INHIBITION OF QUORUM SIGNALLING AND BIOFILM FORMATION IN *CHROMOBACTERIUM VIOLACEUM* IN THE PRESENCE OF CADMIUM, COBALT AND NICKEL DIVALENT CATIONS

by

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DEDICATION

This thesis is dedicated to my grandmother, Dr. Daisy A. Palmer for her unyielding support during its completion.

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LIST OF ABBREVIATIONS

Abbreviation	Description
AHL	N-acyl homoserine lactone
AI	Autoinducer
C6 HSL	N-hexanoyl-L-homoserine lactone
CFU	Colony forming unit
DKP	Diketopiperazine
HSL	Homoserine lactone
ISS	International Space Station
LB	Luria Broth
LSMMG	Low-shear modeled microgravity
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
OD600	Optical density at 600 nm
RPM	Revolutions per minute
RT-PCR	Reverse-transcriptase PCR
RWV	Rotating wall vessel

ABSTRACT

Bacteria are single celled organisms capable of acting as a single unit by sensing and responding to population density via a phenomenon called quorum signaling. Quorum signaling regulates a variety of phenotypes including biofilm formation and virulence factor production. In the soil bacterium *Chromobacterium violaceum* the virulence factor violacein results in a deep purple pigmentation and is one such regulated phenotype. Previously, a number of biological and organic molecules have been described as quorum signaling inhibitors, but to date no metal-based inhibitors have been identified. In this study, we show that quorum sensing is inhibited in *C. violaceum* when in the presence of sub-lethal concentrations of cadmium based salts. Cobalt and nickel salts have also been indicated as inhibitors in other gram negative species. Inhibitory effects of cadmium divalent cations on *C. violaceum* were shown in biofilm formation, pigmentation and virulence factor production, as well as transcript levels for genes involved in these processes. This study represents the first description of heavy metal based quorum sensing inhibition in *C. violaceum*.

1. INTRODUCTION

Quorum signaling allows many single-celled bacteria to act as a single unit. Gram negative bacteria secrete different signaling molecules into their environment. These signals, also called autoinducers (AIs) include N-acyl homoserine lactones (AHLs), diketopiperazines (DKPs) and AI-2 type signals (65). Gram positive bacteria typically use a Two-Component system utilizing oligopeptides as AIs that are secreted through a specialized transport system (45). Constitutive expression of these AIs allows for population-wide regulation of expression of genes that are more useful at high cell numbers, such as virulence factors and genes relating to biofilm formation. Quorum signaling utilizing AHLs is regulated by a positive feedback loop in which the LuxR transcriptional regulator-like protein binds to the *luxI* promoter, ramping up *luxI* transcription and translation (54). This positive feedback loop is highly sensitive (54) and allows for synchronicity in the activation of quorum signaling (45). Both gram negative and gram positive bacteria utilize positive feedback loops in activation of quorum signaling (45), hence the original name "autoinducer".

Chromobacterium violaceum is a gram negative soil bacterium that utilizes N-hexanoyl-L-homoserine lactone (C6 HSL) AIs (41). When C6 HSL levels reach a critical concentration, they reenter the cell and bind to a DNA binding protein, CviR transcriptional regulator, which allows activation of *C. violaceum* quorum regulated genes (60). Such genes include biofilm formation (63), chitinase production (14) and genes involved in the production of violacein (60), a dark purple tryptophan derivative (5) that allows interspecies competition in a mixed culture environment (18), similar to the way *Esherichia coli* utilizes indole (17). *C. violaceum* ATCC 12472 is well

characterized and often serves as a model for gram negative quorum signaling since pigmentation is an easily visualized quorum regulated phenotype. *C. violaceum* 026 (CV026) is a quorum signaling reporter strain that responds to the presence of C6 HSL, but does not produce it (41). The availability of the reporter strain as well as the distinct phenotype of the wild-type organism makes *C. violaceum* an excellent choice for quorum signaling characterization.

Other well characterized AHL-based quorum regulation systems include the Lux system (43) that is responsible for bioluminescence in *Vibrio fischeri* (25) and the Las and Rhl systems in *Pseudomonas aeruginosa* (45). There are also indications of cross species signals that are recognized by members of different species that allow communication between bacteria in mixed-species environments. A common cross-species quorum signal is AI-2, as it is utilized in many different bacterial species (23).

Many biological and organic molecules have been shown to inhibit quorum signaling. Organic inhibitors include furanones (28) which act to destabilize LuxR (40), aspirin (21) and many autoinducer analogs which inhibit LuxI binding sites (55) (51). Biological quorum inhibitors have been found in many places, from enzymes produced by gram positive bacteria that inactivate AHLs (19) to plant extracts (27) (61). To date, no metal-based quorum inhibitors have been identified in *C. violaceum*, and metal-based quorum inhibitors have only been identified in one other species, *Burkholderia multivorans* (62). The previous study started when it was noticed that biofilms do not form in water reservoirs on the International Space Station (ISS), and culminated in the finding that biofilm formation and AHL production were reduced in *B. multivorans* when grown in the presence of nickel and cadmium ions.

When working with heavy metals in biological systems, question of toxicity must be addressed. The binding of cadmium, cobalt and nickel ions to DNA can inhibit gene replication and prevent growth of microorganisms if concentrations are high enough (64). Bacteria utilize several resistance mechanisms to prevent heavy metal toxicity, such as hydrogen sulfide production (37), integration of metals into organometallic compounds (26) and by excluding the metal ions from the cytoplasm (16). Environmental-based resistance comes from chelation of metal ions in soil into various present organic molecules such as citric acid (26).

Cadmium, cobalt and nickel divalent cations are known to be competitive antagonists of calcium and magnesium in DNA binding (64), and have previously been shown to be lethal in *Escherichia coli* in even very small concentrations when magnesium is limited (1). Previous studies have also found that cadmium is effective in regulating gene expression in eukaryotic cells (6). Cadmium (66) (2) (49), cobalt (67) and nickel (35) (13) have also been shown to act as inhibitors, either via competitive inhibition of enzymes that utilize manganese (II) or magnesium (II) cations as cofactors (66) (2) (49), or noncompetitively (35) (67). This study represents the first description of heavy metal based quorum sensing inhibition in *C. violaceum*, and the second description of cadmium based gene regulation in prokaryotes.

2. MATERIALS AND METHODS

Strains and Culture Conditions

The bacterial strains used were *C. violaceum* ATCC 12472, *Pseudomonas aeruginosa* PAO1 and *Serratia marcescens* ATCC 13880. Broth cultures of *C. violaceum* and *S. marcescens* were grown in Luria Bertani (LB) at 30°C while shaking at 150 RPM for 20-22 hours. *P. aeruginosa* was grown in LB broth at 37°C while shaking at 150 RPM for 20-22 hours. Plate cultures of all organisms were grown on LB agar. Plate inoculated *C. violaceum* was grown at 30°C, *P. aeruginosa* was grown at 37°C and *S. marcescens* was grown at 25°C. Cadmium, cobalt and nickel divalent chloride salts were added at concentrations of 0.5 mM, 0.4 mM, 0.3 mM, 0.2 mM and 0.1 mM. Stock solutions of divalent chloride salts were created by dissolving into 1 L of high resistance (18 MΩ/cm^2) "MilliQ" water at concentrations of 10 and 40 mM, then were filter sterilized thorugh a 0.2 μm pore-size filter.

Screening

Twelve metal salts (Table 1) were screened on four different agar media (LB Agar, Tryptic Soy Agar, Mueller-Hinton Agar and Nutrient Agar) for inhibition of quorum signaling using a diffusion assay. *C. violaceum, P. aeruginosa* and *S. marcescens* were plated as a lawn on LB agar. 6 mm punches were removed from the center of the agar plates, and the resulting wells were filled will 10 mM and 40 mM solutions of each metal salt. Plates were incubated at the appropriate temperature for each organism for 24 hours. At the end of incubation, plates were observed for regions of non-lethality as well as inhibition of quorum signaling, indicated by a loss of pigmentation.

Table 1. Metals screened for quorum inhibition.

Cation	Anions
Cadmium	Nitrate, Chloride
Cobalt	Nitrate, Chloride
Copper	Chloride
Iron (II)	Sulfate
Iron (III)	Chloride, Sulfate
Manganese	Chloride, Sulfate
Nickel	Chloride
Strontium	Nitrate

OD600 Calibration Curves

C. violaceum was grown overnight in either untreated, cadmium, cobalt or nickel treated LB. Cultures were diluted into LB broth to differing OD600 values, then serially diluted into LB and plated onto LB agar. Agar plates were incubated for 24 hours and CFUs per mL each OD600 value.

