Plate Assay Design:

Agar plates composed of Marine Broth Agar were first exposed to the electrode. The electrode ink (50µL) was drop cast using the carbon-based inks. The patterns and orientation of the plates were dictated using a predetermined guide with the electrode

drop cast at the center of the plate. The ink was then left to dry for 45 min to 1 hour in the biosafety cabinet. The plate was then left in the bio-safety hood for 6 hours with the fan off to prevent the plate from drying out. After that time, we took 25µL of the 10^{-4} dilution of cells and drop cast 3 spots directly around the electrode and drop cast 2 more spots on the outside of the plate at a distance that would assure that the metal ion concentration would be minimal. The plates were then left to dry for 45 min to 1 hour. Once dry, the plates were then placed in an incubator set at 20°C for two days.

Imaging of Plates:

Images of the plates were taking with a UVP Gel imaging system with CCD camera. The images were taken two days after the electrode and bacteria were drop cast. A white light slide was placed under the plates and projected light through the plate to the camera for brightfield images. For the brightfield images the exposure time of the camera was set to 300 milliseconds. To examine the luminescence emitted from *S. woodyi,* we turned off the light and set the exposure time of the camera to 5 minutes. A ruler was included in the images to allow for a proper scale reading of both colony size and electrode spacing.

Calculation of Metal Ion Concentration in Agar Plates:

Inductively Couple Plasma-with optical emission spectroscopy (ICP-OES) was used to determine the density of the heavy metals in the agar plates. In order to prep the agar samples, we first would cut agar samples both near and far from the electrode at the center of the plate. For each sample, we would cut out approximately 1cm x 1cm pieces

of agar. These samples were then placed in pre-massed test tubes, which were then measured again to get a mass of agar that was removed. Three samples were taken near the ink and three more were taken from the edge of the plate. After that, each test tube had 3mL of RO water added to it. Each test tube was then placed in a microwave for 10 seconds to melt the agar samples. Once microwaved the samples were vortexed for 10 seconds. Once all the samples were melted and mixed, they were placed in a fridge overnight (though this step was not necessary for the analysis). The following day the samples were injected into the ICP-OES and analyzed while looking for the elements: Ca, Na, Mg, Ag, Cu, Zn. The concentrations of each ion were properly calibrated using external standards. The parameters used were 1 kW power, 15.0 L/min plasma flow, 200 kPa nebulizer pressure, 3 replicates taken per sample, and a pump rate of 10 rpm.

Results and Discussion

For the first set of experiments we grew the biofilms of *S. woodyi* on MB plates with metal containing electrodes with all 3 metals. Based on the design of the experiment, these electrodes were functional and discharging current into the agar support. We confirmed this by taking open circuit measurements (using a Gamry 1000 Potentiostat) of the Zn and Ag electrodes over 80 hours. These data are shown in Figure 2 and confirm that not only was there a reducing and oxidizing potentials generated by these systems but also these open circuit potentials decreased by ½ after 40 hours of operation.

