Treating Seawater Biofilms with Violacein Extracts

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Janthinobacterium lividum **RB10 produces violet compound violacein that is an indole derivative from tryptophan. Violacein is a natural product that has been studied for its antimicrobial, antitumorogenesis, antifungal, and antiparasitological properties. Despite the effectiveness of the compound on the treatment of a variety of pathogenic and disease causing agents, the mechanism of how violacein works as an antimicrobial is uncertain. Here, the bacteria was isolated from the environment, identified using 16S RNA, and the growth of culture was optimized to increase yield of violacein. The compound was then tested to treat seawater biofilm development showing change in biofilm morphology.**

Introduction

Natural product research is borne out of a need to find effective chemotherapeutics for pathogens and conditions causing morbidity and mortality. Bacteria have long been utilizing chemicals in concert with quorum sensing to impede competition with other bacteria. One agent that was found to have chemopreventative properties is violacein [3-(1,2-dihydro-5-(5-hydroxy-1H-indol-3-yl)-2-oxo-3H-pyrrol-3-ilydene)-1,3-dihydro-2H-indol-2-one], an antibiotic dihydro-indolone [1]. These bacteria have also been shown to enable protection against diseases such as the fungal pathogen, chytridiomycosis, that causes decline in amphibians[2]. Mechanisms of how violacein prevent and protects against tumorogenesis has been investigated and was shown to induce apoptosis in TF1 leukemia cells through a nonclassical mechanism of cell death using a calpain based cascade [1]. Violacein-producing bacteria has been studied for the treatment against gram-positive bacteria such as *Bacillus* and *Micrococcus* in 5.8μM concentrations though the mechanism of how the compound works in bacteria is not yet understood [3, 4].

L-tryptophan is a precursor to a number of indole-containing secondary metabolites including the violacein biosynthetic pathway **(Fig. 1)**. The pigment is formed through the oxidation and coupling of L-tryptophan [5]. Though the *Chromobacterium* genus has been the most-studied violacein-producer, the pigment is produced by other bacteria species such as *Altermonas luteoviolacea*, and *Pseudoaltermonas spp*., and betaproteobactium *Janthinobacterium lividum*. Yield and conditions of production were noted to be variable for bacteria other than *C. violaceum* though yield was shown to be enhanced with the addition of tryptophan and glycerol [6]. Temperature of the violacein-producing bacteria was also suggested to be a key factor of pigment production [7].

The goal for this mini project is to both enhance the speed of which violacein appears by altering growth conditions and to harvest the compound for treatment against marine biofilm communities. Reaction of these diverse bacteria communities to violacein would allow understanding of how the biofilm protects against antimicrobial agents.

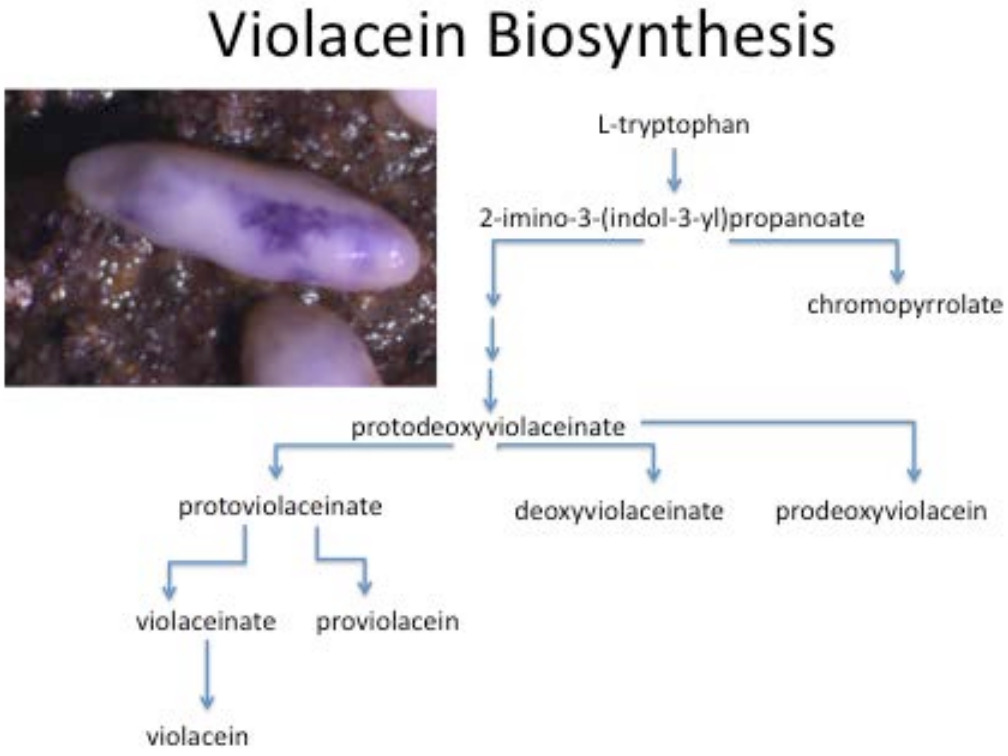

Figure 1: Violacein biosynthetic pathway. L-tryptophan serves as a precursor for violacein and derivatives. Inset picture on left shows growth of violacein-producing bacteria on grain of white rice from garden soil.

