On Feasibility of Fluorescence-based Bacteria Presence Quantification: *P.Aeruginosa*

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Abstract: Introduction: Wound healing typically occurs in the presence of bacteria at levels ranging from contamination to colonization to infection. The role of bacteria in wound healing depends on multiple factors, including bacterial concentration, species present, and host response. Thus, the determination of bacterial load is of great importance. However, existing clinical bacteria load assessment methods (biopsy or swabbing combined with culture methods) are slow, labor- and time-consuming. *Pseudomonas aeruginosa* is a known pathogen implicated in numerous healthcare-associated infections and can express fluorescent metabolites during proliferation. In particular, the siderophore pyoverdine produces a fluorescent emission between 450-520 nm when excited at 400nm and can be measured quantitatively via fluorescence spectroscopy. The current project aims to investigate the possibility of quantifying bacterial presence using fluorescence measurements. Methods: Cultures of *P.aeruginosa* (PA01) were grown at various temperatures (ambient temperature, 30, 37-43°C), inoculum starting condition ($5*10^7$ -$5*10^8$ $CFUmL^{-1}$), and initial nutrient's concentration (0.6, 1.5, 3.0 gL^{-1}) in Tryptic Soy Broth media. Media optical density (OD, as a proxy of bacterial concentration) and fluorescence (ex. 400nm, em. 420- 520nm) were measured hourly for 10 hours. Results: Cultures remained metabolically active in the whole temperature range, producing pyoverdine fluorescence (emission max at 455nm). We have correlated optical density with a fluorescent signal to establish a dependence between fluorescence and growth stage. Noticeable pyoverdine accumulation started approximately 3 hours after the beginning of the log growth phase and experienced saturation at the beginning of the stationary phase. Three distinct regimes (a sigmoid curve) were observed: linear dependence of fluorescence on OD for low concentrations, more rapid nonlinear dependence, and saturation when approaching the stationary phase. Conclusions: The sigmoid dependence of bacterial fluorescence on their concentration persisted through variations in temperature and inoculum starting condition; thus, it may have the potential for determining culture growth phase progression. These results, combined with classical knowledge on disease progression, could also lead to an advanced infection diagnosis before current pathogenesis observation techniques.

1 INTRODUCTION

Wound healing occurs in the presence of bacteria (e.g., *Staphylococcus, Streptococcus, Pseudomonas* species, and *Coliform* bacteria, including aerobic and anaerobic types), at levels ranging from contamination to critical colonization to infection. The role of bacteria within wounds depends on multiple factors, including bacterial concentration, species present in the wound, and host response.

There are several distinct levels of bacteria presence in the wound: contamination, colonization, and infection. These levels delineate from the number of microorganisms present per gram of tissue, which can be highly variable and can range from less than 1 to 10^8 or 10^{10} colony-forming units (CFU).

The increased bacterial burden may be confined to the superficial wound bed or present deep within the wound or even surrounding tissue.

Contamination and colonization by low microbial concentrations are considered normal and are not believed to inhibit healing. However, critical colonization and infection are associated with a significant delay in wound healing.

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With the ubiquity of microorganisms within the natural environment, lacerations and skin tissue lesions can lead to health-threatening issues if infectious pathogens can take hold within a compromising injury. With that in mind, the importance of assessing the stage of infection and identifying primary microorganisms involved can assist with wound treatment. It can also help prevent disease progression if pathogens are properly characterized early on.

The gold standard collection method is to do a tissue biopsy or needle aspirate of the wound's leading edge after debridement. However, the practical standard method for bacterial load determination for skin infections is to culture (e.g., pour or spread plate) a microbiological swab of the wound surface. Obviously, this method suffers from multiple shortcomings. Among them, a) specimen can be contaminated by normal skin or mucosa flora, b) swabs frequently yield too small a specimen for accurate microbiologic examination (Washington. 1996), and c) the duration of incubation for certain cultures can be long. While most aerobic and anaerobic bacteria will grow overnight, some mycobacteria require as many as 6 to 8 weeks before colonies are observed.