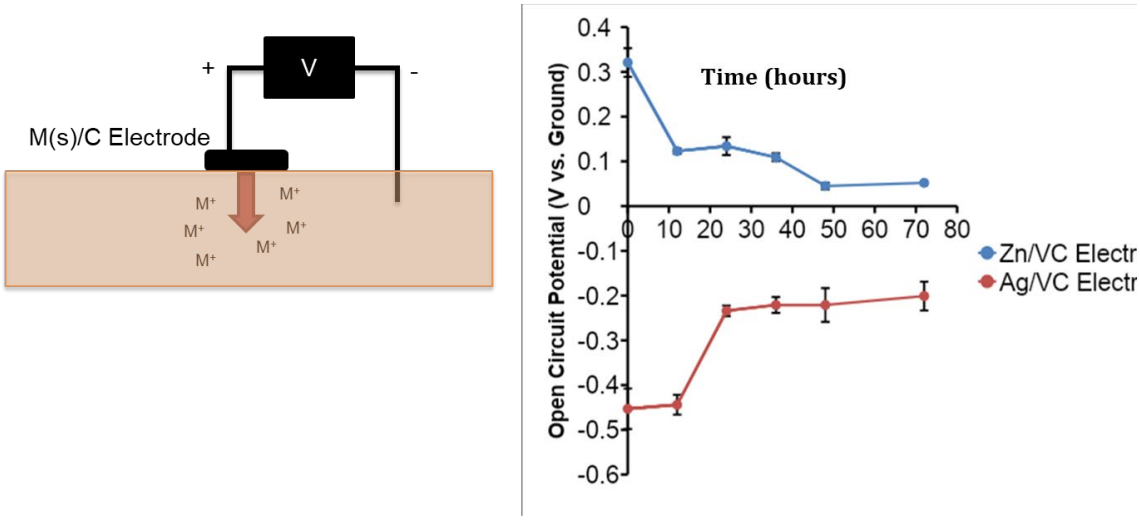


Figure 2. Image of the general measurement of the open circuit potential using the drop cast electrodes (Right) and the resulting open circuit potential voltage over time from zinc and silver electrodes.

In general, time 0 on the graph in Figure 2 was when S. woodyi was drop cast onto the agar plates. After growing for two days with the operating electrodes or a control electrode without metals, we took brightfield and long exposure luminescence images of the colonies (Figure 3).

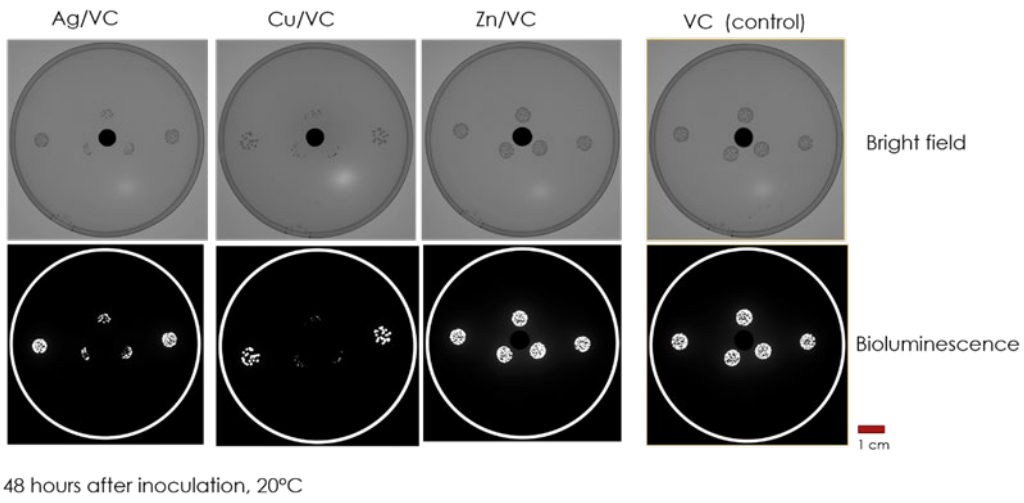


Figure 3 Brightfield and luminescence images of *S. woodyi* colonies grown over 2 days with Ag/C, Cu/C, Zn/C electrodes compared to images of colonies with a carbon only electrode.

In the first set of experiments we observed that silver (Ag/VC) ink and copper (Cu/VC) ink both resulted in a zone of inhibition around where the ink was placed extending approximately 10-12 mm from the edge of the electrode.. The zinc (Zn/VC) ink however, did not result in any inhibition of growth around the electrode. The images shown in Figure 3 confirm that the silver and copper inks also prevented the cells from growing around the electrodes in the zone of inhibition and from expressing bioluminescence. It was here that we decided to further investigate the concentrations of zinc that were present in the agar since there was not inhibition of *S. woodyi* growth. We took samples of the agar both near the electrodes and at the edge of the plates and used ICP-OES to analyze the ions in the agar to show that there was indeed toxic metals in the agar and being to rule out an electricidal effect. The presence of an actual effect on bacterial inhibition is continues to be explored because of its potential application in wound care (Reza Asadi & Torkaman, 2014).

