"THE BIOFILM REVOLUTION"

BOZEMAN SYMPOSIUM

June 19 – 21, 2003

CENTER FOR BIOFILM ENGINEERING

MONTANA STATE UNIVERSITY

BOZEMAN, MT

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DR. LAWRENCE FUNT
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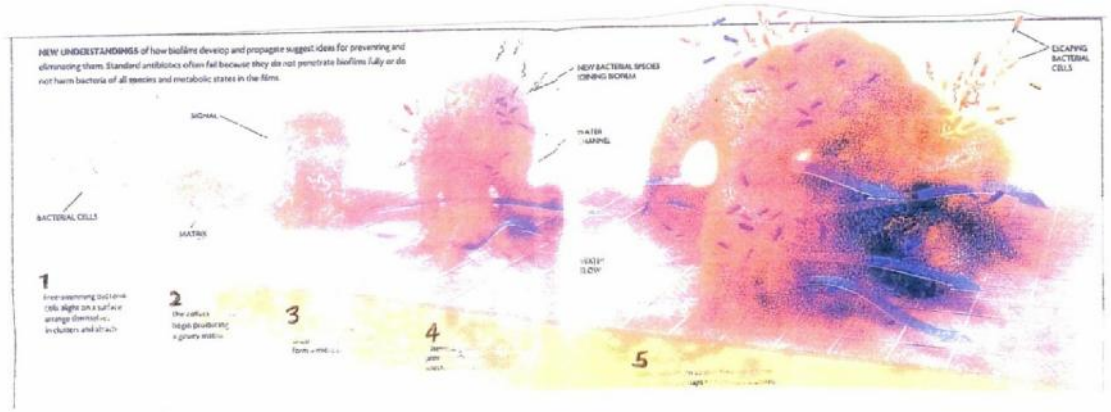
MONTANA STATE UNIVERSITY CHAIRMAN:
J. WILLIAM COSTERTON, PhD

Report of Studies Using Direct Observation to Prove the Efficacy of EXXCL Oral Blue Antimicrobial Technology in Killing Both Planktonic and Biofilm Organisms

“THE BIOFILM REVOLUTION”

HOW BIOFILMS FORM

NEW UNDERSTANDINGS OF HOW BIOFILMS DEVELOP AND PROPAGATE SUGGEST IDEAS FOR PREVENTING AND ELIMINATING THEM. STANDARD ANTIBIOTICS OFTEN FAIL BECAUSE THEY DO NOT PENETRATE BIOFILMS FULLY OR DO NOT HARM BACTERIA OF ALL SPECIES AND METABOLIC STATES IN THE FILMS.



1. **Free-swimming bacterial cells alight on a surface, arrange themselves in clusters, and attach.**
2. **The collected cells begin producing a gooey matrix.**
3. **The cells signal one another to multiply and form a micro-colony.**
4. **Chemical gradients arise and promote the coexistence of diverse species and metabolic states.**
5. **Some cells return to their free-living form and escape, perhaps to form new biofilms.**

Table of Contents

REPORT

Title : THE EFFICACY OF COMPOUND A IN KILLING PLANKTONIC AND BIOFILM CELLS, USING MODERN METHODS SUITABLE FOR FDA SUBMISSIONS.

Prepared for Dr. Larry Funt, Coalition of Health-Care Professionals

Respectfully Submitted :

J. William Costerton, PhD., Consultant

Experimental work conducted by Bacterin Inc., Guy Cook, President

Dated : 05.28.01

Abstract of Report #1

Prologue

"The pivotal difficulty in the traditional methods (*used for determining the efficacy of antimicrobial agents in killing bacteria growing in biofilms*) is their reliance on the "colony-forming-units" (CFU) that produce colonies on agar, after the sessile (biofilm) population has been removed from the surface and separated (as much as possible) into individual cells. This removal is incomplete, and the separation steps are inadequate, so that efficacy data based on "scrape-and-plate" culture methods are misleading and tragic consequences have already affected hundreds of recipients of medical devices incorrectly characterized as being colonization resistant."

"Fortunately, at a time when biofilms are being recognized as being responsible for all device-related infections and fully 65% of all bacterial infections (Costerton, et al., 1999), the scientists and engineers of the Center for Biofilm Engineering (CBE) have developed new technologies. These new methods, which are made possible by the superior resolving power of the Scanning Confocal Laser Microscope (CSLM), are unequivocal because they rely on direct observations and avoid the extrapolations traditionally used in this field. The FDA has recently invited several proponents of these new direct technologies to Washington, D.C. to help the agency draft new guidelines for the assessment of the efficacy of antibacterial agents in killing biofilm bacteria."

Aim of The Present Study

"The Coalition of Health-Care Professionals (COHP) already has data proving that its Compound A kills planktonic cells of many bacterial species. This study was designed to use the new methods of direct observation, to determine whether this agent kills biofilm cells with similar efficiency."