Other methods (e.g., Polymerase Chain Reaction or PCR) have been proposed. However, they mostly address the quantification step, while swabbing remains the primary specimen collection technique.

Thus, other methods of bacterial load quantification, preferably close to real-time, are required. This could be accomplished by monitoring the potential site of infection for unique metabolites that can be quantified easily. Endogenous fluorescence (FL) has great potential as a remote and non-invasive modality. It can be performed remotely, thus decreasing the risk of contamination.

Most clinically important strains (both gram-positive and negative) clearly show a distinctive double-peak of tryptophan fluorescence (emission peaks at 340 nm, with two excitation maxima; at 230 nm and 280 nm) (Dartnell, 2013) with comparable intensity between the strains studied. However, the use of UVC light *in vivo* is quite problematic.

Fluorescence extending through the 400-500nm emission range, from excitation of around 350 nm, is also reported for many cells due to cellular metabolites such as NAD(P)H. However, it is highly variable, dependent on the microbial strain and metabolic state, is not always detectable (Estes, 2003), and is much less intense than the tryptophan signal (Dartnell, 2010).

In addition to that, it is known that most clinically relevant bacteria (*S. aureus, S. epidermidis, Candida, S. marcescens, Viridans streptococci, Corynebacterium diphtheriae, S. pyogenes, Enterobacter, and Enterococcus*) produces red FL (from porphyrins (Kjeldstad, 1985)) when excited at 405nm. In contrast, *P.aeruginosa* produces a bluish-green FL (from pyoverdin (Cody, 1987)) while excited at 405nm. Considering the polymicrobial nature of most chronic wounds, it is possible to use this endogenous fluorescence to characterize bacterial load.

Fluorescence has been previously used to differentiate between different species. In particular, the fluorescence of extracellular pyoverdines has been used to distinguish between cultures of certain strains of *Pseudomonas* (Shelly, 1980 a and b). Leblanc et al. (Leblanc, 2002) successfully distinguished between different species of bacteria using principal component analysis (PCA) of the autofluorescence of the aromatic amino acid, nucleotide, and NADH components of the cell. Giana et al. (Giana, 2003) successfully discriminated between the clinically important *Escherichia coli*, *Enterococcus faecalis*, and *Staphylococcus aureus*.

However, both porphyrins and pyoverdine are mostly extracellular compounds produced by bacteria. Moreover, their production and fluorescence can be affected by numerous factors. Thus, quantification of bacteria presence using porphyrin- and pyoverdine-based fluorescence is not straightforward.

The goal of the current project is to establish the feasibility of fluorescence imaging of bacterial load under clinical parameters and investigate the possibility of quantifying bacterial presence using fluorescence.

In this article, we will present the results of bacteria fluorescence quantification on the cultures of *P.aeruginosa* (PA01). The bacterium *Pseudomonas aeruginosa* is an increasingly prevalent human pathogen, responsible for 12% of hospital-acquired urinary tract infections, 10% of bloodstream infections, and 8% of surgical wound infections. In the UK, 7.6% of acute hospital patients acquire healthcare-associated infections, around a sixth of which are caused by methicillin-resistant *Staphylococcus aureus* and another sixth by *Clostridium difficile* (Smyth, 2008).

Pseudomonas aeruginosa is known to express fluorescent metabolites during proliferation. These metabolites include pyoverdine and pyocyanin and are thought to play a role in the virulence of other hemolytic pathogens as well. Of pyoverdine

specifically, 400 nm light is known to produce a fluorescent emission between 450-500 nm. It can be measured quantitatively based on concentration within an appropriate growth media such as tryptic soy broth. When correlated to optical density, this fluorescent signature can be compared to the cell quantity and growth stage. Additional factors, such as temperature and initial starting concentration, also play a role in cell growth and pyoverdine expression.