Electrode Type	**Region 1**	**Region 2**
Ag/VC	$[Ag^{+}] = 4.5 \pm 0.6$ ppm (n = 3)	$[Ag^{+}] = 1.4 \pm 0.2$ ppm (n = 3)
Cu/VC	$[Cu^{2+}] = 116 \pm 28.9$ ppm (n = 3)	$[Cu^{2+}] = 1.9 \pm 0.3$ ppm (n = 3)
Zn/VC	$[Zn^{2+}] = 3.7 \pm 0.6$ ppm (n = 3)	$[Zn^{2+}] = 0.6 \pm 0.2$ ppm (n = 3)
VC	$[Ag^{+}] = 0.0 \pm 3.6$ ppm (n = 3), $[Cu^{2+}] = 3.4 \pm 2.1$ ppm, (n = 3) $[Zn^{2+}] = 0.9 \pm 0.5$ ppm (n = 3)	$[Ag^{+}] = 0.0 \pm 0.9$ ppm, (n = 3) $[Cu^{2+}] = 2.8 \pm 0.8$ ppm, (n = 3) $[Zn^{2+}] = 0.4 \pm 0.1$ ppm (n = 3)

Table 1. ICP-OES data from the first set metal ion analysis from agar plates. Region 1 refers to the samples taken that were 10-12 mm to the dropcast ink edge and region 2 (100cm from the edge of the electrode).

The data collected from the ICP-OES runs confirms that there were significant concentrations of toxic metal ions within the plates. We are also able to observe how much of each metal was released from the electrodes into the agar. By comparing the metal results to the VC-only control results, we confirmed that silver and zinc are able to transfer a moderate amount of metal ion from the ink to the agar to a concentration that inhibited the growth of *S. woodyi*. Copper electrodes released a significantly greater density of ions from the ink. We are also able to see that the agar itself only seems to allow less than 2ppm of a given ion to the edge of the plates where control biofilms were spotted. Thus, we can have control and experimental colonies on the same plate. This tells us that once away from the ink, the ions are heavily restricted in their mobility across the agar.

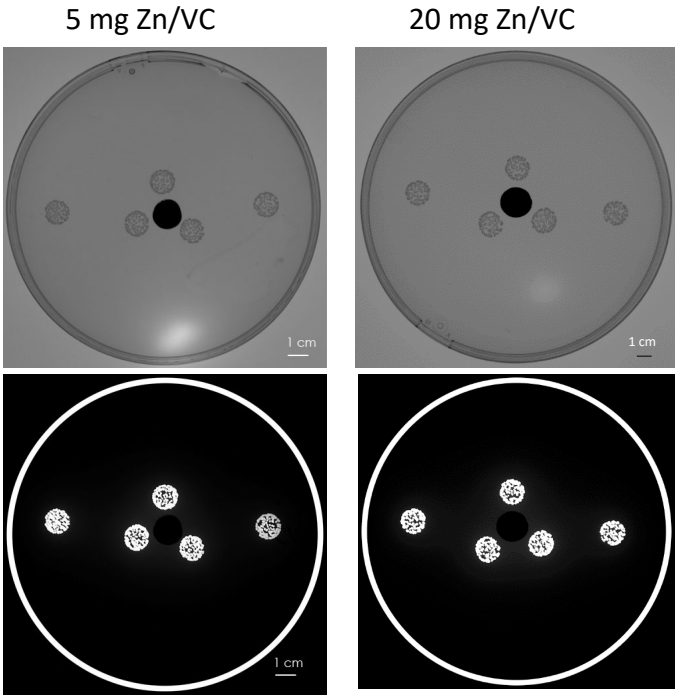


Figure 4. Brightfield and luminescence images of *S. woodyi* colonies grown over 2 days with 5 mg or 20 mg loadings of Zn(s) in carbon inks.