Growth Curve

Overnight cultures of *C. violaceum* were diluted to an OD600 of 0.2 and then plated into microtiter plates containing LB and cadmium, cobalt or nickel chloride salts at a final volume of 200 uL in triplicate. Plates were incubated in a plate reader for 48 hours, with OD600 readings taken every 20 minutes. OD600 values were then standardized to CFUs before analyzing on a graph.

Violacein Extraction

Overnight cultures of *C. violaceum* were diluted to an OD600 of 0.2 and inoculated into test tube broth cultures containing LB and cadmium, cobalt or nickel

chloride salts at a final volume of 2 mL. Cultures were grown for 21-22 hours at 30°C in a shaking incubator at 150 RPM. Following the incubation, 1 mL of culture was assayed for turbidity by obtaining the OD600 value, and 0.2 mL of culture were assayed for violacein production as previously described (7). 200 uL of cells were lysed by treating with an equal volume of 10% SDS for 10 minutes. 0.5 mL water saturated butanol was added to the lysate and vortexed vigorously for 15 seconds. The lysate was centrifuged for 10 minutes at 13,200 xG to separate the aqueous and organic phases and to pellet cell fragments. 200 uL aliquots of the organic phase were then analyzed for violacein production by reading absorbance values at 585 nm in a plate reader. Violacein production was standardized by comparing to the CFUs in the culture, which were obtained by standardizing the OD600 values to the calibration curves obtained previously.

Biofilm Growth

Microtiter plates containing LB and cadmium, cobalt and nickel chloride salts were inoculated with *C. violaceum* overnight cultures diluted to OD600 values of 0.2. Microtiter plate cultures were then grown at 30°C in a shaking incubator at 150 RPM for 20-22 hours. Biofilms were stained as previously described (47). Planktonic cells were washed from the wells in sterilized PBS, then biofilms were stained using 225 uL 1% crystal violet for 10 minutes. Unbound stain was removed by rinsing with deionized water, and bound stain was eluted with 30% acetic acid. The eluate was then diluted into deionized water and read in a plate reader at 550 nm.

Chitinase Production

Chitinase production was quantified as previously described (Shen et al, 2010). *C. violaceum* was grown in 50 mL cultures with 3 mg/mL chitin powder from shrimp shells (Sigma C8908) as well as cadmium, cobalt and nickel chloride salts for 3 days at 30°C under shaking at 150 RPM. Three 1 mL aliquots of each culture were placed into sterile 1.5 mL Eppendorf tubes, and centrifuged at 13,000 G for 5 minutes. The chitinase-containing media was removed and transferred to a new sterile Eppendorf tube and centrifuged again to ensure near complete removal of cells. 0.5 mL media was transferred to a new sterile Eppendorf tube and mixed with 0.5 mL of 6% chitin azure (CA) (Sigma 3020) to create a final CA concentration of 3%. The media was then incubated at 30°C for 1 hour. Following the incubation, the CA and media were centrifuged for 5 minutes at 13,000 G. 200 uL aliquots of the media were transferred to a microtiter plate and the absorbance of released Remazol Brilliant Violet 5R was read at 560 nm. The assay was confirmed using stock chitinase from *Streptomyces griseus* (Sigma C6137) to rule out interference between the metal ions and the chitinase enzyme.

Motility

C. violaceum was assayed for motility by using an inoculating needle to stab LB agar deeps with 0.4% agar with LB and cadmium, cobalt or nickel salts, and then incubating at 30°C for 20-22 hours. Motility was determined by growth throughout the agar.

Microgravity

Since the original study (62) discovered their model in water reservoirs on the ISS, this study was expanded to determine the effect of nickel, cobalt and cadmium on *C. violaceum* in Low-Shear Modeled Microgravity (LSMMG) (46). 50 mL cultures of *C. violaceum* were inoculated into disposable rotating wall vessels (RWVs) with 0.5 mM cadmium, cobalt or nickel. The RWVs were rotated at 22 RPM in either the LSMMG or control (normal gravity) orientation (46) for 20-22 hours (Figure 1). Post incubation, cultures were analyzed for violacein production as previously described in this paper.

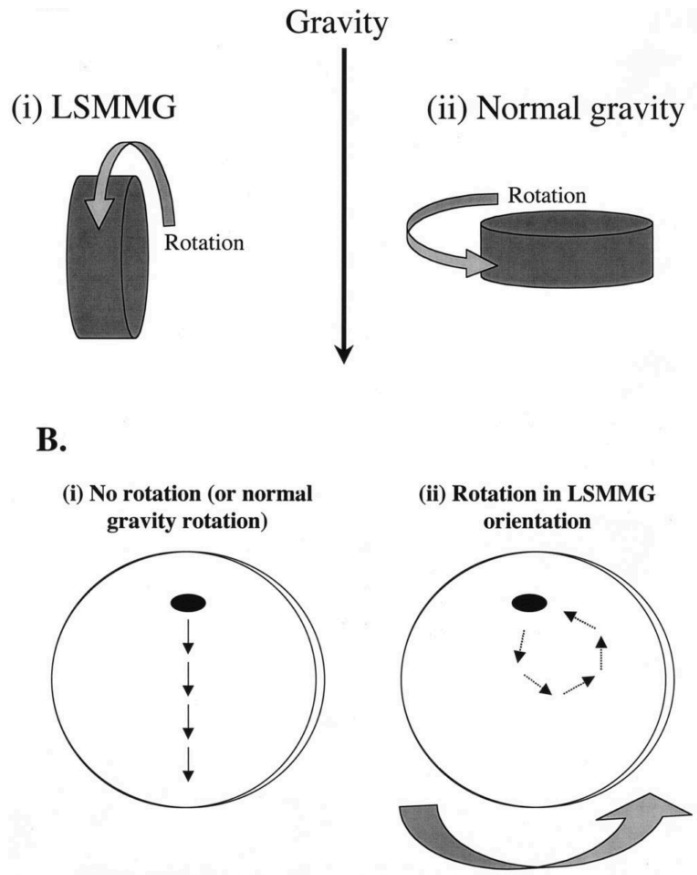

Figure 1. LSMMG System. Low-shear modeled microgravity RWVs normal gravity orientation and microgravity-inducing orientation. (46)

RT-PCR

Cultures of *C. violaceum* were grown for 20-22 hours with cadmium, cobalt or nickel divalent salts in a shaking incubator at 30°C and 150 RPM. Following the 20-22 hour initial incubation period, cultures were diluted 1:100 into fresh tubes of the same media and then grown to an OD600 of 0.2 for cobalt, nickel and controls, and 0.1 for cadmium. Total RNA was stabilized using RNAprotect Bacteria Reagent (Qiagen 76506) and extracted using the Qiagen RNeasy Mini Kit (Qiagen 74104) using the bacterial protocol utilizing lysozyme lysis with the addition of Proteinase K. RT-PCR was performed using ThermoScript RT-PCR System (Invitrogen 11146-016) and GoTaq Green Master Mix (Promega, M712). Five uL samples were then run on a 2% agarose gel in TAE. DNA fragments were stained with GelRed. All primers used are listed in Table 2. RT-PCR products were also analyzed for concentration using the High Sensitivity DNA Assay on a Qubit 3.0 Fluorometer.

Table 2. Primer Sequences used for RT-PCR.

Gene Name	Gene Function	Primer Sequence	Size
CviI	Autoinducer synthetase, LuxI homologue	5'-TCCGACAACTTGGTATTGTGC-3' 5'-CCTCCAACATGAAAGCATGC-3'	84 bp
CviR	LuxR family transcriptional regulator, LuxR homologue	5'-CAATGGACATTGCTGGAGTG-3' 5'-GGCTCAGGATGTTGTCAAGG	87 bp
pykF	Pyruvate Kinase, Housekeeping	5'-CTGCGCATCGGCAAGTTT-3' 5'-GCATTGCCTTCCGTTTCAT-3'	85 bp

3. RESULTS

Screening

Screening of twelve metal ion salts revealed that three heavy metal cations were effective in inhibiting quorum sensing in *C. violaceum, P. aeruginosa* and *S. marcescens*: cadmium (Figure 2), cobalt (Figure 3) and nickel (Figure 4) chloride and nitrate salts. Quorum inhibition was evident for three metal species in *S. marcescens*, for two metals in *P. aeruginosa* and in one species in *C. violaceum*, as determined by inhibition of pigment production. Because of their reduced reactivity, chloride salts as opposed to nitrate salts were chosen for use in the working solutions. *C. violaceum* was chosen as the modeling organism due to its status as a model organism for gram negative quorum sensing.