Materials and Methods

Isolation and Identification of the violacein-producing bacteria:

Garden soil collected from MBL street yard, Mill Pond soil, School and Millfield corner soil, and school street marsh was added to a petri dish with 5ml of 250µM cycloheximide until soaking. Grains of white rice were added to the soil and left to grow at room temperature until turned purple. Colony was picked from the rice and streaked on nutrient agar plates. An individual colony from MBL street yard was boiled for 10 min in 20µl ddH_2O in a 250µl tube at 100°C. Using the Promega Go-

Taq Green 2X Mix, 2µl of the boiled colony was added to the reaction with 2µl of 16S_8F primer (15 pmol), 16S_1492R (15 pmol) primer and nuclease-free water (Microbial Diversity Course Manual). Thermocycler program with 95°C for 2min -- 25 cycles of 95°C for 30 sec, 46°C for 30 sec, 72°C for 1.5 min -- 72°C for 10min, and 4°C for ∞. After running 5µl of PCR reaction on 1% agarose gel with 2 µL of SYBR to check DNA band, PCR reaction was cleaned using the Promega Wizard PCR Preps DNA purification system. Finally, the DNA was quantified using the Nanodrop, 20uL was added to 96 wellplate in duplicate for sequencing. Sequencing results were checked against NCBI blast for coverage of 88% for *Janthinobacterium lividum* RB10.

Isolation of Crude Violacein Pigment:
Culture was seeded on cotton mats in 3x2L flasks with 330ml of nutrient broth supplemented with 250µM tryptophan and 1% glycerol, then grown at 15°C in the dark for 5 days. The remaining cell culture was wrung out of the cotton mats, next mats were rinsed with distilled water. The culture was spun down to pellet the cells and the supernatant was added to a 170 ml frit column with 20 grams of Dianon HP-20 resin (Supelco). Resin was washed with 25% acetone, 75% acetone, and 100% acetone. All eluted fractions were collected. In parallel, cotton mats were extracted and masticated resulting in ~1200 ml of methanol using 300 ml Methanol to soak the cotton at a time. The violacein:methanol mixture was then placed in a water bath with Lab Armor beads at set to 80°C temperature to let the methanol boil off in the chemical hood. Once the volume reached 400 ml, the violacein solution was added to a round bottom flask attached to a distillation column. Evaporated methanol was collected in a flask and the violacein was concentrated down to 250ml. The spectra of the violacein was checked on a Cary 60 UV-Vis spectrophotometer (Agilent Technologies) with peak signatures of 260.3nm and 577nm and concentration was determined using the Beer-Lambert equation $A\varepsilon L=C$ and calculated using the molar extinction coefficient $\varepsilon=1.7x10^4$ L/mol*cm at 577nm [8].

Zone of Inhibition Cultures (not shown):
Biofilms were plated on seawater complete from six sources from the Marine Resource Center in Marine Biology Center (MRC). Sources include: 1) Squid tank, 2) H1B tank, 3) Seahorse drainpipe, 4) Toadfish tank, 5) Murius seaworm tank, 6) Dogfish tank. A dilution of 1/100 of the biofilm in seawater complete was made and 100 µl was plated on the SWC plates. Filter paper was autoclaved then soaked with 10µl of 100 % methanol, 10µl of 50 % methanol, 10µl crude violacein solution, 10µl 25% acetone elution, 10µl 75% acetone elution, and 10µl water sample. The filter paper was allowed to dry in the oven at 38°C for 1 min before placed on plate of biofilm bacteria.

UV treatment of violacein-producing bacteria:
J. lividum was UV treated with 10,000, 20,000, 40,000 microjoules/cm^2 at 254nm wavelength using a Stratalinker 1800 crosslinker (Stratagene) on 1ml of confluent culture grown for 4 days at 15°C in open 1.5ml tubes. Serial dilution of the treated

stocks was performed and 10^{-3} and 10^{-4} were plated in duplicate on nutrient agar plates for a colony count. The plates were grown at 22°C for 72 hours.

Light microscopy of seawater biofilm:
Images of the squid and the murius biofilms in tank water were taken on a Zeiss Axio Scope.A1 light microscope coupled to x-cite series 120Q with either transmitted light or reflected light with 420/480 filters.

