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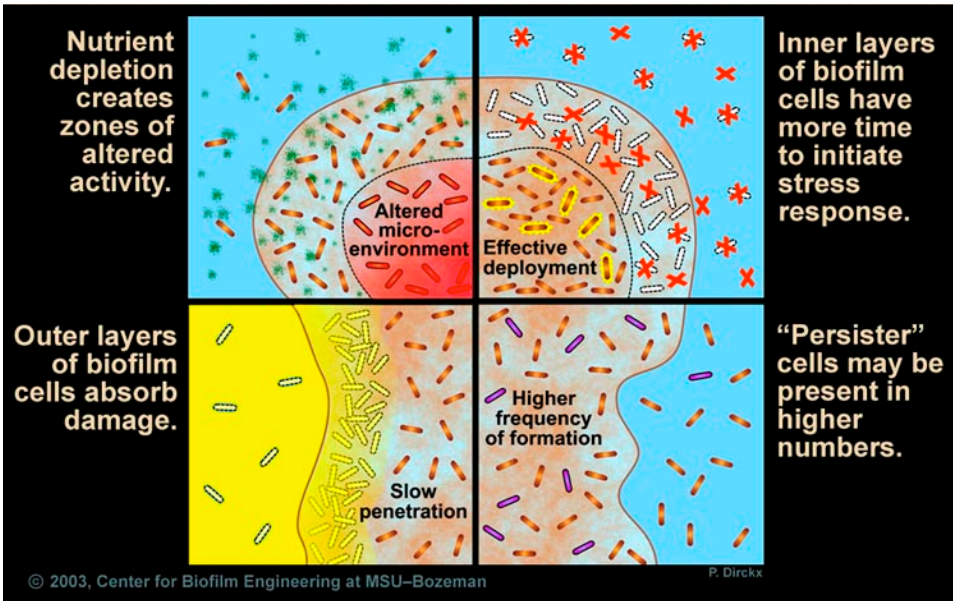
Preliminary Report on Activity of Biocidin against Multiple Species of Biofilms

In this study we are determining whether Biocidin® is effective against biofilms of *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Klebsiella pneumonia*e and *Staphylococcus aureus*.

Generally it thought that bacteria live as individual cells and currently most of the susceptibility tests available in the diagnostic laboratories are targeting mainly these single cells. However, bacteria in their natural habitat do not live as single cells, they live as communities of bacteria that can sense each other by using chemical signaling molecules. These communities of bacteria are commonly known as biofilms. Biofilms are responsible for 80% of all infections and for most of the chronic infections. Biofilms are complex, dynamic structures that react to stimulus in a coordinate behavior via intercellular and intracellular communication. Biofilms are also 10-1000 times less susceptible to antimicrobials than their planktonic counterparts (1). However, when bacteria disperse from a biofilm, antibiotic sensitivity is restored (2). The increased tolerance to antimicrobials by biofilms is thought to be due to several factors (Fig. 1) including:

A. The presence of an extrapolymeric substance (EPS) which functions as a protective barrier and delays the penetration of the antimicrobials and in some circumstances inactivates their activity (3)
B. The presence of an heterogeneous cell population with difference growth rates population (4),
C. The biofilm phenotype, a subpopulation of the community contains active mechanisms that are expressed to combat the detrimental effects of antimicrobial agents (5).
D. The presence of persister cells, a tolerant population of cells that avoid killing, as they do not grow or die in the presence of antibiotics and are not formed as a response to antimicrobials; they represent specialized survivor cells whose production is regulated by the growth stage of the population, both in open and closed systems (6).