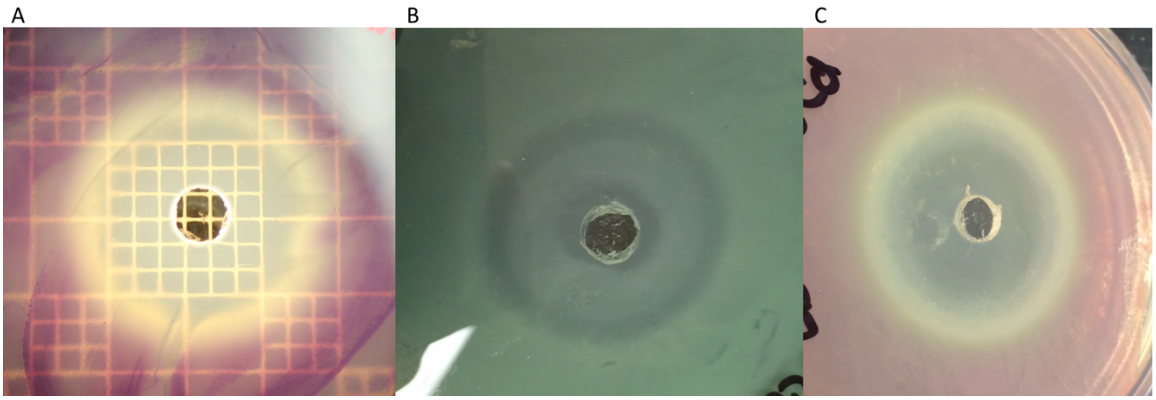

Figure 2. Cadmium screening. Cadmium induced inhibition of quorum signaling in A) *C. violaceum* (40 mM $CdCl_2$) B) *P. aeruginosa* (10 mM $CdCl_2$) and *S. marcescens* (40 mM $CdCl_2$).

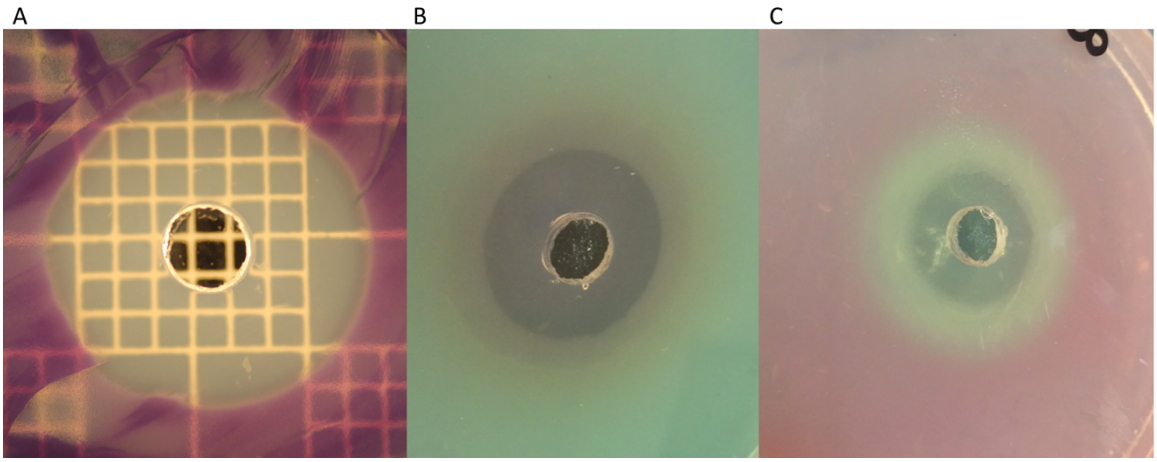

Figure 3. Cobalt screening. Cobalt induced quorum signaling inhibition in A) *C. violaceum* (40 mM $CoCl_2$) B) *P. aeruginosa* (40 $CoCl_2$) and C) *S. marcescens* (40 $CoCl_2$).

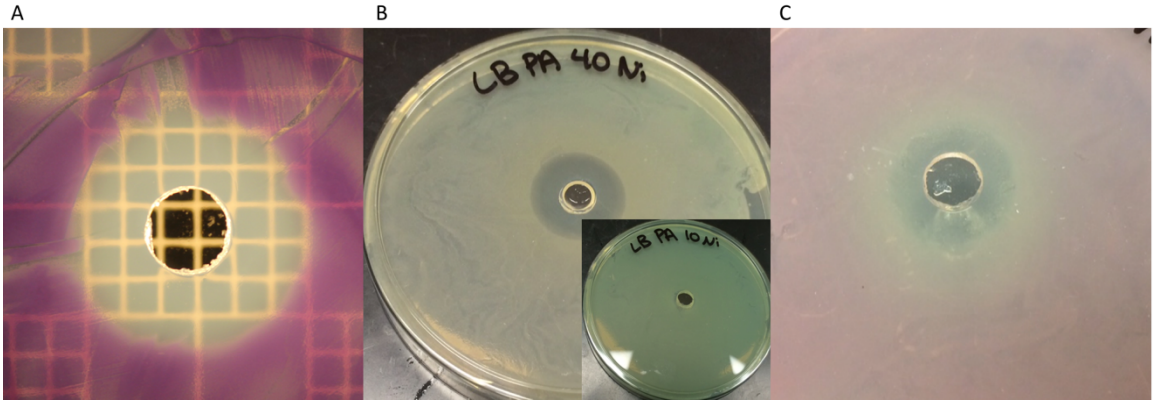

Figure 4. Nickel screening. Inhibition of quorum sensing from exposure to nickel divalent cations in A) *C. violaceum* 40 mM $NiCl_2$), B) *P. aeruginosa* (40 mM $NiCl_2$ and 10 mM $NiCl_2$ inset) and C) *S. marcescens* (40 mM $NiCl_2$). Nickel at < 10 mM concentration does not result in any inhibition in *P. aeruginosa* (B, inset) but at concentrations < 40 mM, quorum signaling is completely inhibited. A similar phenotype was not seen in *S. marcescens* or *C. violaceum*.

OD600 Calibration Curve

OD600 calibration curves revealed that cell densities are read differently in the presence of metal ions (Figure 5). For this reason, all OD600 values were converted to CFU/mL to compare data between treatments.