Scanning electron microscope:
SEM was completed in the Central Microscopy Facility at Marine Biology Lab using the Zeiss NTS Supra40VP 0.1-30kV. Fixation of 100µl of murius and squid tank biofilms with 10ml of PBS buffer plus 4% glutaraldehyde at pH 7. Samples were fixed to 0.2µM filter after vacuum then washed 3 times in PBS only buffer. Samples were dehydrated with 50%, 70%, 85%, 95% ethanol. Final dehydration steps with three intervals of 10min soak in 100% ethanol on ice. Samples were dried using a critical point drier then sputter coated in palladium.

454 pyrosequencing:
DNA was extracted from the biofilm was using the MoBio Biofilm kit (MoBio Laboratory) according to protocol. SSU rRNA for Nextgen sequencing were amplified using reverse primer (adapter, linker, SSU rRNA complimentary oligo 907R): 5'-CTATGCGCCTTGCCAGCCCGCTCAG|GG|CCGYCAATTCMTTTRAGTTT-3' and forward primer for squid tank (adapter, barcode*, linker, SSU rRNA oligo 515F):
5'CGTATCGCCTCCCTCGCGCCATCAG|TTGTAAGGT*|GA|GTGYCAGCMGCCGCGGTAA3'
or forward primer for Murius tank (adapter, barcode*, linker, SSU rRNA oligo 515F):
5'CGTATCGCCTCCCTCGCGCCATCAG|ATAACCGTT*|GA|GTGYCAGCMGCCGCGGTAA3'.
Extracted DNA (1:100 dilution) was added to the PCR reaction according to the Microbial Diversity lab manual with Phusion 2x HF MasterMix, DMSO, reverse and forward primers, and nuclease-free water. Sequencing results were analyzed using Qiime open software.

Violacein treatment of biofilms:
Biofilm in tank water was added to directly to tissue culture treated well plates and incubated overnight at room temperature. Next day the wells were dumped and 1ml of seawater complete was added to each well. Wells were treated in quadruplicate with 100µl of methanol, 50µl methanol, violacein was added in final concentrations of 10µM, 5µM, and 2.5µM. Results were checked the following day after incubating at room temperature and images of the treated biofilms were taken on a Zeiss Discovery V8 dissection light microscope at 10x magnification.

Results
Optimization of violacein began with selecting a variety soil from different sampling locations. The fastest and darkest pigment producer was from the yard where one of the Microbial Diversity course directors lived on MBL Street. This colony was

then isolated further on a variety of nutrient-rich media plates such as Luria-Bertaini, R2A, and nutrient agar. Colonies appeared after 2 days on Luria-Bertaini and nutrient agar, though colonies appeared yellow when growing at room temperature and in 30°C. Growing at 15°C, nutrient agar produced purple colonies faster than LB agar (3 vs 5 days). With the addition of 1% glycerol and 250uM of L-tryptophan the colonies produced darker colored colonies after 2 days growth at 15°C compared to without additional reagents.

Growing the bacteria in culture took 5 days before the culture would turn purple due to *J. lividum* forming a biofilm in the media. Large culture growth by embedding sterile cotton mats in sterile 2L bottles with nutrient media with the added glycerol and L-tryptophan **(fig. 2)** that showed purple coloring after 48 hour incubation [9]. The mats were extracted after 5 days to harvest the violacein. Yield of violacein from after crude methanol extraction and low was about 10mg.

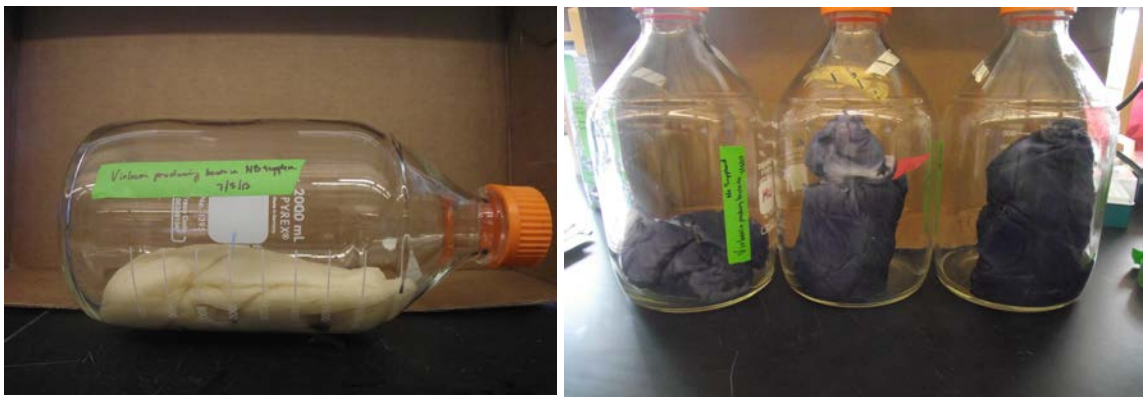

Figure 2: Violacein optimization. 1% Glycerol and 250μM L-tryptophan were added to the nutrient broth media to enhance pigment development. Cotton mats were used to allow bacteria to become sessile and produce violacein faster than with liquid cultures.