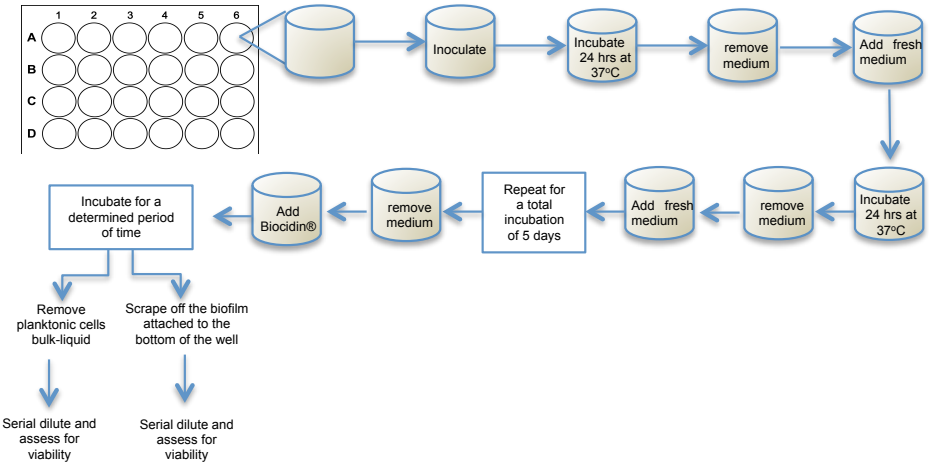


Due to the biofilm resilience and the inability of the current antimicrobial therapies to resolve the infections. Biocidin® could be one of those alternatives.

Since starting testing Biocidin® for its antimicrobial activity we have found that the minimum inhibitory concentrations against planktonic cells are:

	Minimum inhibitory concentration (MIC)	
	Average	Standard Deviation (SD)
E. coli (ATCC 11775)	6.25%	0.2%
S. aureus (ATCC 6538)	12.5%	1.1%
P. aeruginosa (ATCC 10752)	12.5%	0.6%
K. pneumonia (ATCC	12.5%	0.5%
C. albicans (ATCC 20260)	12.5%	0.3%

Once MICs were established for each bacterial species to be studied, we initiated biofilm testing using a method developed in our laboratory.



Biocidin® efficacy was determined against biofilms. Various concentrations of Biocidin® diluted in saline (0.85% sodium chloride) were tested and viability was assessed. Overall, biofilms were less susceptible than the bulk-liquid (planktonic) populations. This was expected as, once cells disperse from a biofilm into the bulk-liquid they become susceptible to antimicrobials once again.

Table 2. % Death following exposure to various concentrations of Biocidin® for a period of 4 hours at 37°C with aeration

		0% biocidin®	**25% Biocidin®**	**50% Biocidin®**	**75% Biocidin®**	**100% Biocidin®**
S. aureus	Biofilms	0%	92.9%	88.4%%	95.0%	89.7%
	Planktonic	0%	99.2%	60.0%	91.9%	99.9%
K. pneumonia	Biofilms	0%	90.7%	78%	82.7%	99.8%
	Planktonic	0%	99.1%	55.9%	91%	99.97%
P. aeruginosa	Biofilms	0%	92.1%	99.99%	99.96%	N/A
	Planktonic	0%	93.3%	99.99%	99.97%	N/A
C. albicans	Biofilms	0%	99.96%	99.99%	99.98%	99.99%
	Planktonic	0%	95.6%	96.3%	95.9%	99.7%

Subsequently, biofilms were exposed to a fixed concentration of Biocidin® for a period of 24 hours and cell viability was monitored.