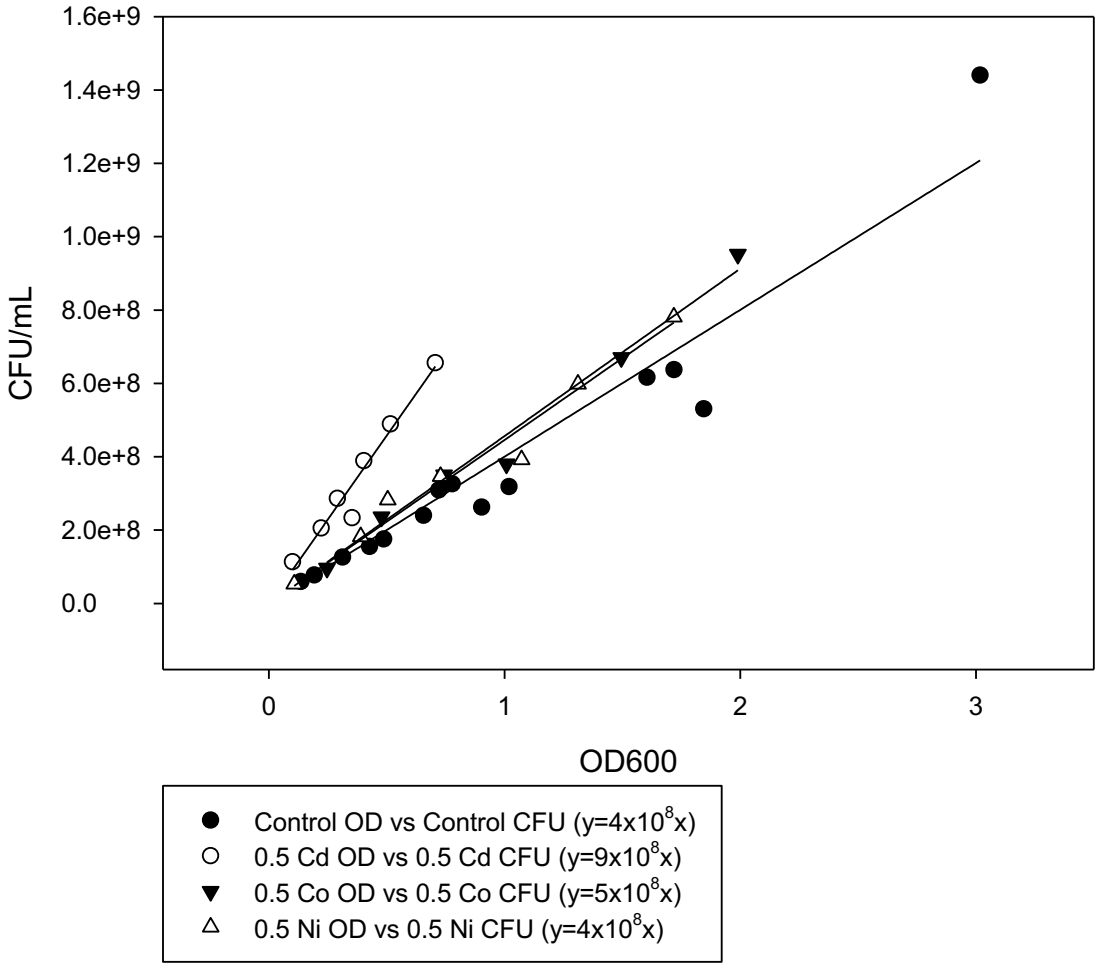

Figure 5. OD600 calibration curve. OD600 calibration curves for each metal treatment revealed that cell cultures treated with cadmium, cobalt and nickel are read differentially from untreated cultures.

Growth Curve

Forty-eight hour growth curves revealed that although cell growth is differential using different metal ions, between 20 and 22 hours the cell numbers are approximately the same (Figure 6). Hence, 20-22 hours was chosen as the optimal incubation time for characterization of quorum inhibition utilizing cadmium, cobalt and nickel salts. All metals tested were also seen to be non-lethal at concentrations of 0.5 mM, indicated by cells reaching the same CFU count or higher at stationary phase.

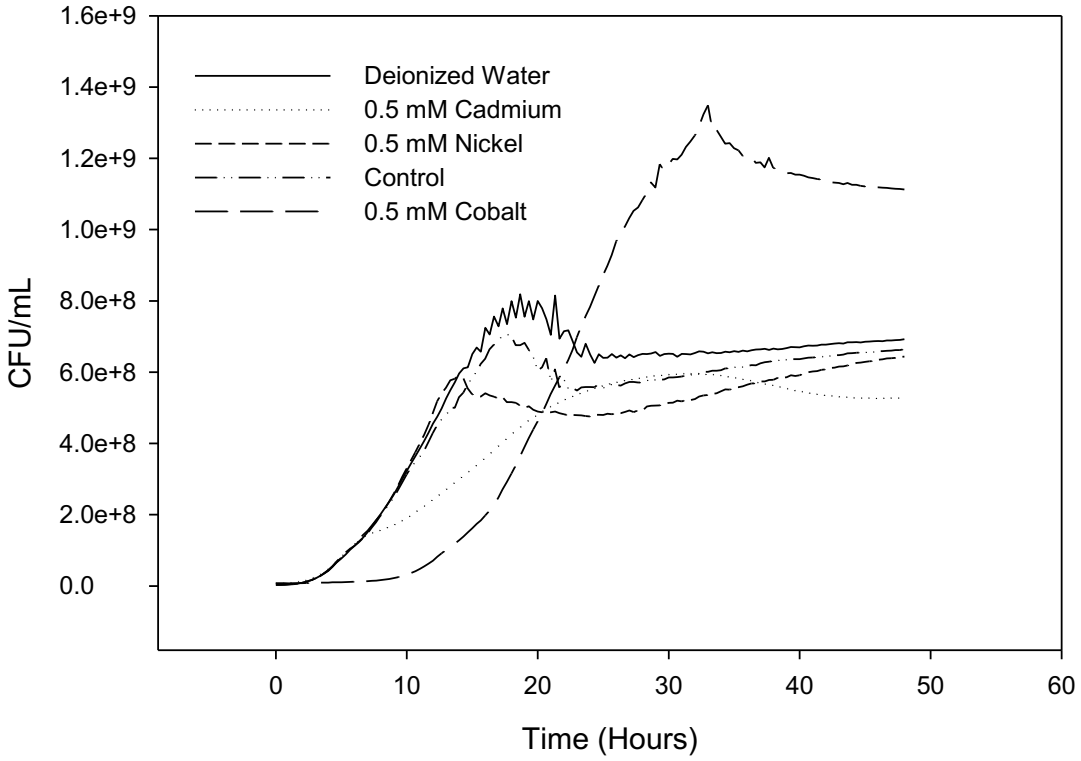

Figure 6. Growth curves. 48-hour growth curve of *C. violaceum* in the presence of cadmium, cobalt and nickel salts. Controls were generated using deionized water and no treatment (Control). All metals were utilized at 0.5 mM concentrations.

Violacein Extraction

Violacein extraction revealed that cadmium had a statistically significant inhibitory effect on violacein production (Figure 7), while nickel only had an inhibitory effect at relatively higher concentrations (Figure 8) and cobalt had no effect (Figure 9). Statistical analysis was performed using One-Way ANOVA (Dunnett's Test).

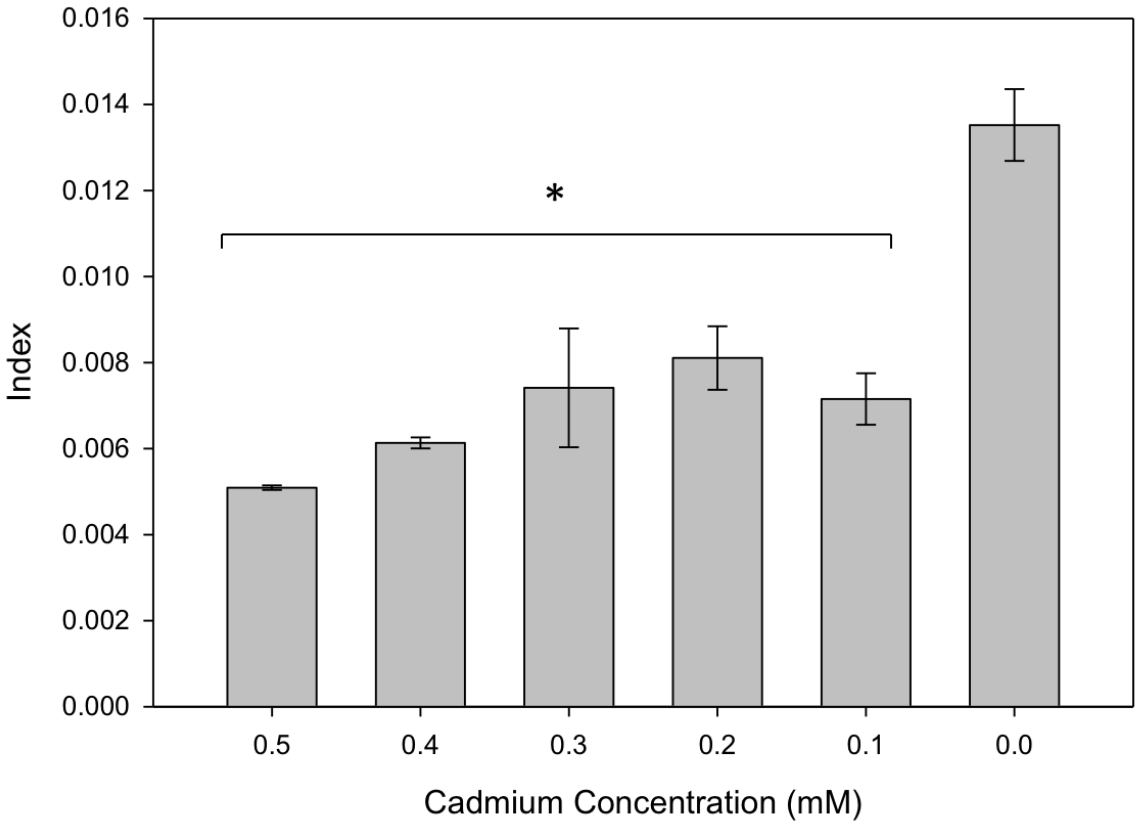

Figure 7. Violacein production in the presence of cadmium ions. Violacein extraction of *C. violaceum* grown at varying concentrations of cadmium chloride salts. Index was calculated by dividing the A550 value read as a quantification of violacein production by the log(CFU/mL), and can be interpreted as the amount of violacein produced per cell. All differences are statistically significant from untreated samples ($p < 0.05$) (Dunnett's Test). Error bars indicate standard error.