While testing growth conditions, *J. lividum* was grown over a three-day period in a liquid culture that was turbid but was faintly producing violacein. Culture was treated with increasing amounts of UV based on a paper from Walker et. al. [10]. UV protection should be conferred to bacteria that produce pigment. Giving almost triple the amount of UV that was used in the Walker paper, the results show that the growth of the colonies were largely unaffected with the increased UV exposure **(Fig. 3).** In fact, the cfu count of untreated is lower than for the 40,000μJ/cm^2 that may be due to the choice of media that cultures were grown (nutrient broth with added glycerol and L-tryptophan). Nutrient broth may confer protection against UV, so the assay should be retried with minimal media.

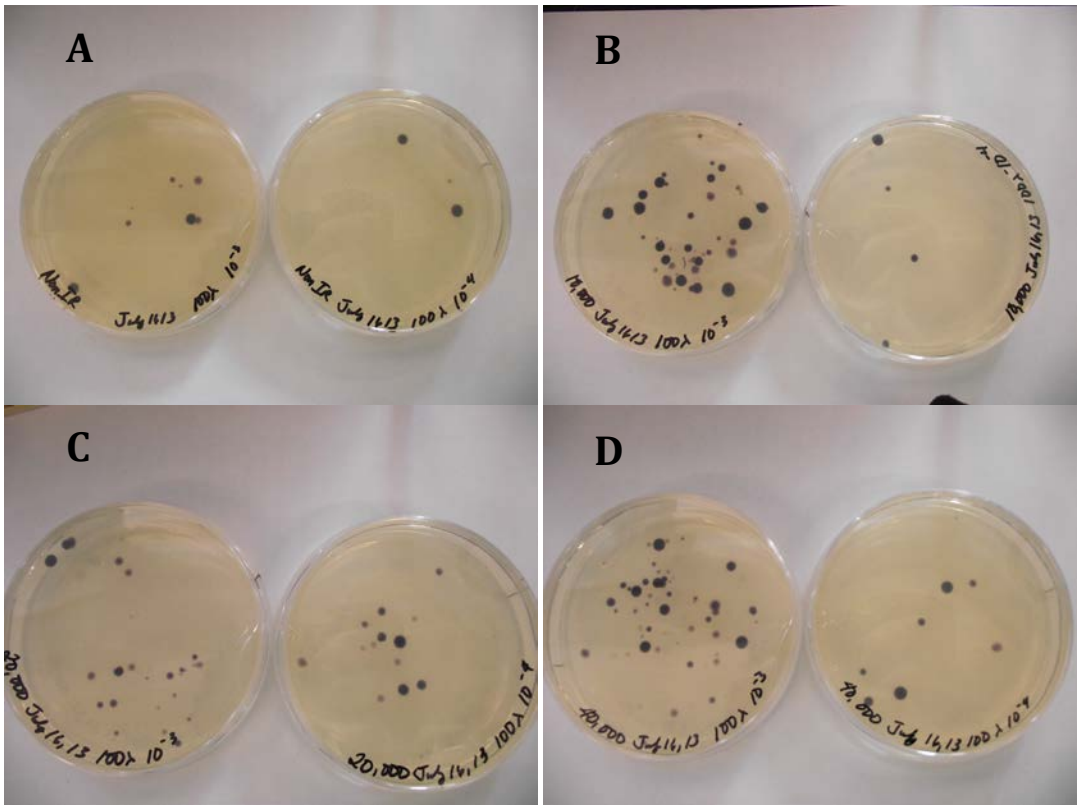

Figure 3A-D: UV treated violacein-producing bacteria. Panels of plated of *J. lividum* RB10 on nutrient agar with 10^{-3} (left) and 10^{-4} (right) serial dilutions after treating with UV at 254nm wavelength. A) untreated cell culture with $1.9x10^5$ cfu/ml; B) 10,000 μJ/cm^2 culture with $3.8x10^5$ cfu/ml; C) 20,000 μJ/cm^2 culture with $7.6x10^5$ cfu/ml; D) 40,000 μJ/cm^2 culture with $5.5x10^5$ cfu/ml.

Communities in the squid and Murius sampled biofilms as identified through 454 sequencing were largely represented by the Proteobacteria (62%) and the Bactroidetes families (19%) in Murius tank **(fig. 4).** Proteobacteria (67%) and the Bactroidetes (24%) families were also dominant in squid tank. Both of these families are gram-negative and generally gram-negative bacteria were shown to be less susceptible to violacein.