2 METHODS

2.1 Culture Preparation

Many of the materials and consumables used through this study for the manipulation of microbial cultures, including pipette tips, Petri dishes, and culture tubes, were sourced from Sarstedt unless otherwise listed. Tryptic Soy Broth (cat. 1054590500) was purchased from Millipore-Sigma, and phosphate-buffered saline tablets were purchased from Bio Basic (cat. PD0435) and were used as indicated.

Bacterial strains used for this study were supplied from ATCC and maintained at Ryerson University by the Wolfaardt lab group. PAO1 and PA14 are commonly used for studying the basic biology and genetics of *P. aeruginosa*, and PA01 was chosen as the representative strain for this study.

2.1.1 Inoculum Preparation

Several colonies of *Pseudomonas aeruginosa* (PA01) were collected from a maintained 3 gL^{-1} Tryptic Soy Agar (TSA) culture plate using an inoculation loop and deposited into a 50 mL conical tube containing 10 mL of 3 gL^{-1} Tryptic Soy Broth (TSB) under aseptic conditions. The tube was then sealed and placed onto a shaking incubator set to 37 °C and left to incubate to the late-exponential growth stage, between 16-20 hours after initial cell deposition. The following day, the overnight solution was rinsed by centrifuging two 1 mL portions of overnight serum collected into 2 mL microtubes, replacing the supernatant solution with sterile phosphate-buffered saline (PBS), and by resuspending the microbial pellet within the solution. The process was repeated twice to ensure proper rinsing of cells, and then both rinsed aliquots were combined within a single 2 mL microtube for sample inoculation.

Table 1: Inoculum Parameters.

Test microorganism	*Pseudomonas aeruginosa (PA01)*
Growth media	3 gL^{-1} Tryptic Soy Broth (TSB)
Incubation period	16-20 hours (Overnight)
Incubation temperature	37 °C
Rinse solution	1x Phosphate Buffered Saline (PBS)
Centrifuge duration	4 min at 9000 x g
Expected inoculum load	10^5-10^7

2.2 Culture Quantification

2.2.1 Microbial Reference Quantification

Following inoculum preparation, cells were enumerated by serially diluting the prepared inoculum (100 µL of the previous dilution was added to 900 µL of PBS per step) and spot plating 0.1 µL of the 10^5, 10^6, and 10^7 dilutions onto 3 g L^{-1}TSA plates. The plates were then left to incubate at ambient temperature for 7 days prior to counting developed colonies of PA01. The PA01 colonies grown during spot plating are depicted in Fig 1. The Petri dish was imaged by a camera with 425nm long pass filter and 405nm excitation. The bluish-green fluorescence of pyoverdine is clearly visible.

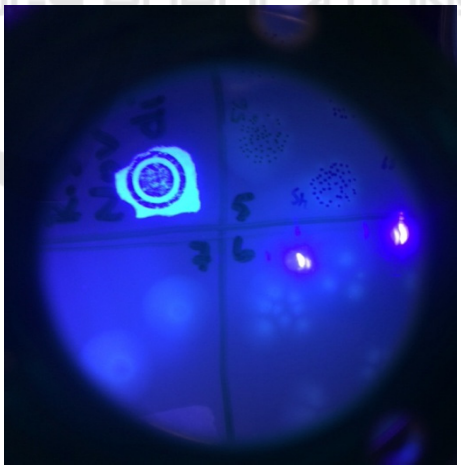

Figure 1: The PA01 colonies grown during spot plating. The colonies are imaged by a camera with 425nm long pass filter and 405nm excitation. Two bright spots are caused by specular reflection of the excitation light.

Table 2: Microbial Quantification.