Since the first set of trial with the zinc metal did not seem to inhibit or disrupt the bioluminescence of the *S. woodyi* biofilms, we decided to try again with the zinc metal in the ink, now with greater amounts of zinc in the ink which should generate higher concentrations of zinc ions in the agar support. We used 20mg as we believed that 20mg was as much metal as the ink could hold before the metal no longer mixed with the ink. We used 5mg as a comparison/control to the first set of experiments. As seen in the Figure 4, there was no noticeable difference in the bioluminescence between the 20mg zinc ink and the 5mg zinc ink. We also confirmed that there was no significant increase in the agar plate by using ICP-OES of metal extracted agar samples (Table 2).

Electrode Type	**Region 1**	**Region 2**
20mg Zn/VC	$[Zn^{2+}]$ = 8.7± 1.1ppm (n = 3)	$[Zn^{2+}]$ = 0.0 ± 0.1ppm (n = 3)
5mg Zn/VC	$[Zn^{2+}]$ = 3.7 ± 0.6 ppm (n = 3)	$[Zn^{2+}]$ = 0.6 ± 0.2 ppm (n = 3)

Table 2. Results from the ICP-OES analysis of agar samples from the plates with zinc ink. Region 1 refers to the samples taken that were 10-12 mm to the dropcast ink edge and region 2 (100cm from the edge of the electrode).

We found that by using a higher a higher loading of zinc metal in the ink that it did not add a proportional concentration of ions to the agar. For using four times the amount of metal, we only saw roughly two times the concentration of ions near the ink and roughly the same number of ions at the edge of the plate. We also noticed that when we made the ink with the 20 mg of zinc metal, the ink itself was having difficulty mixing and holding the metal that was added. At the same time, we still needed to find the concentration of zinc ion that would cause the bioluminescence from *S. woodyi* to become inhibited or shut off without cell death.

In order to experiment with higher concentrations of zinc ion, we decided to mix Zn(II) directly into the agar support at predetermined concentrations. While this does remove the ability of an electrochemical experiment to run on the same plate as a control, it does allow us to understand at what concentration the Zn(II) ions would inhibit the growth of the organisms. The control for these experiments were MB plates with no additional Zn(II) ions.