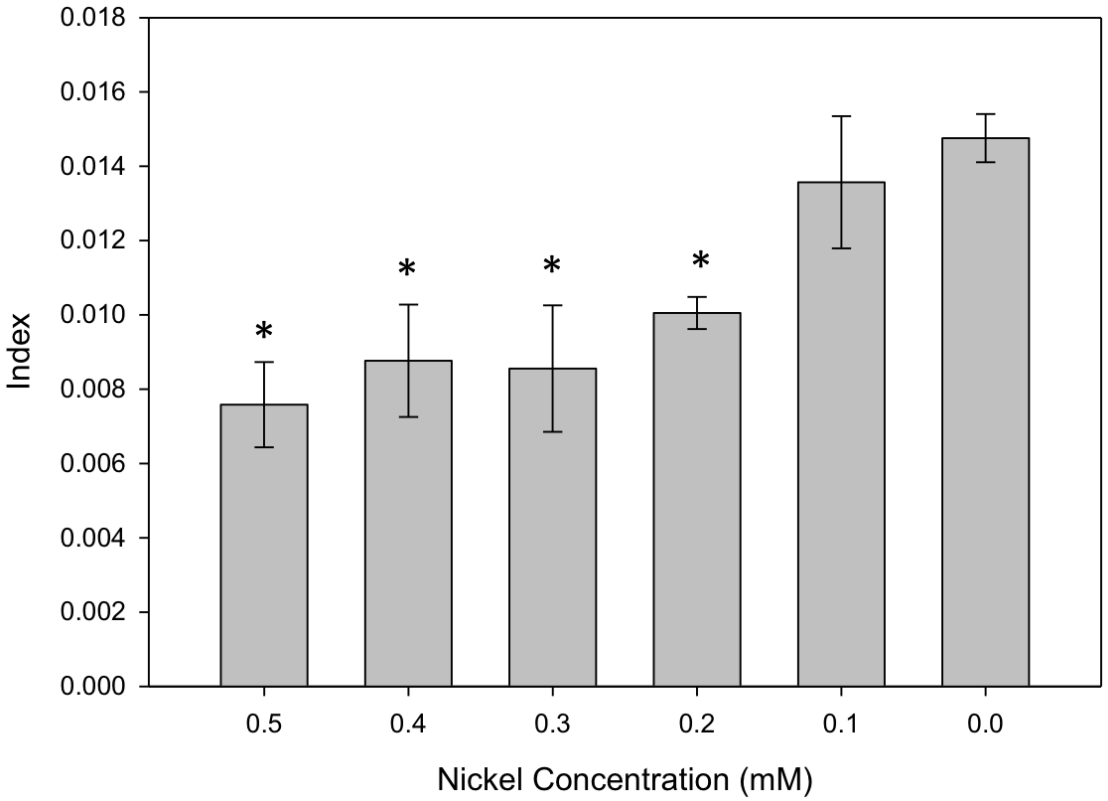

Figure 8. Violacein production in the presence of nickel ions. Violacein extraction of *C. violaceum* grown at varying concentrations of nickel chloride salts. Index is calculated as in Figure 7. Differences marked with an asterisk (*) are statistically significant from untreated samples ($p < 0.05$) (Dunnett's Test). Error bars indicate standard error.

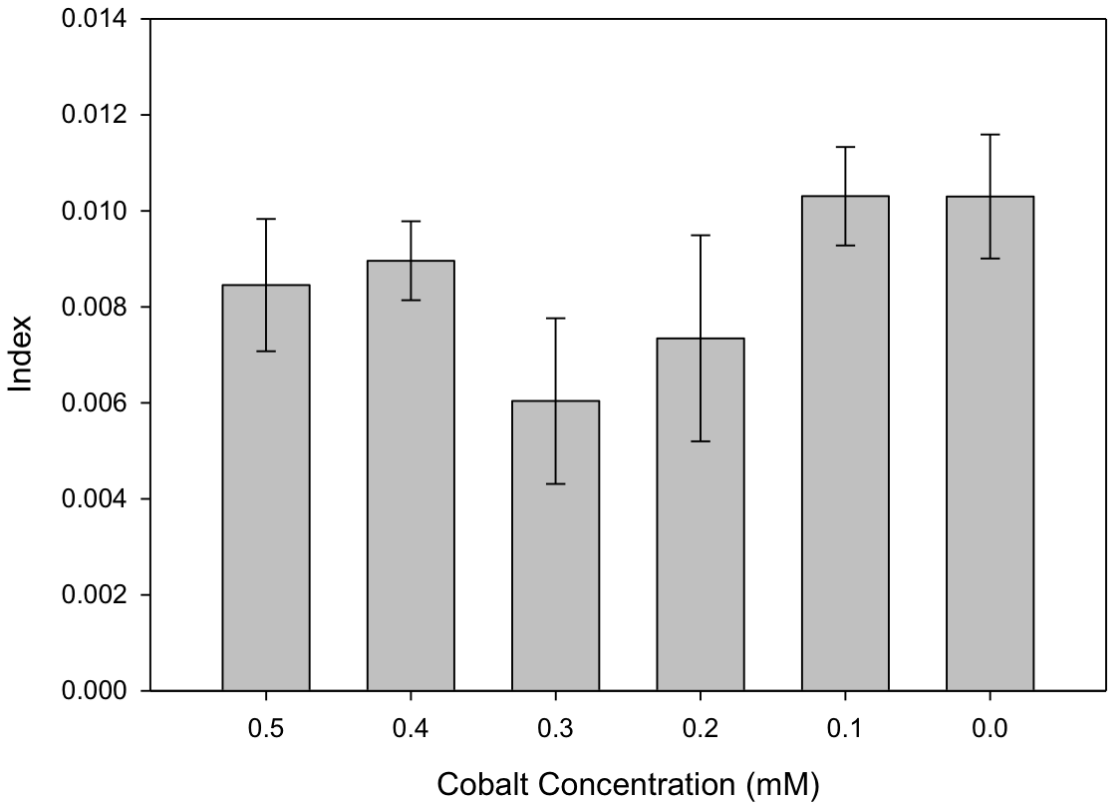

Figure 9. Violacein production in the presence of cobalt ions. Violacein extraction of *C. violaceum* at varying concentrations of cobalt chloride salts. Index is calculated as in Figure 7. No differences are statistically significant from untreated samples (Dunnett's Test). Error bars indicate standard errors.

Biofilm Growth

Crystal violet staining indicated that cadmium was effective at reducing biofilm production in *C. violaceum* (Figure 10). Cobalt was only effective at minor inhibition at very low concentrations (0.1 mM) (Figure 11). This minor difference is unlikely to be a useful inhibition. Nickel had no inhibitory effects (Figure 12).