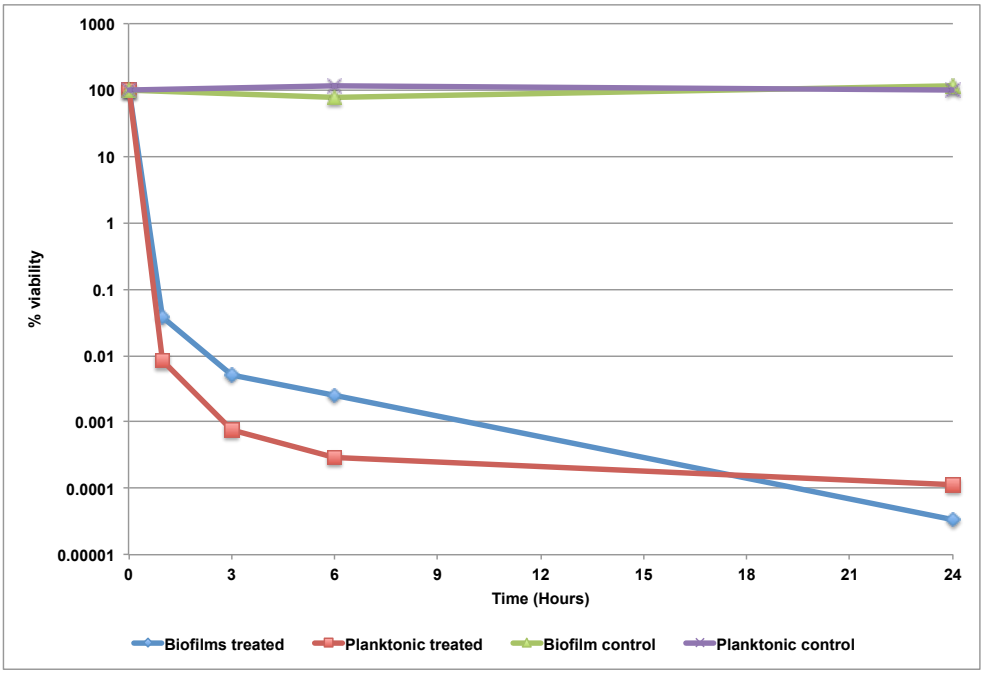


Figure 1. *P. aeruginosa* biofilms exposed to 50% Biocidin® for a period of 24 hours. At 24 hrs, most of the biofilm and planktonic populations were eradicated.

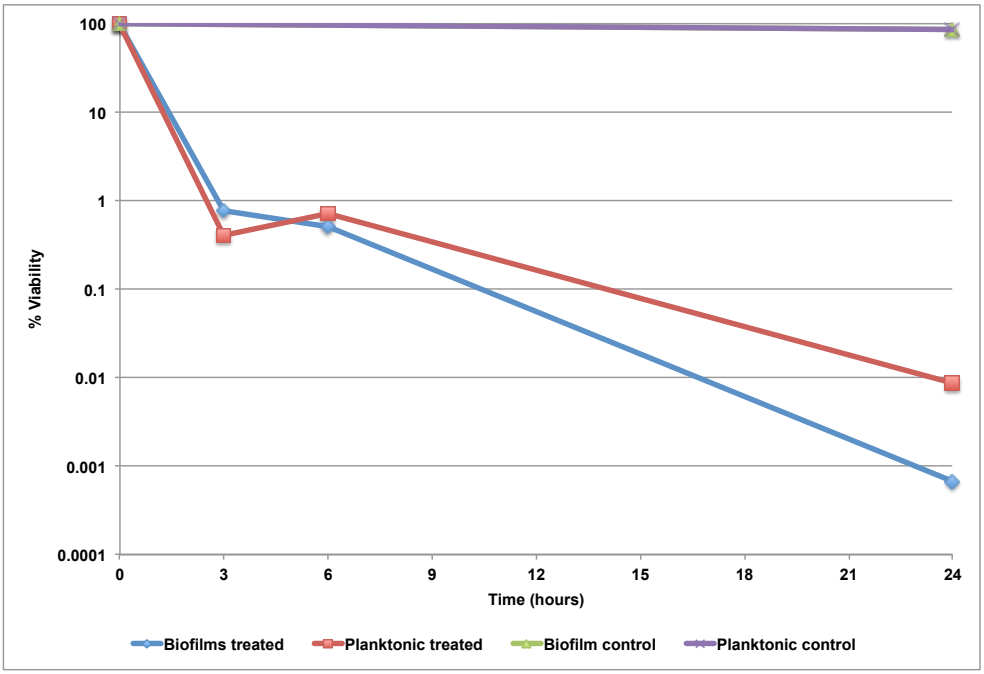


Figure 2. *E. coli* biofilms exposed to 50% Biocidin® for a period of 24 hours. At 24 hrs, most of the biofilm and planktonic populations were eradicated.