Quantification Method	Spot plate technique
Quantification period	Immediately after rinsing cells
Maximum dilution factor	10^7
Growth media	3 gL^{-1} Tryptic Soy Agar (TSA)
Incubation temperature	25 °C (ambient Temperature)
Incubation period	7 days

2.2.2 Microbial Rapid Quantification

To allow for the rapid quantification of PA01 bacterial cell concentration within liquid media, optical density (OD) measurements were calibrated to colony-forming unit counts obtained from microbial reference quantification. Since plate counting requires multiple days and significant resources to determine the bacterial concentration at any point in time, rapid quantification of bacteria concentration can be achieved by measuring the absorbance or scattering of light. For this purpose, standard OD measurements at 600nm were performed using parafilm-sealed macro-cuvettes (cat. BR759035, Millipore-Sigma) with a 10 mm light pathlength, within a BioPhotometer (S/N 6131 21925, Eppendorf AG). The concentration of bacterial cells suspended within 3 mL of 3 g L^{-1} TSB was then calibrated using multiple concentration points between 10^0-10^2 CFU and graphed to produce a standard calibration curve. For spectroscopic blanking purposes, a sterile TSB control was maintained at 4 °C for each trial performed.

2.2.3 OD-Correlated Fluorescence Spectroscopy

Fluorescence spectroscopy was performed using an LS 50 B Luminescence Spectrometer (S/N 50801, Perkin-Elmer Ltd.) on macro-cuvettes containing 3 mL TSB inoculated with PA01, as defined by the microbial quantification methods above. Fluorescence emission scans were achieved using 400nm (2.5 nm slit width) as the excitation wavelength, while emission was recorded in 420-520nm (2.5 nm slit width) range with 0.5nm increments for 1 minute. OD measurements at 600 nm were taken prior to each fluorescence microscopy scan. The typical raw fluorescence spectrum is depicted in Fig 2.

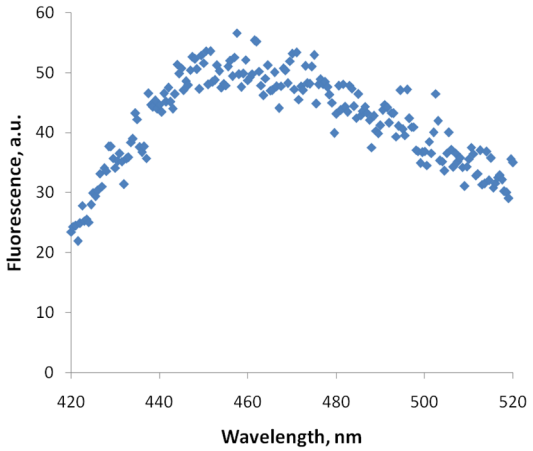

Figure 2: An example of a raw fluorescence spectrum of the PA01 sample.

A spectral integral was used over the whole 420-520nm range to quantify the fluorescence signal:

$$fl = \int_{420}^{520} S(\lambda) d\lambda \quad (1)$$

Here, S(λ) is a fluorescence signal measured at a particular wavelength λ. To eliminate background fluorescence and dependence on various starting conditions instead of absolute values, the normalized fluorescence ration was used,

$$FL = \frac{fl - fl_c}{fl_c} \quad (2)$$

where fl_c is the fluorescence spectral integral for a sterile TSB control sample.

2.3 Experimental Protocol

Initial trials were performed by inoculating macro-cuvettes containing 3 mL of 3 g L^{-1}TSB with 30 µL (1/100 dilution) PA01 inoculum, prepared and quantified using the above methods. Samples were then incubated at a predetermined temperature (ambient temperature, 30°C, 37-43 °C) within a standing incubator for 8-10 hours. Prior to incubation and after each following 1 h interval, OD-correlated fluorescence spectroscopy was performed on the prepared samples to determine the concentration of PA01 cells within the growth medium and the quantity of fluorescence emitted by each sample.

To investigate the effect of the initial bacterial concentration, another set of trials were performed at 37 °C, where the starting concentration of PA01 was altered by inoculating the macro-cuvettes with 150 (1/20), 75 (1/40), and 37.5 µL (1/80) of quantified PA01 inoculum.

Finally, the impact of nutrient availability was investigated by altering the initial concentration of tryptic soy broth (0.6, 1.5, and 3.0 gL^{-1})) while keeping the number of cells inoculated and temperature the same (30 µL (1/100 dilution) PA01 inoculum and 37 °C, respectively).