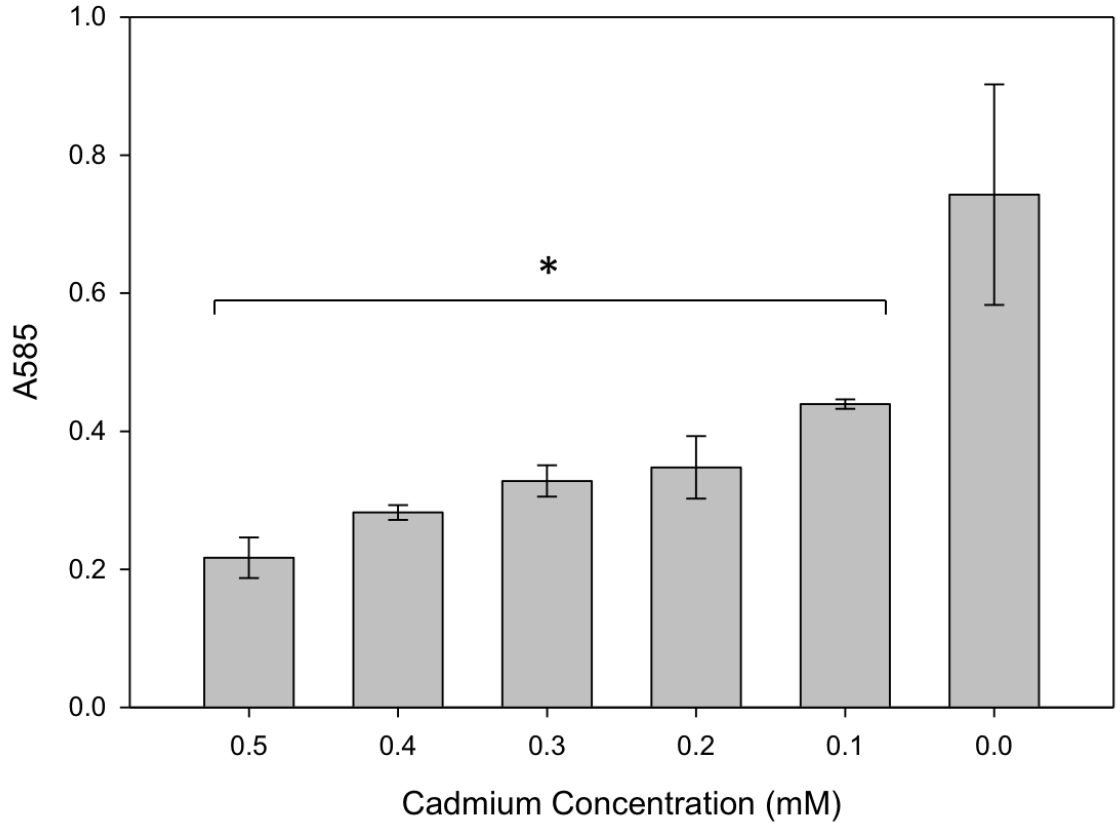

Figure 10. Biofilm growth in the presence of cadmium ions. Biofilm production of *C. violaceum* when grown in varying concentrations of cadmium divalent cations. All reductions in biofilm formation are significant (* = $p < 0.05$) (Dunnett's Test). Error bars indicate standard error.

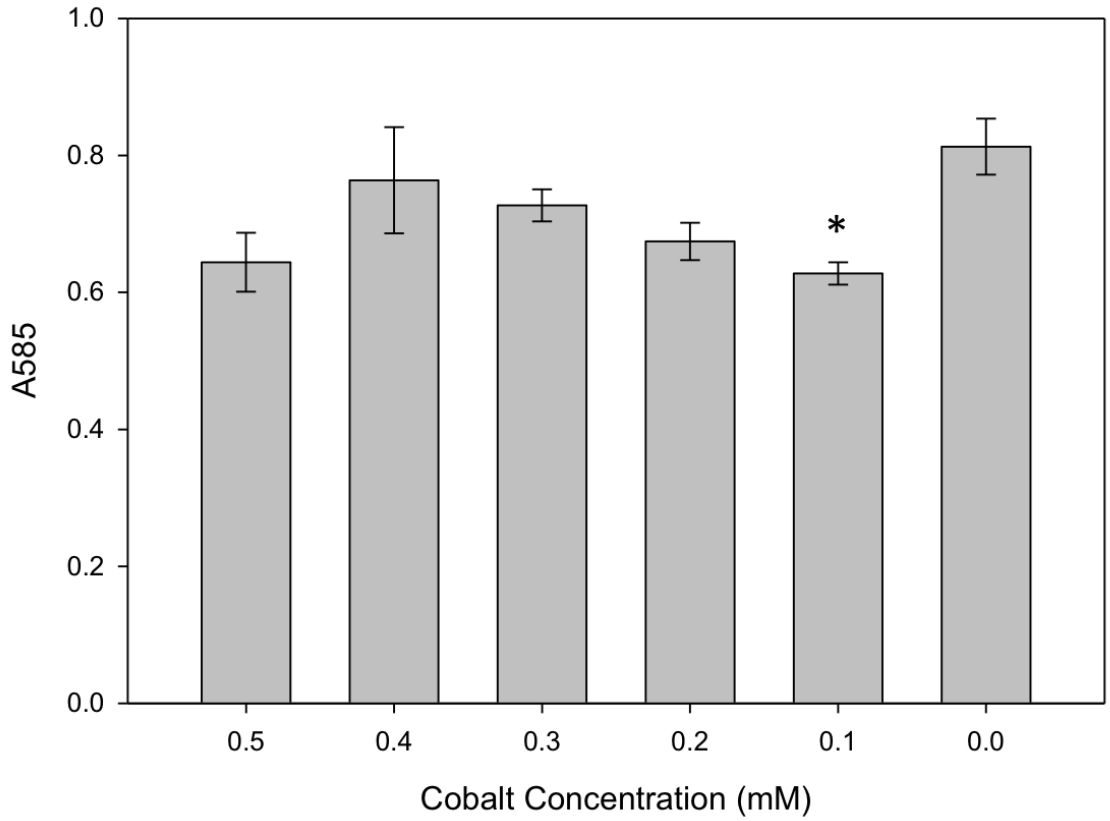

Figure 11. Biofilm growth in the presence of cobalt ions. Biofilm production of *C. violaceum* when grown in varying concentrations of cobalt divalent cations. Reductions marked with an asterisk (*) are statistically significant ($p > 0.05$) (Dunnett's Test). Error bars indicate standard error.

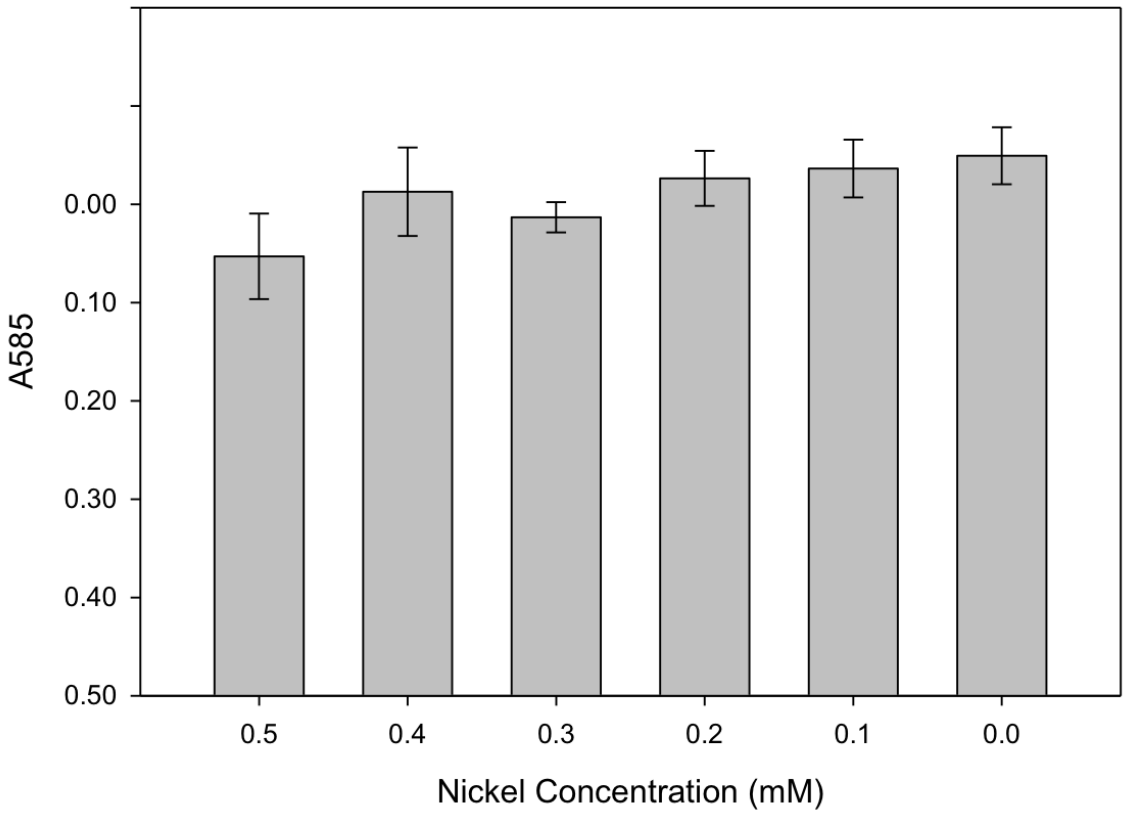

Figure 12. Biofilm growth in the presence of nickel ions. Biofilm formation of *C. violaceum* in the presence of nickel divalent cations. No differences in biofilm formation are significant ($p < 0.05$) (Dunnett's Test). Error bars indicate standard error.

Chitinase Production

Chitinase production was inhibited in *C. violaceum* when grown in the presence of cadmium, cobalt and nickel divalent cations (Figure 13). Controls verify that the presence of cadmium, cobalt and nickel in the spent media do not interfere with the assay.