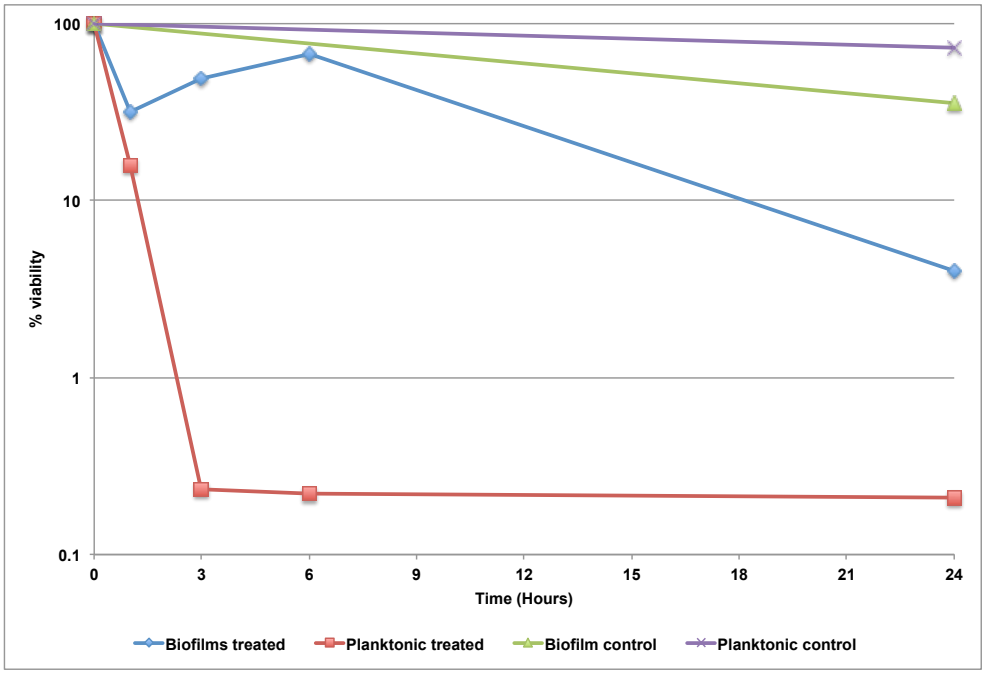


Figure 3. *K. pneumoniae* biofilms exposed to 25% Biocidin® for a period of 24 hours.

Biofilms of *C. albicans* were also exposed to Biocidin® but to 25% instead of 50% for a period of 24 hrs. However, instead of solely monitoring cell viability on agar plates (Fig. 5), I used a live/dead stain (Invitrogen) and monitor the Biocidin® efficiency using confocal microscopy. Images were taken at 200x magnification zoom 5 using a confocal scanning laser microscope (Fig. 4). In addition, I analyzed the images using the COMSTAT image analysis software (Table 3) and Luminance Analyzer v1.0 (written at Binghamton University) (Fig. 6) for fluorescence intensity measurements.

Cell viability was reduced by >99% in the bulk-liquid population and by >90% in the biofilm population (Fig. 5). A significant reduction was observed in biofilm thickness, on the total biomass and surface area of biomass (Table 3). Exposure to Biocidin® resulted in a decrease of live cells or membrane non-impaired to 50%, from an original 80% live stained population (Fig. 6).