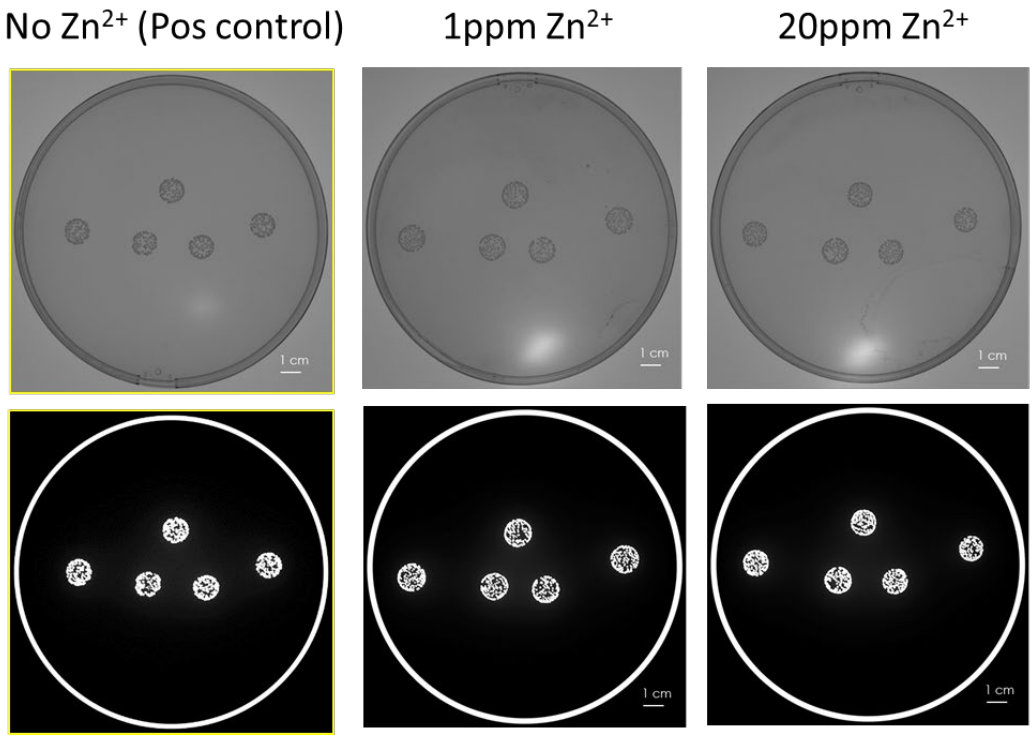


Figure 5. Brightfield and luminescence images of *S. woodyi* colonies on MB agar plates with 1 ppm and 20 ppm zinc(II) in the plates as opposed to dropcasting a metal ink and inducing metal diffusion.

In order to get higher concentrations of zinc to be exposed to the organism, we added specified concentrations of zinc ion into the agar mix. We decided to start at 1ppm because it was close to the concentrations achieved with the metal ink drops. We chose 20 ppm as we thought it would be a high enough increase of the zinc ion concentration to affect the bioluminescence. The images in Figure 5 which show the results from these experiments confirm that 20 ppm of zinc(II) did not inhibit growth nor decrease the luminescence of the colonies. From this information we then decided to take a rather large increase in the concentration to try to get an idea of the range of zinc ions that the organism would be able to tolerate.

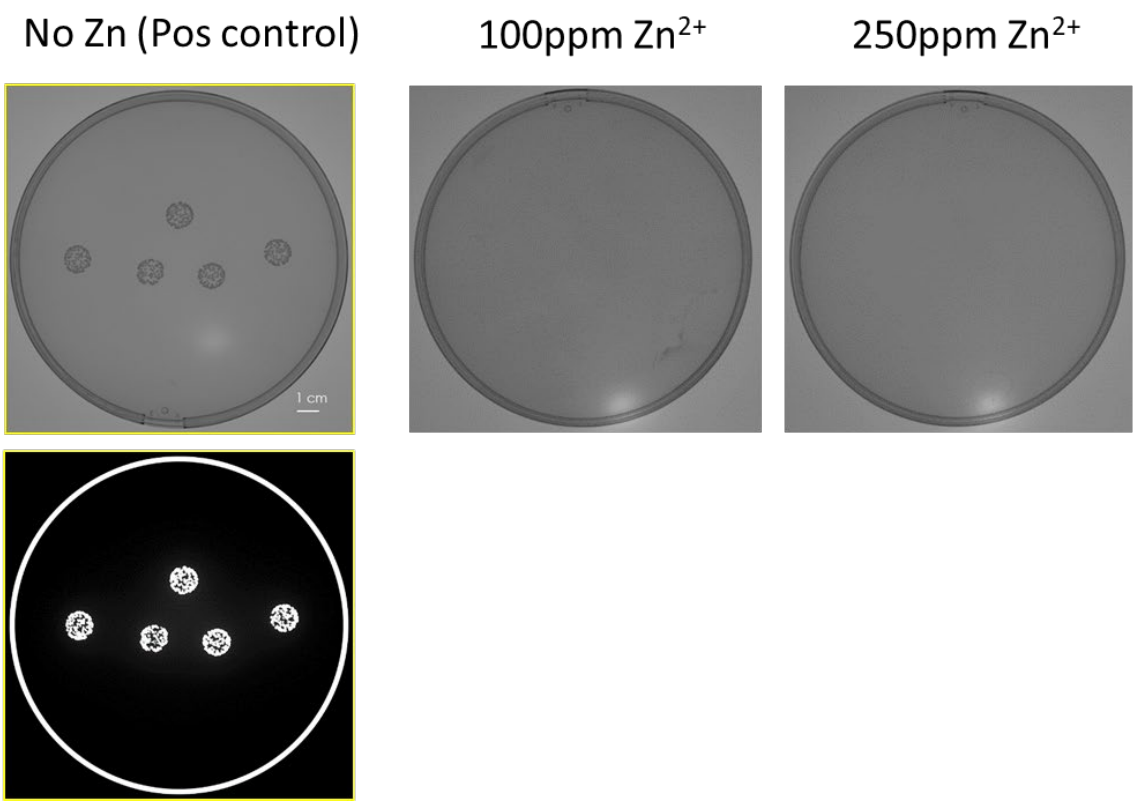


Figure 6. Brightfield and luminescence images of *S. woodyi* colonies on MB agar plates with 100 ppm and 250 ppm zinc(II) in the plates as opposed to drop casting a metal ink electrode and inducing metal diffusion.