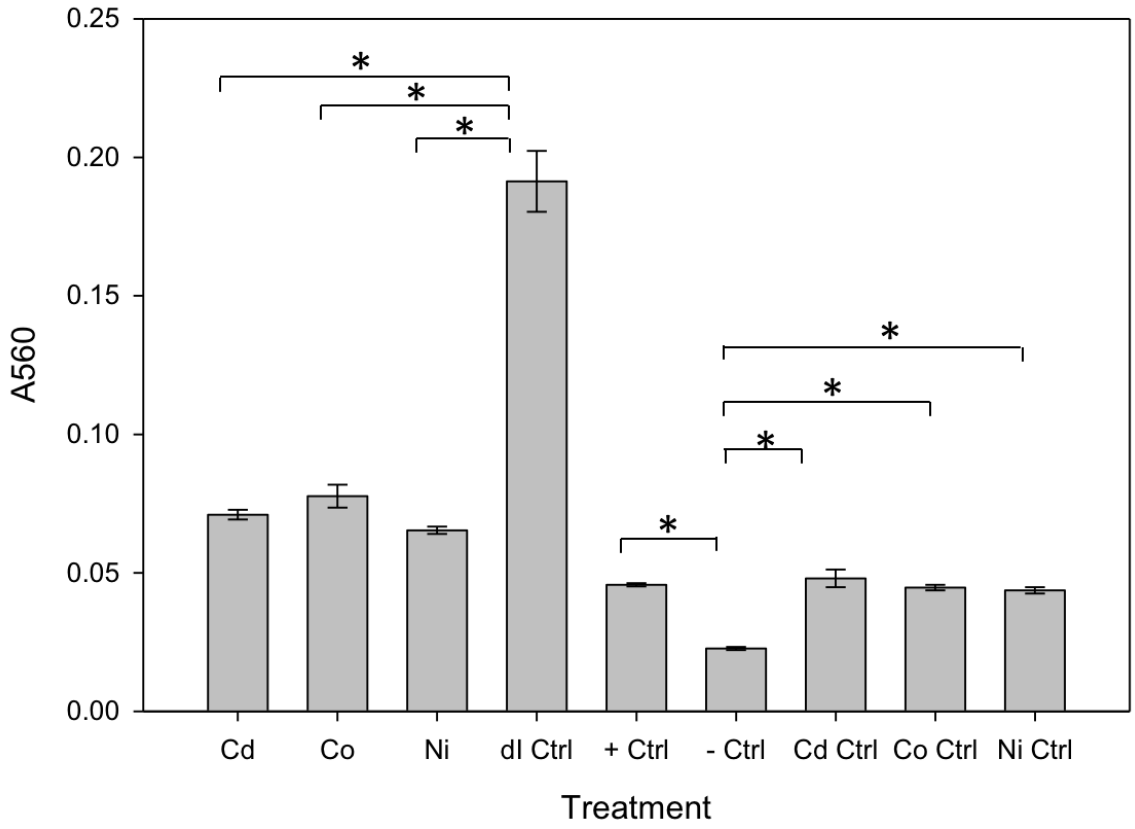

Figure 13. Chitinase production. Chitinase production of *C. violaceum* grown in the presence of cadmium, cobalt and nickel divalent cations. Differences marked with an asterisk (*) are significant ($p < 0.05$) (Dunnett's test). Error bars indicate standard error.

Motility

Motility in *C. violaceum* was inhibited completely at high concentrations of cadmium (0.5-0.3 mM) and greatly reduced at lower concentrations (0.2 and 0.1 mM) (Figure 14). Motility was not inhibited at any concentration of nickel (Figure 15). When grown in the presence of cadmium, motility was inhibited only anaerobically (Figure 16).

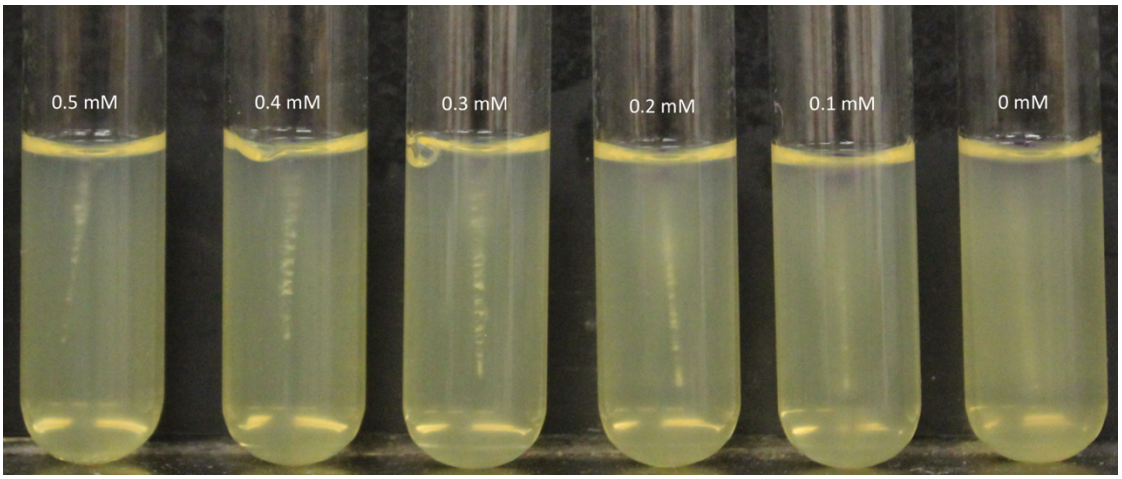

Figure 14. Cadmium inhibited motility. Inhibited motility in *C. violaceum* in the presence of cadmium divalent cations.

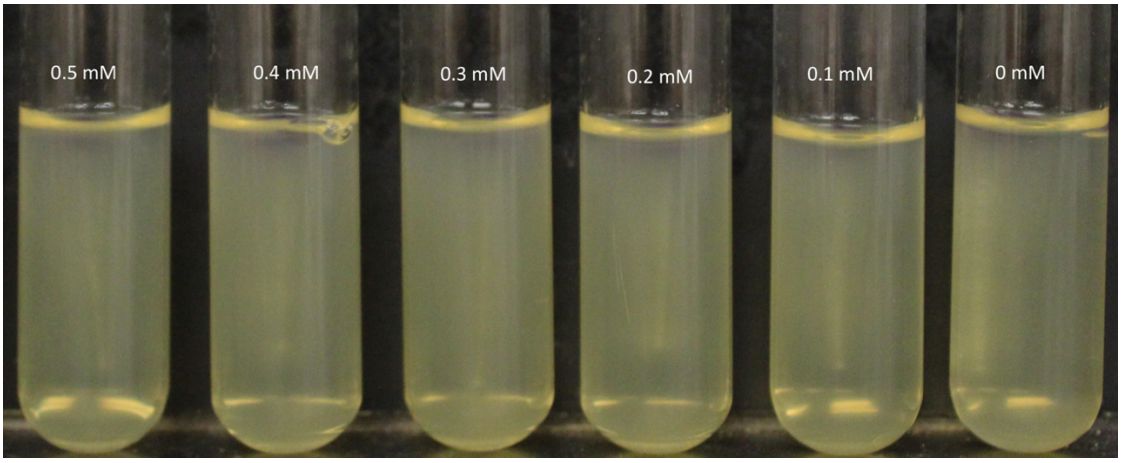

Figure 15. Nickel inhibited motility. Uninhibited motilty in *C. violaceum* in the presence of nickel divalent cations.

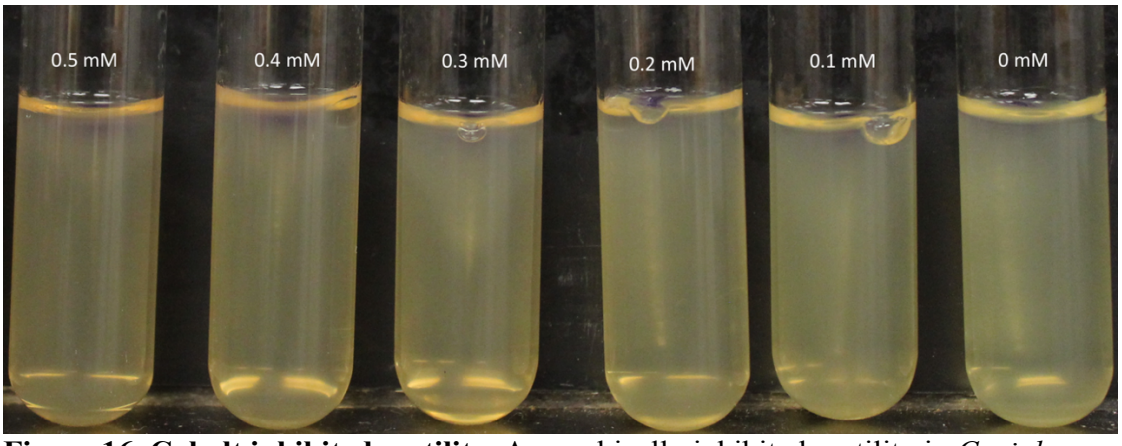

Figure 16. Cobalt inhibited motility. Anaerobically inhibited motility in *C. violaceum* in the presence of cobalt divalent cations.