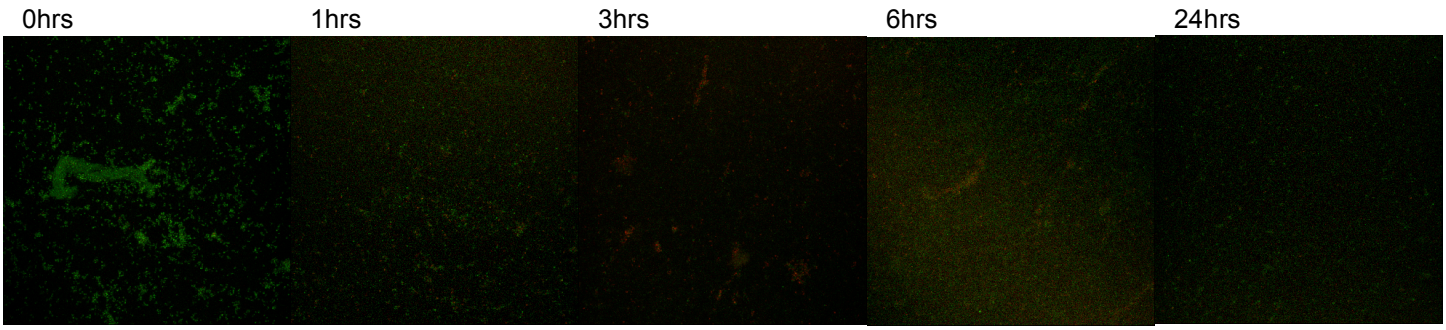


Figure 4. *C. albicans* biofilms exposed to 25% Biocidin® for a period of 24 hours

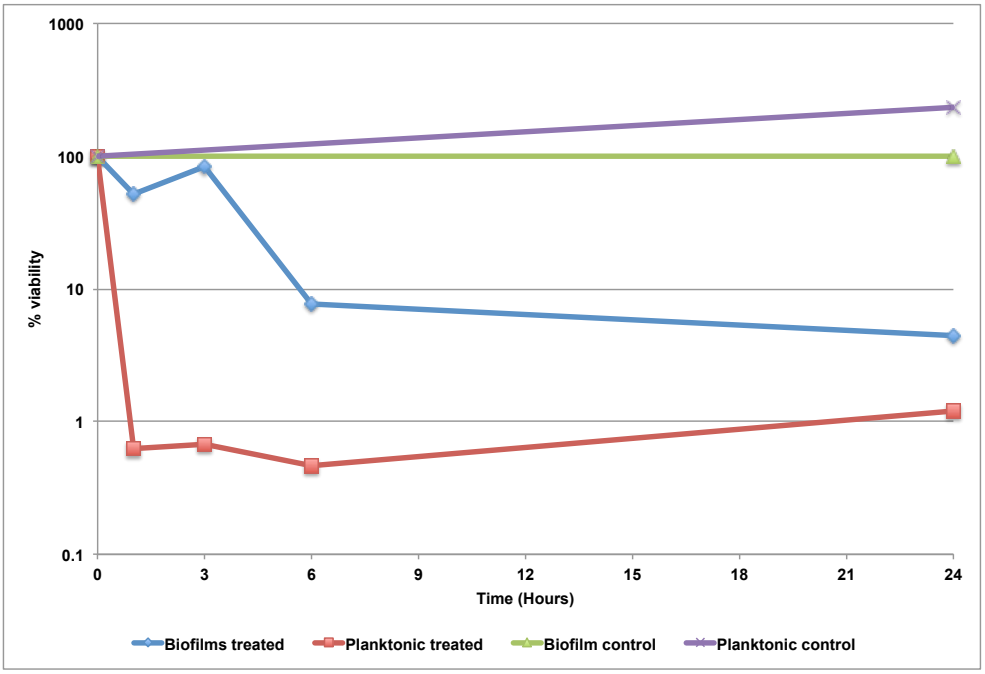


Figure 5. *C. albicans* biofilms exposed to 25% Biocidin® for a period of 24 hours.

Table 3. COMSTAT analysis of CSLM images of *C. albicans* exposed to Biocidin for a period of 24 hrs.