3 RESULTS

Calibration procedure for PA01 shows the following dependence between bacteria concentration N ($CFUmL^{-1}$) and optical density OD (R^2=0.991):

$$N = (5 * 10^9 * OD)^{0.97} \quad (3)$$

To estimate the carrying capacity *K*, we have grown the culture in 3 g L^{-1}TSB for 26 hours and found that the optical density saturates at approximately 0.462 value, which according to Eq.3, corresponds to K=1.21*10^9 $CFUmL^{-1}$.

We performed three types of experiments. All results are presented in normalized fluorescence (FL) vs. optical density (OD), a proxy for the bacterial concentration.

3.1 Dependence on Temperature

Firstly, we studied growth and fluorescence at several temperatures (ambient temperature, 30C. 37C, 38C, 39C, 40C, 41C, 42C, 43C), while maintaining inoculation and nutrient's initial concentrations the same (30 µL (1/100 dilution) PA01 inoculum and 3.0 gL^{-1}TSB, respectively). The results are depicted in Fig 3.

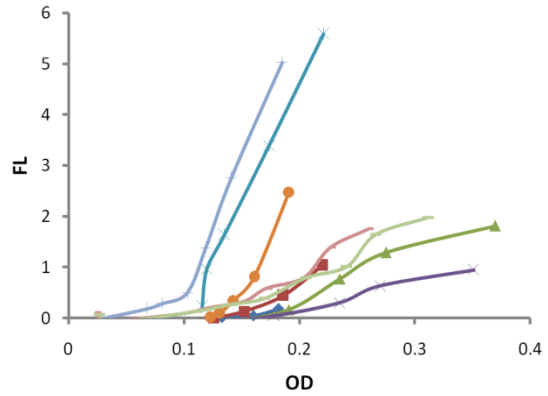

Figure 3: Dependence of fluorescence (FL) on optical density (OD) for various temperatures: ambient temperature (blue rhombs ◊), 30C (red, squares □), 37C (red, line), 38C (green, triangles Δ), 39C (green, line), 40C (blue, star *), 41C (blue, |), 42C (purple, cross x), 43C (brown, dot). All other parameters (inoculation and nutrient's concentration) were kept the same.

3.2 Dependence on Inoculums Concentration

To investigate the effect of the initial bacterial concentration, another set of trials were performed at 37 °C, where the starting concentration of PA01 was altered by inoculating the macro-cuvettes with 150 (1/20), 75 (1/40), and 37.5 µL (1/80) of quantified PA01 inoculum. The results are depicted in Fig 4. In Fig 5, one can see the zoomed area with low OD.

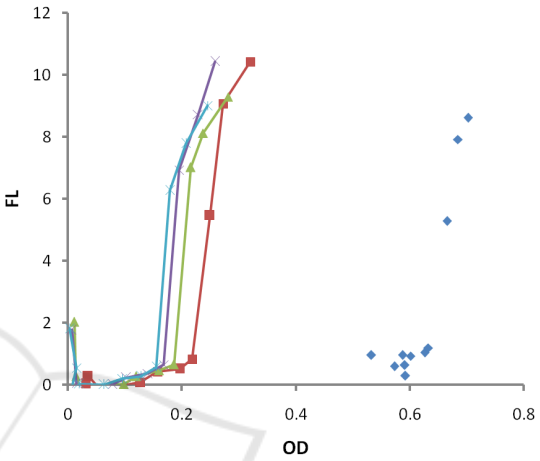

Figure 4: Dependence of fluorescence (FL) on optical density (OD) for various starting concentrations: original stock (blue rhombs ◊), 1/20 (red, squares □), 1/40 (green, triangles Δ), 1/60 (purple, cross x), 1/80 (blues, star *).