We decided to go to increase the concentration to > 100 ppm Zn(II) in order to determine a range in which to start narrowing down where the bioluminescence of *S. woodyi* would shut off. From the results shown in Figure 6, we see that both 100 ppm and 250ppm where high enough concentrations that the *S. woodyi* was unable to grow. It should be noted that this level of zinc tolerance is unprecedented for a marine bacterium. So, while >100 ppm Zn(II) does effectively shut off the bioluminescence of the organism, we are searching for a concentration that the organism can tolerate yet is high enough to turn off the organism's bioluminescence. We were interested of the minimal concentration of Zn(II) that would enable colony growth.

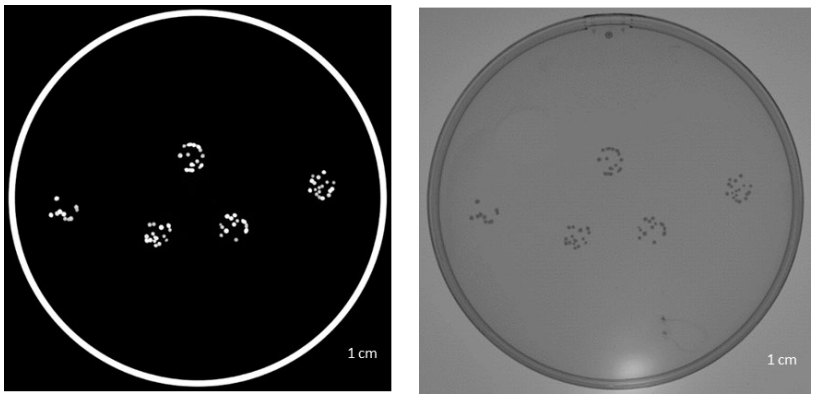


Figure 7. Brightfield and luminescence images of *S. woodyi* colonies on MB agar plates with 35 ppm zinc(II) in the plates as opposed to drop casting a metal ink electrode and inducing metal diffusion.

Our experiment using 35ppm Zn plates (Figure 7) allowed us to finally see where colony growth was still possible but unfortunately the cells were also still luminescent at this concentration which means that *S. woodyi* might be a poor candidate for being a whole cell bioreporter for toxic metals. However, it's metal tolerance mechanisms for Zn(II) might be very informative in what pathway this organism can survive 35 ppm of Zn(II) ions. Comparing the 20 ppm Zn(II) plates and the 35 ppm Zn(II) plates, we do see a noticeable difference in the levels of bioluminescence. However, the colonies that did grow did so at a lower density than previous plates. The lack of cell growth most likely account for the lowered overall luminescence. Yet, *S. woodyi* is still luminescent, so there might still be a range of Zn(II) ions that will inhibit bioluminescence yet still allow the organism to grow.