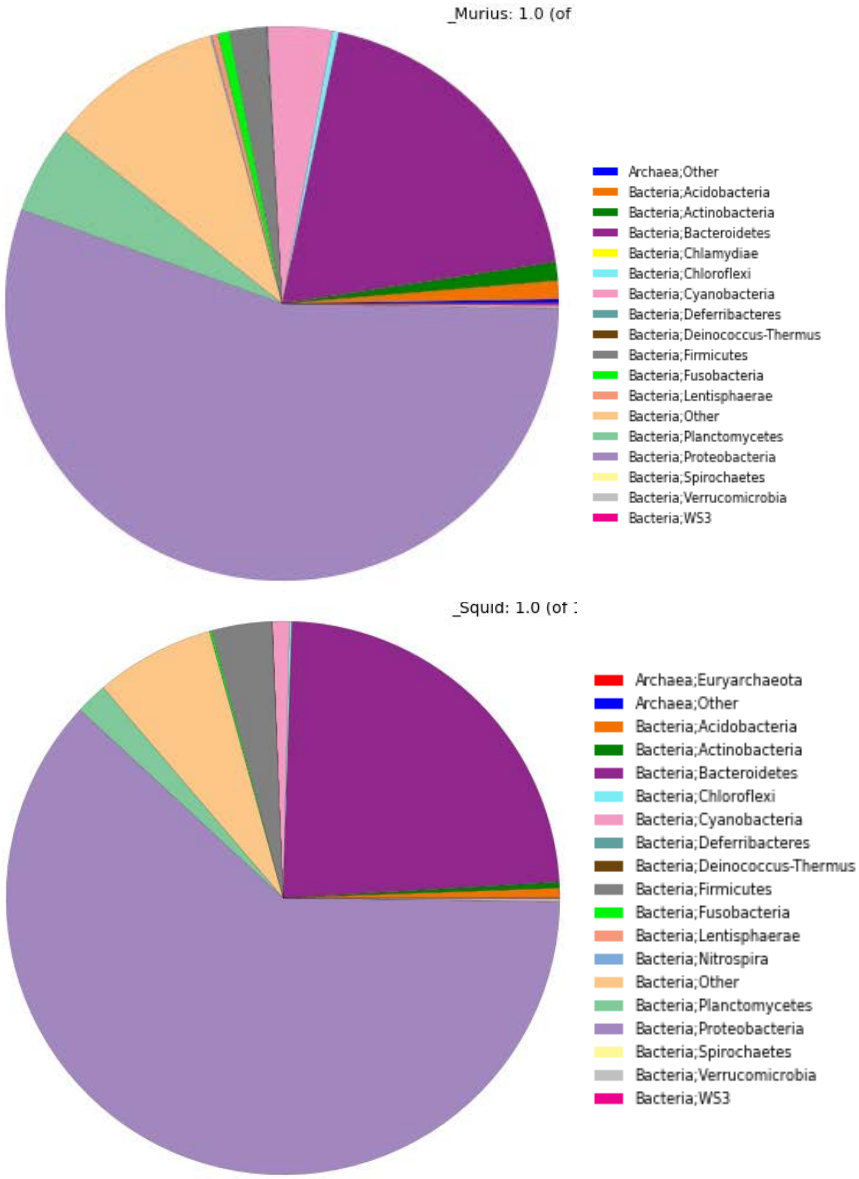

Figure 4: Communities represented in marine biofilm. 454 pyrosequences of murius (top chart) and squid tanks (bottom chart) find that the gram-negative Proteobacterium and Bacteroidetes families are the dominant population in the sampled biofilms.

Light microscopy of the biofilm samples that were to be tested with violacein were taken showing a mixed population of bacteria for both the Murius seaworm mucus and for the squid biofilm that formed at the interface of seawater and air **(Fig.5).** Representative SEM image of the Murius biofilm shows network of extracellular matrix allowing the bacteria to adhere to debris found in the biofilm and attachment in the case of squid to the side of the tank **(Fig. 6).** The biofilms were also stained with Congo red to detect protein aggregation, but the results from this stain were inconclusive.