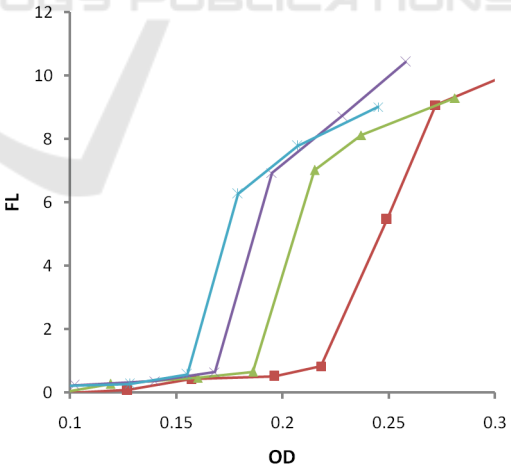

Figure 5: Dependence of fluorescence (FL) on optical density (OD) for various starting concentrations: 1/20 (red, squares □), 1/40 (green, triangles Δ), 1/60 (purple, cross x), 1/80 (blues, star *).. Zoomed area with low OD.

3.3 Dependence on Initial Nutrients Concentration

Finally, the impact of the initial nutrient's concentration was investigated by altering the initial nutrient's concentration (0.6, 1.5, and 3.0 gL^{-1} Tryptic Soy Broth (TSB)) while keeping inoculation and temperature the same (30 µL (1/100 dilution) PA01 inoculum and 37 °C, respectively). The results are presented in Fig 4.

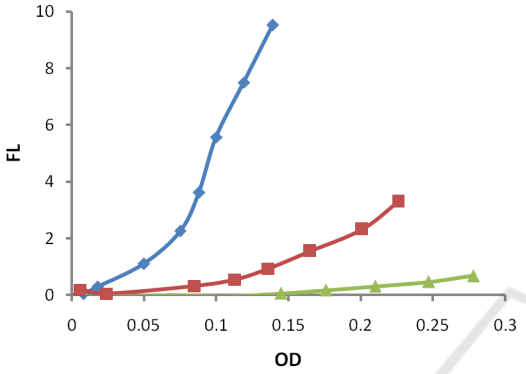

Figure 6: Dependence of fluorescence (FL) on optical density (OD) for various starting nutrient's concentrations: 3gL^{-1} (green, triangle, Δ), 1.5 gL^{-1} (red, squares □), 0.6 gL^{-1} (blue, rhomb ◊)

4 DISCUSSION

Our data supports the view that *P.aeruginosa* is a versatile and opportunistic microorganism. It remains metabolically active even at temperatures approaching 43 °C.

Our preliminary results support the nutrient-dependent siderophore production model developed in (Saiko, 2021). According to the developed approach, siderophore production in a resource-limiting environment has three distinct phases:

I. Slow siderophore production at low bacteria concentrations where resources are abundant ($S > S_{th}$)
II. Rapid accumulation of siderophores upon reaching a specific nutrient's concentration S_{th}. Linear dependence on the bacteria concentration N.
III. Upon reaching resource limits, the bacteria focus on growth solely, which will result in saturation of compound accumulation (while bacterial concentration still growth)

The transition into rapid siderophore accumulation regime (Phase I -> Phase II) occurs at N_{th}:

$$N_{th} = N_0 + \gamma(S_0 - S_{th}) \quad (4)$$

here N_0 and S_0 are the initial bacterial and resource concentration, accordingly. S_{th} is the resource concentration below which bacteria start producing siderophore rapidly. During that phase II, the siderophore accumulation depends on the bacterial concentration linearly:

$$C = \frac{\xi}{\gamma}(N - N_0) - \xi(S_0 - S_{th})) \quad (5)$$

Finally, when the bacteria population approaches the carrying capacity, K ($K=(N_0+\gamma S_0)$), the bacteria divert all resources to replication only, thus reducing siderophore production.

All these phases were observed in our experiments.

We found that at the early stages of the growth, where nutrients are abundant, the siderophore production is relatively small.