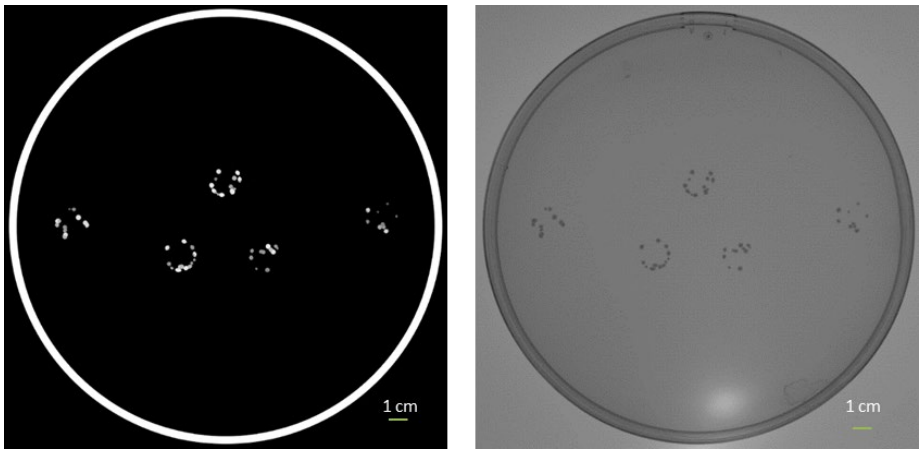


Figure 8. Luminescence and brightfield images of *S. woodyi* colonies on MB agar plates with 50 ppm zinc(II) in the plates as opposed to drop casting a metal ink electrode and inducing metal diffusion.

In the images of the 50 ppm zinc plates we once again see decreased bioluminescence along with smaller colony sizes. It is likely that the decreased colony sizes are responsible for the decreased bioluminescence. However, since we have not yet reached a concentration where the *S. woodyi* can form colonies, but not exhibit bioluminescence we tried more Zn(II) concentrations in this range.

The plates that contained 70 ppm zinc ion in the agar (Figure 9) showed a greater number of colonies grown, though less dense. As the colonies decease so to is the bioluminescence of the *S. woodyi.* It is around this concentration that we begin to see the limits of the heavy metal tolerance of the *S. woodyi*. At the ion concentration of 85 ppm the organism was unable to grow (Figure 9). From the data presented we are able to conclude that the concentration of zinc ion that would enable the *S. woodyi* to grow, but not exhibit its bioluminescence would be somewhere between 70 and 85 ppm.

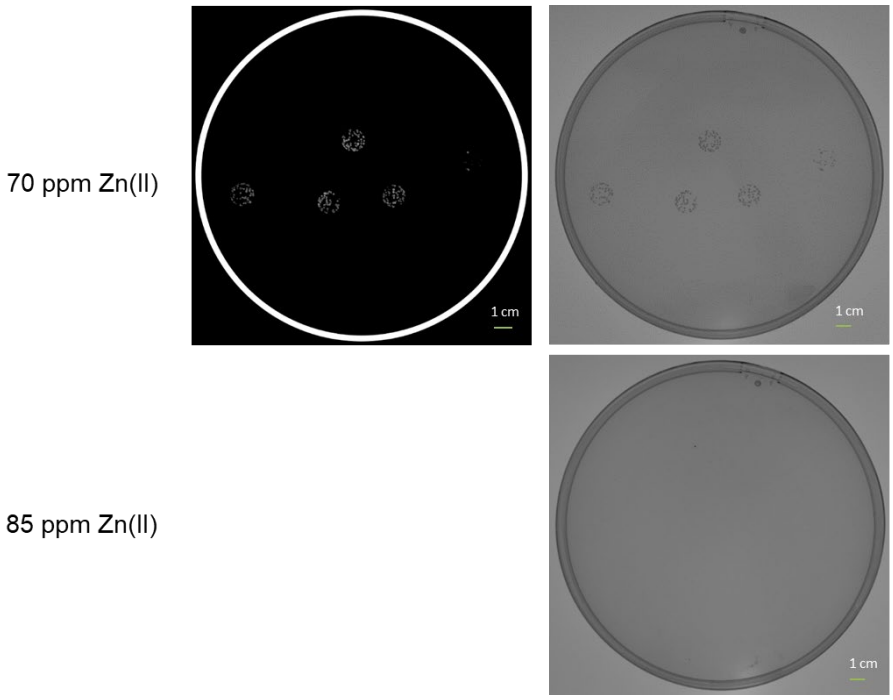


Figure 9. Luminescence and brightfield images of *S. woodyi* colonies on MB agar plates with 70 ppm zinc(II) (top images) and 85 ppm Zn(II) (bottom images) in the plates.