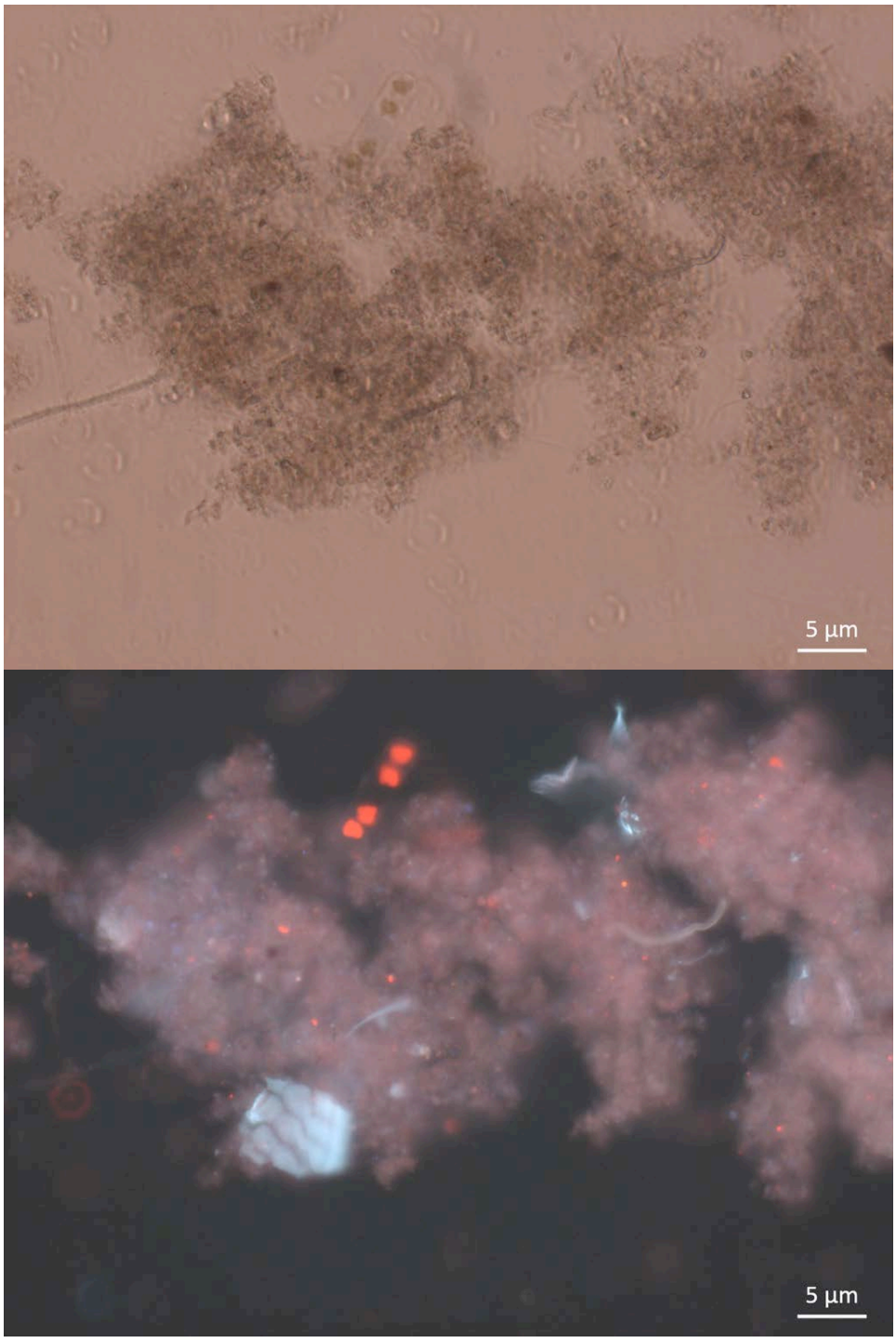

Figure 5: Light Microscopy of Squid biofilm: Transmitted light at 200x magnification (top) and the same field with 420/480 reflected light showing some of the bacteria diversity. Brightly red colored spots show possible cyanobacteria.