	0hrs		**1hrs**		**3hrs**	
	average	sd	average	sd	average	sd
Total biomass (µm^3/µm^2)	0.1794	0.0747	0.0093	0.0122	0.0127	0.0138
Portion of slice occupied by bacteria (%)	1.82	1.24	0.45	0.66	0.71	0.80
Average thickness (µm)	0.3398	0.1125	0.0047	0.0053	0.0052	0.0048
ughness coefficient (dimensionless, range: zero-infinit	1.9328	0.0358	1.9973	0.0037	1.9972	0.0029
Surface area of biomass in this image stack (µm^2)	2.53E+05	1.16E+05	1.70E+04	2.29E+04	2.39E+04	2.62E+04
Surface to biovolume ratio (µm^2/µm^3)	2.34	0.16	2.94	0.28	2.96	0.25
Maximum thickness (µm)	33.92	7.41	12.50	4.27	15.63	2.27
	6hrs		**24hrs**		**24hrs control**	
	average	sd	average	sd	average	sd
Total biomass (µm^3/µm^2)	0.0074	0.0041	0.0027	0.0019	0.4839	0.2558
Portion of slice occupied by bacteria (%)	0.48	0.32	0.19	0.13	4.43	1.98
Average thickness (µm)	0.0013	0.0016	0.0003	0.0004	1.0053	0.5021
ughness coefficient (dimensionless, range: zero-infinit	1.9991	0.0010	1.4998	0.9999	1.8313	0.0972
Surface area of biomass in this image stack (µm^2)	1.42E+04	8.40E+03	5.28E+03	3.73E+03	6.36E+05	4.44E+05
Surface to biovolume ratio (µm^2/µm^3)	3.19	0.29	3.31	0.13	2.56	0.23
Maximum thickness (µm)	7.98	5.21	2.01	2.25	39.79	4.20

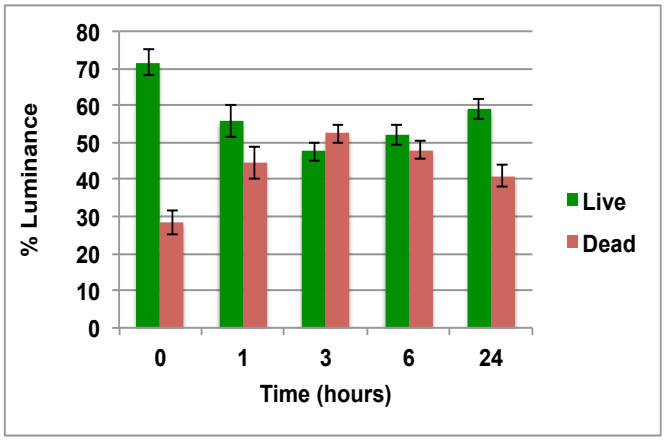


Figure 6. *C. albicans* biofilms exposed to 25% Biocidin® for a period of 24 hours stained with live/dead stain, observed by CSLM and analyzed using Luminance Analyzer v1.0

Overall, Biocidin® seems to be extremely effective against planktonic microbial populations at concentrations of 25% or above. Biofilm populations are more resilient and an increased concentration of Biocidin® needs to be used to be as effective. Although 90% of cells in both planktonic (bul-liquid) and biofilm populations of all microorganisms tested are killed with 25% Biocidin following 4 hrs of treatment. Continuous exposure to Biocidin® leads to a significant decrease of cell viability. Upon exposure to Biocidin® (50%) for a period of 24 hrs biofilm and planktonic population cell viability, of *E. coli* and *P. aeruginosa,* is reduced to the point of eradication. In addition, exposure of either *K. pneumonia* or *C. albicans* to 25% Biocidin® for a period of 24 hrs leads to a significant cell viability reduction, of the planktonic population, although not to the point of eradication.

The next phase of our study will determine the effectiveness at reduced concentrations over 5-7 day periods.

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