Microgravity

Violacein extraction of cultures grown in microgravity revealed that in the presence of cadmium, growth in the bioreactors further decreases violacein production, whereas with nickel and cobalt, violacein production is increased (Figure 17). This indicates that divalent metals negatively impact quorum signaling in microgravity environments, such as the ISS.

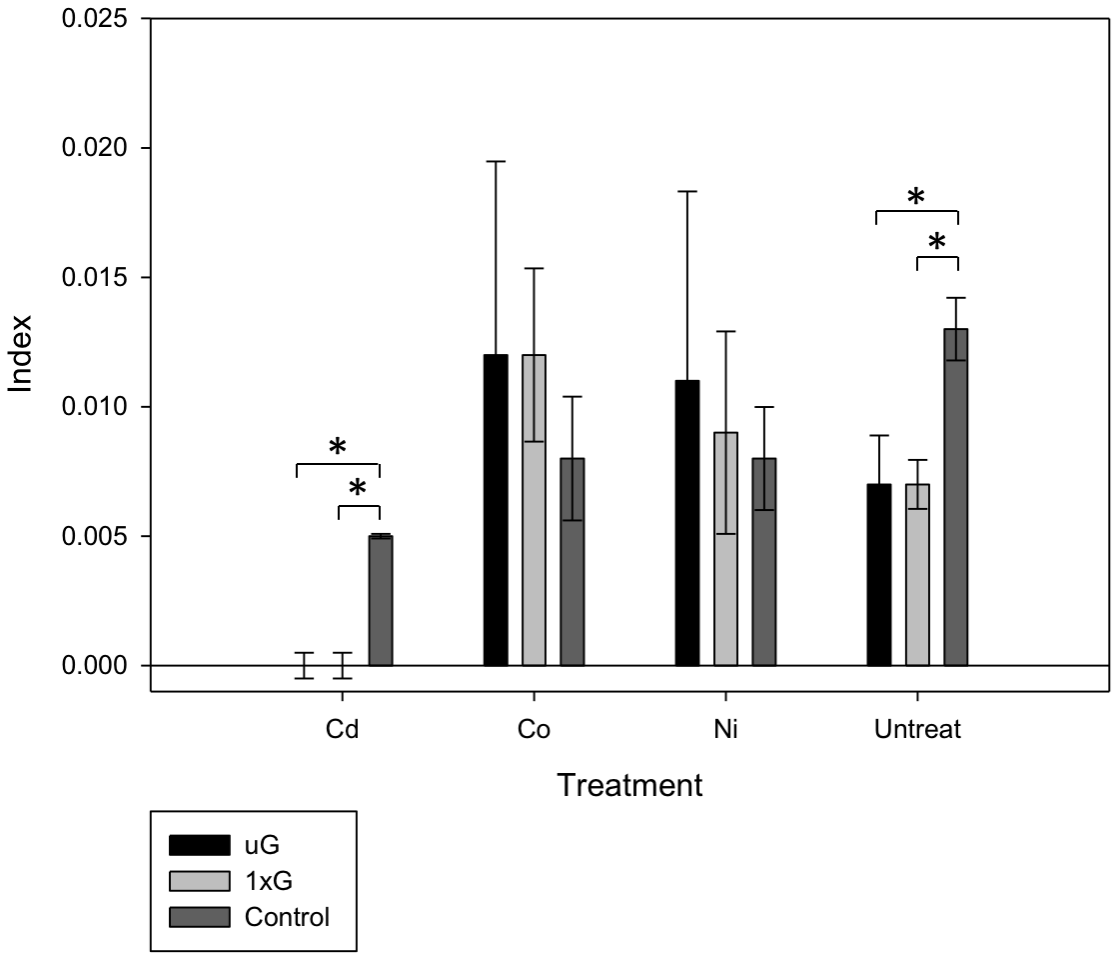

Figure 17. Quorum sensing in microgravity. Violacein production in *C. violaceum* grown in the microgravity bioreactors. Asterisks (*) indicate significant differences (Dunnett's Test). Error bars indicate standard deviations.

RT-PCR

RT-PCR results indicate that quorum signaling inhibition occurs in the positive feedback loop, as indicated by the reduced CviI concentration in cultures of *C. violaceum* inoculated with cadmium divalent cations (Table 3).

Table 3. Concentration of PCR product obtained from Two-Step RT-PCR.

Treatment	Gene	Concentration (ng/µL)
Cadmium	*CviI*	9
	CviR	12.1
	pykF	14.3
Control	*CviI*	14.1
	CviR	11.6
	pykF	12.3

4. DISCUSSION

Heavy-metal based inhibition of quorum sensing is a novel idea, therefore additional work is required to completely elucidate the mechanisms by which inhibition occurs. Typical quorum inhibitors are stable, low molecular weight molecules that are not lethal to the cell and are unable to be metabolized (51). It is also believed that quorum inhibitors must be specific for the quorum regulator protein, typically a LuxR homologue, but as both this study and a previous study (62) have shown, LuxI homologue transcription can be inhibited by sub-lethal cadmium and nickel ions.

Characterization of several phenotypes that are known to be quorum regulated in *C. violaceum* shows that quorum signaling inhibition is global and likely occurs at the level of induction. Inhibition of violacein production is the most obvious phenotype, resulting in a complete loss of pigmentation in *C. violaceum* grown in the presence of 0.5 mM $CdCl_2$, but quantification of pigment loss revealed that nickel also has a limited effect. In the presence of cadmium, chitinase activity, flagella production and biofilm production were all significantly decreased without lethal effect on the cell populations.

Cadmium, cobalt and nickel have all been shown to be inhibitors of enzyme function (2) (13) (49) (66). Considering this and the positive feedback loop caused by interactions between CviR transcriptional regulator protein and the *cviI* promotor region (60), it is likely that cadmium acts by inhibiting this positive feedback loop, preventing increase in the number of *cviI* transcripts produced once critical cell density has been reached. As a result, quorum signaling is not activated.

Cadmium, cobalt and nickel are also known to be competitive antagonists to calcium and magnesium in DNA binding (1) (33) (64). Previous research has shown cadmium to have binding specificity for certain regions of DNA (64), while cobalt and nickel have been shown to be able to bind DNA when compatible ligands are present (56) (3). Given the reduced amount of *CviI* transcript when *C. violaceum* is treated with cadmium as well as the quorum sensing inhibition by nickel and cobalt that are seen in *P. aeruginosa* and *S. marcescens* but not in *C. violaceum*, it is possible that DNA binding somewhere within the *cviI* gene may be the mechanism of inhibition by heavy metals, although enzyme binding is more likely.

Bacteria contain multiple mechanisms of resistance to heavy metal toxicity such as efflux pumps, metallothioneins, and biofilm formation (58). Efflux pumps act by actively pumping harmful substances out of the cell (34). *C. violaceum* codes for many efflux pumps, including transporters for several trace metals, including cadmium, cobalt and nickel (29). Once such transporter is *zntA*, which provides resistance to xenobiotics (8). Metallothioneins act as metal ion chelators that soak up excess heavy metal ions within the cytoplasm of a cell (24).

Metallothioneins are typically specific to a single metal ion, determined not by the shape or residues for the metal binding sites, but by the Irving-Williams series and by the buffered concentrations of each metal as required by the cell (24). Metallothionines bind cadmium (6) (30) (42) (48), cobalt (11) (22) (10), and nickel (4) (44). Another type of protein, NikR, also regulates nickel levels within cells to physiologically relevant levels in anaerobic resipiration (20).

Biofilm formation is the most universal resistance mechanism utilized by bacteria. Biofilms have been shown to increase resistance to heavy metal toxicity by sequestering the cells in a metal free environment (36). Biofilm resistance to heavy metals has been characterized in many bacteria, including *P. aeruginosa* (15), *Xylella fastitiosa* and *E. coli* (36). While addition of cadmium to potable water systems is ill-advised, the addition of cadmium, cobalt and nickel to non-potable water systems, such as nuclear reactor cooling systems or other machinery systems through which water must pass, may prevent the build-up of biofilm, preventing corrosion of metal components. This would lead in turn to lower maintenance costs and a reduction in the number of systems failures.

This study is the first characterization of metal ion based inhibition of quorum signaling, as well as the first implication of regulation of quorum signaling by inhibiting the positive feedback loop in *C. violaceum*.

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