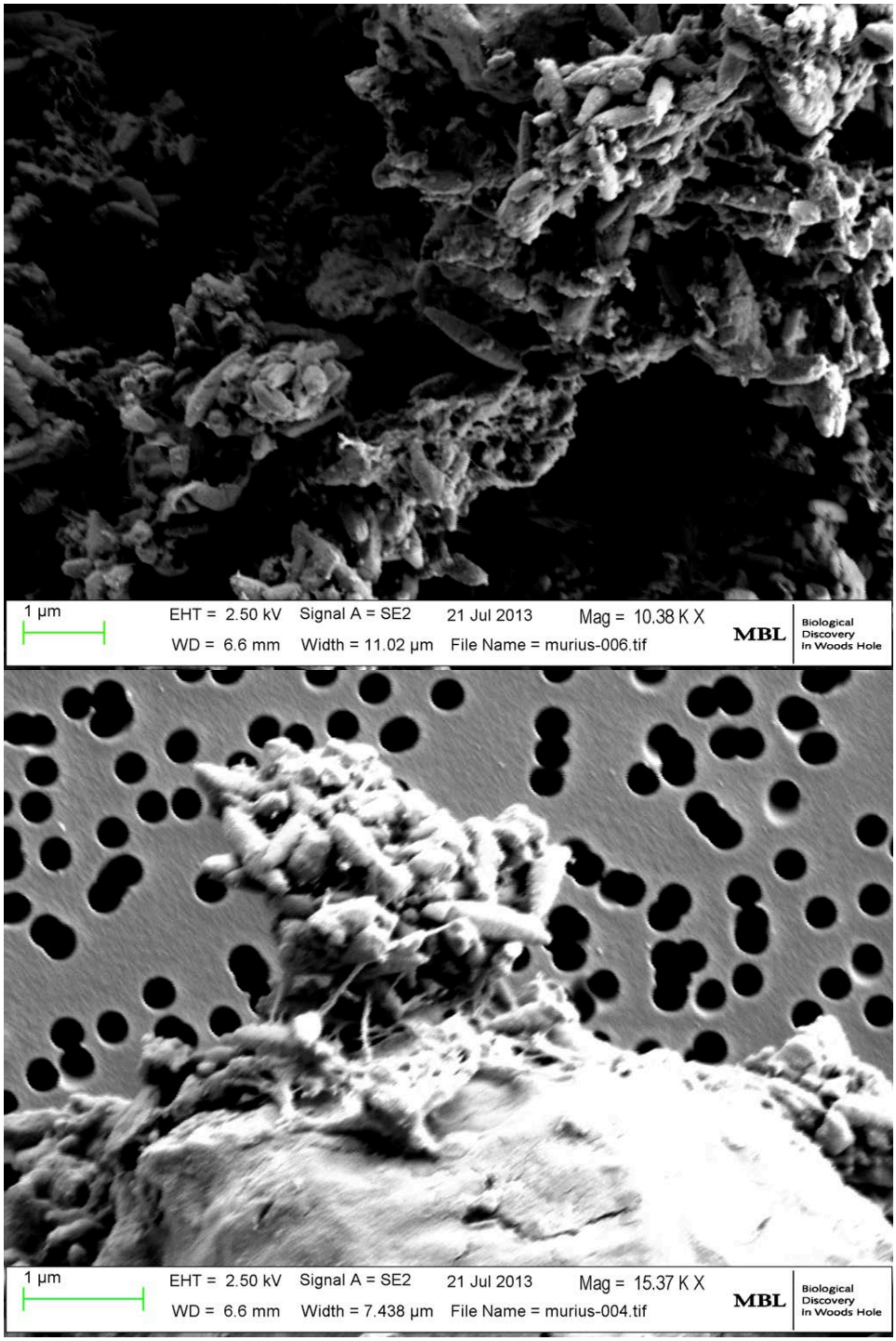

Figure 6: Seawater bacteria extracellular matrix network. Murius tank biofilm (top) shows large clusters of rod-shaped bacteria at 10,380X magnification imbedded in matrix netting. Higher magnification image (bottom) at 15,370X shows bacteria in mesh attached to biofilm debris.