In order to confirm if the death and/or inhibition of bioluminescence was caused by an electricidal effect instead of the metal ions, we infused the plates with Vulcan carbon (a highly conductive form of graphite). The agar plates without vulcan carbon had an electrical resistance of approximately 1-2MΩ (determined with single point resistance measurements with fixed probes and conductivity meter). So, it rather unlikely that any electricity was flowing at a distance greater than 1mm from the electrode region. The addition of vulcan carbon to the agar medium decreased the electrical resistance of the agar support to 1-2 kΩ and the presence of Vulcan carbon did not inhibit the growth of the colonies (data not shown). If there was going to be an electrical effect present, it was going to be seen with plates that had significantly less electrical resistance. However, we did not see any significant difference in range of inhibition (Figure 10) or

bioluminescence when compared to the plates that did not have the Vulcan carbon (Figure 3). Thus, we can conclude that there is not an overt electricidal effect that is inhibiting or shutting off the bioluminescence of S. woodyi. Future experiments will build on the biofilm assay we developed during this work to confirm if and electricidal effect is responsible for bacterial growth inhibition.

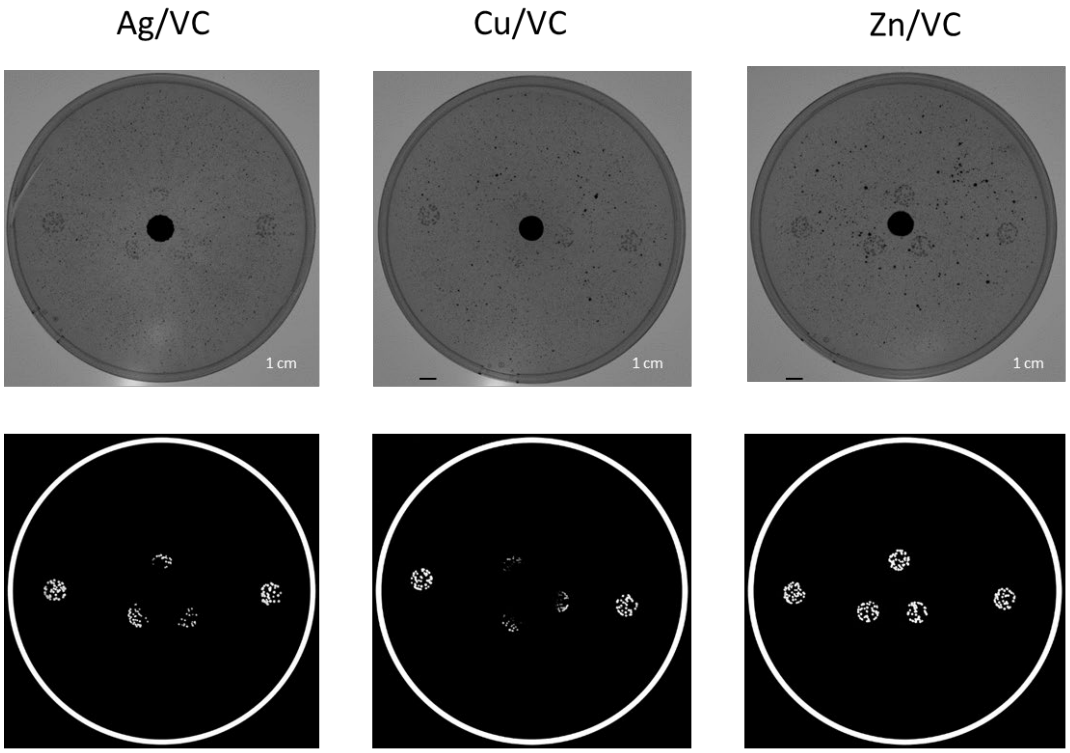


Figure 10. Brightfield and luminescence images of *S. woodyi* colonies grown over 2 days with Ag/C, Cu/C, and Zn/C electrodes with 10%(w/w) loadings of Vulcan carbon into the agar supports.

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