We also found that the infliction point Nt_h is affected by the initial bacterial concentration N_0 (Fig 5) and the initial nutrient concentration S_0 (Fig 6). There is a clear linear dependence of the infliction point N_{th} on the initial inoculums concentration N_0. The higher the initial concentration, the higher the infliction point N_{th} is (Fig 5). Also, we found a clear linear dependence of the infliction point N_{th} on the initial nutrient's concentration S_0. The higher the initial concentration S_0, the higher the infliction point N_{th} is (Fig 6).

In all our tests, upon reaching approximately $K/2$ bacteria concentration (OD=0.23), the siderophore accumulation slope starts decreasing (see Fig 3 and Fig 4). This finding agrees with the nutrient-dependent siderophore production model (Saiko, 2021), and available experimental data from other groups (Bren, 2013). Thus, our results support the view that under starvation, bacteria will focus on growth only (Bren, 2013) and stop diverting resources to siderophore synthesis.

Despite promising results in culturing media, the possibility of quantification of *P.aeruginosa* presence based on pyoverdine fluorescence within wounds requires further challenges to be solved. *P.aeruginosa* is a particularly difficult model. Firstly, the fluorescence of bacteria can be impacted by other factors. Specifically, *Pseudomonas* fluorescence is determined by two factors (Meyer, 1978): a) iron bonded to pyoverdine quenches fluorescence, b) pyoverdine production is affected by iron availability.

Thus, *P.aerugenosa* fluorescence can be diminished near blood vessels (due to fluorescence quenching and/or decreased pyoverdine production).

Secondly, it is known (Smith, 2006) that *P. aeruginosa* isolated from acute infections differ substantially in phenotype from those isolated from chronic infections. It was found (Morgan, 2019) that *P.aeruginosa* isolated from chronic human wounds were frequently defective in virulence functions and biofilm formation. In addition to that, *P.aeruginosa* has an extensive "quorum sensing" (QS) system with three autoinducers. These QS sub-systems act hierarchically and regulate cell survival, biofilm formation, and virulence (Gellatly, 2013).

Thirdly, *P.aeruginosa* can sequester iron in ways other than pyoverdine production. It can (i) produce another siderophore (pyochelin); (ii) utilize a wide range of siderophores synthesized by other organisms (Cornelis, 2002); (iii) acquire Fe(II) through the *Feo* system (Cartron, 2006). *P. aeruginosa* can also utilize heme-iron by expressing two different heme-uptake systems, namely *phu* and *has* (Ochsner, 2000).

Finally, a weak fluorescence signal from bacteria *in vivo* can be masked by strong autofluorescence from nearby tissues. Thus, the proper selection of the excitation wavelength and emission filter may be required. Therefore, quantification of *P.aeruginosa* presence through pyoverdine fluorescence *in vivo* seems quite challenging at this stage.

There are certain limitations regarding the extrapolation of our results *in vivo*. They were obtained in a resource-limiting environment, which may or may not be the case *in vivo*. Thus, future studies on animal models are required.

In future work, we plan to investigate porphyrins production by another clinically relevant bacteria, *S.aureus*.

5 CONCLUSIONS

We found that a fluorescent emissive signature between 420-520 nm for PA01-produced pyoverdine can be observed when excited with light at 400 nm in a wide range of conditions.

Our temperature-dependence studies demonstrate the production of fluorescent siderophores at temperatures between ambient and 43 °C. Results also point towards a local maximum in fluorescence expression for *P.aeruginosa* around 40- 41 °C, although further experimentation would be required if this is to be determined.

We found that the sigmoid dependence of bacterial fluorescence on their concentration persisted through variations in temperature and inoculum starting condition. This preliminary data supports the hypothesis that siderophore production in *P.aeruginosa* is governed by nutrient-dependent mechanisms.

Starting nutrient concentration data also indicates a positive relation between nutrient exhaustion and fluorescent metabolite expression. This result agrees with previous findings (Bren, 2013) and indicates that siderophore production may become inhibited in situations with high-nutrient concentrations.

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