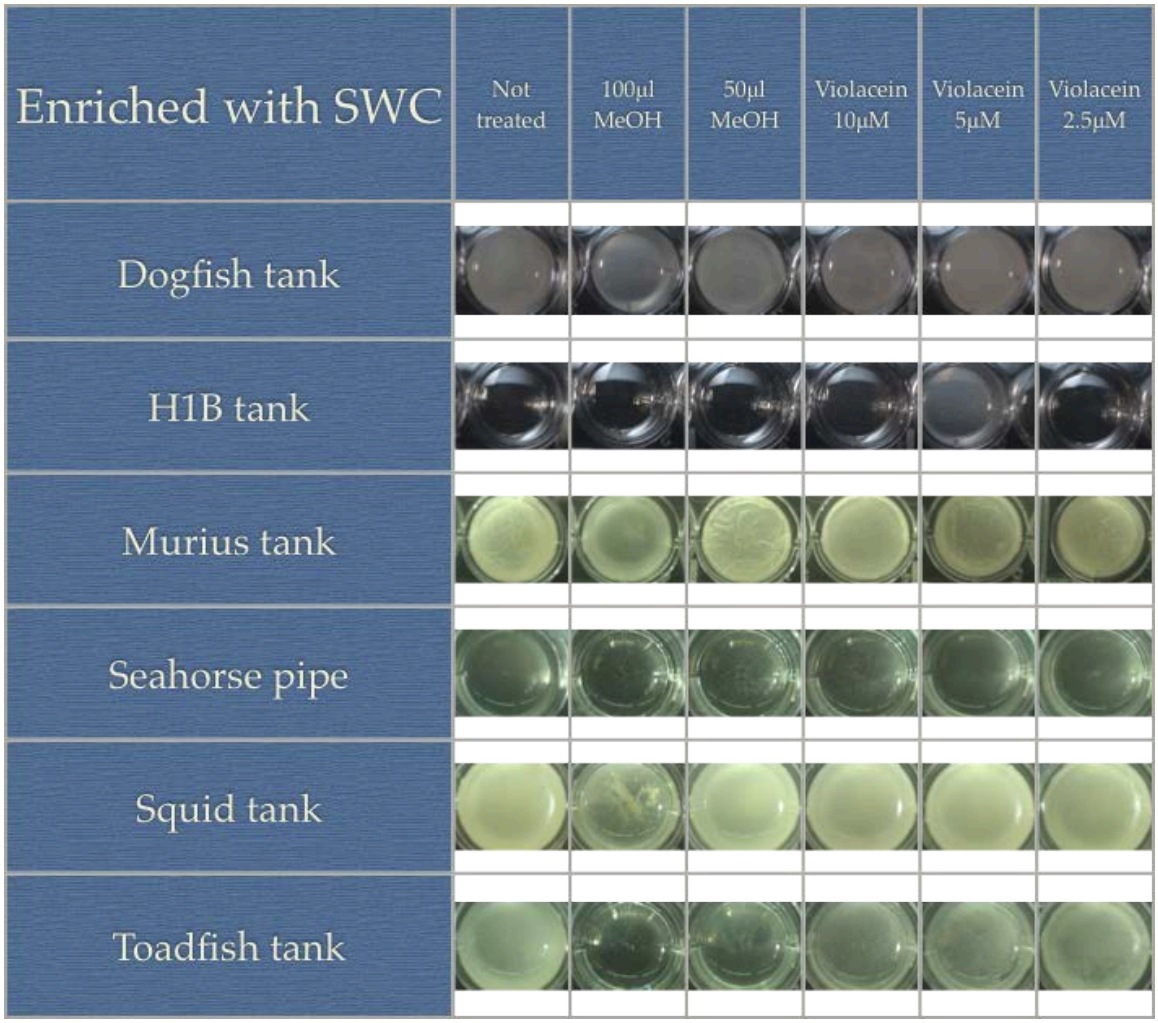

Figure 7: Biofilm treated with crude violacein. The six inoculums were added to wells showing bacteria clearing with 100µL methanol or biofilm morphology changes with increasing concentrations of violacein.

To test the crude extracts, the wells were seeded with 10µM, 5µM, and 2.5µM of violacein after adding seawater complete media to each well **(Fig. 7)**. Volumes of the crude treatment were 108µl, 54µl, and 27µl dissolved in water and residual methanol. This pigment is extremely hydrophobic and has a low solubility in water, so in order to keep the pigment soluble not all of methanol was evaporated down. To control for the addition of methanol as a confounding factor with the violacein, 100µl of methanol and 50 µl was added to the wells. Wells with 100µl of methanol show some clearing of the biofilms when compared to the nontreated wells, whereas 50µl of methanol in well was largely unaffected. Though mass spectrometry of the samples was not performed, the probability of violacein remaining in methanol only is very unlikely after boiling the pigment for 2 days and distilling the methanol for 1 day. The temperature was never raised to boiling so some water may remain. Wells with the highest concentration of violacein did not

show clearing which was expected after the 454 findings, but the treated biofilm surface morphology is smooth and not wrinkled like for the untreated biofilms of Murius, squid, and toadfish. Wells with the highest concentration of violacein were not as turbid as the non-treated wells or wells with 50µl of methanol that suggests that violacein is having a undetermined effect on the mixed biofilm population. Further investigation of bacteria isolates of colonies should be cultured and inoculated with violacein treatment then counted with crystal violet to count cells. Additionally, violacein extracts were seeded on filter paper after 100µl of cultured biofilms from the six tanks were plated on seawater complete agar (not shown). No zones of inhibition were found using the extracts or with the positive controls of 100% methanol and 100% acetone on the mixed biofilm populations.

Conclusion

The original idea was to learn how to test growth conditions and determine inhibition of bacteria growth using violacein. Though the results were largely negative, the biofilm morphology did change with the addition of the highest concentration of the pigment. Further purification of the crude pigment extract and checking with mass spectrometry will allow for higher concentrations of the compound to be added to cultures without the potential of methanol being a confounding factor. With more time, treating mixed biofilm populations with higher concentrations of violacein, then 454 sequencing of the surviving culture could be done to identify the bacteria populations that remain in the face of treatment. After the post-treated population is identified, metabolic comparison with bacteria cultures that are susceptible to violacein in order to find candidate drug targets. Screening isolated bacteria cultures from the seawater with violacein should be completed to test effective inhibitory concentrations for individual strains. Ultimately, understanding the mechanism of how violacein inhibits growth of other bacteria would allow for the further development of chemotherapeutics.

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