Results

"When we exposed *de facto* biofilms of 8 bacterial species, and 1 fungal species, to Compound A we obtained complete killing of the sessile cells in all cases." (these species included: E. coli, C. albicans, E. cloacae, E. faccalis, K. oxytoca, K. pneumoniae, P. aeruginosa, P. mirabilis, and S. aureus) "All of these preparations of *de facto* biofilms from static liquid cultures contained some planktonic cells, that had also settled onto surfaces, and these direct observations confirm the traditional microbiological data that show that Compound A kills planktonic cells of all these species."

Conclusions

"Certain conclusions can be based strictly on the data presented in this report. These include confirmation that Compound A is effective in killing planktonic cells of all of the 8 bacterial and one fungal species included in these studies. These data also show that Compound A kills sessile cells of all these species when they are growing in aggregates in liquid media, which constitute *de facto* biofilms in that they are present in slime-enclosed masses... The agent killed sessile cells, even when they were growing in the middle of very substantial aggregates.... These data indicate that Compound A is effective in killing bacteria in aggregates derived from biofilms, such as the aggregates that are released by mechanical cleaning of surfaces (teeth or pipes) that are heavily colonized by bacterial biofilms. We test a very large number of putative anti-biofilm biocides, and the only agents that have been successful against these entrenched organisms have been oxidizing compounds whose use in the medical context is contraindicated."

"The principals of COHP have shown me data from other sources, whose origins I trust and whose methods I recognize, and I can reach another broader set of conclusions when I combine these data with those in this report. I believe that Compound A has excellent efficacy in killing planktonic bacteria and yeast, of virtually all species. I believe that this agent also has a unique capability of killing sessile bacteria and yeast in aggregates that constitute *de facto* biofilms, and that provide complete protection from killing by most of the anti-biofilm agents we have tested. I think that the agent is bacteriacidal, in itself, but that it also exerts a physical effect on biofilms, because it often changes the state of the biofilm within which the target organisms are living, and rolls it up or fragments it."

Title: The Killing and Solubilizing Effects of Solution A on Homogeneous and Heterogeneous Planktonic Organisms, Sessile Organisms, Biofilm Organisms, and Biofilm Structures

Submitted by: Beierle Lab Technologies
John W. Beierle, Ph.D.,
Consultant

From March 18, 2001 to June 25, 2001

Abstract of Report #2

Introduction to the Problem

"Biofilms have been recognized as moderate to heavy aggregated masses of bacteria for over a hundred years. The "biomats" coating on sand filters of drinking water supplies were noted on Germany in the 1800's. Very slowly mankind began recognizing biofilms in their numerous varieties and forms."

"Eventually recognition, discovery, and problem solving began. This is what is occurring in the world of biofilms" (today). The intricate interactions of microbes within the biomass allow for communication between single celled creatures, which exposed the dogma of simplicity relating to bacterial interactions. Controlling biofilms is now a recognized major problem. To address the biofilm problem, we utilized Solution A, a proprietary mixture with remarkable antimicrobial properties. These properties extend beyond killing free floating or planktonic cells and indeed have disruptive killing and even solubilizing effects on sessile cells within biofilms."

The studies described here were defined with both single homotypic cells as well as mixture(s) of multiple or heterotypic cell types under a variety of conditions."

Materials and Methods

"Oral bacteria were obtained from clinical isolates and never use past the seventh passage. Other bacteria were obtained from the American Type Culture Collection (Virginia). Oral flora was obtained from patients with heavy plaque and/or periodontitis. Samples were obtained from plaque, saliva, and sub-gingival sources. No attempt was made to discriminate each individual species."

Results

"Petri dish biofilms were made up of homogeneous, and heterogeneous, mixed oral flora. Biofilm buildup increased day by day and by the end of two days, an extensive mass was noted... exposure of Solution A to the two day mixed culture biomass showed an extensive breakup of the adherent mixed culture mass."

"Debris was centrifuged into pellet and resuspended in media in a test tube. The tubes were incubated for five days and no growth was detected demonstrating that complete destruction of all living forms occurred along with destruction and solubilizing of the biofilm masses of extracellular material (ECM) in which the bacteria were imbedded.

Summary Statement

"These studies reveal that Solution A rapidly and effectively demonstrated its complete destructive capacity of the ECM holding the mass together, and the kill of the bacteria within that mass of gelatinous material."

"The destruction of total biofilms is so extensive that only a few globules of material are left... Collecting debris by centrifugation and resuspending the disrupted cell matrix pellet in (1) enriched media and (2) media containing purple broth base was performed for growth analysis to detect any viability. No growth was noted even after 10 days incubation in either media. The purple broth based color indicator media is so sensitive that as little as 100 microbes initial innoculum would be detected in as little as 12 hours, whereas no growth was detected after 250 hours incubation."

Conclusion

"Solution A is effective in killing both sessile and planktonic cells rapidly and effectively. Furthermore, Solution A kills cells within the biofilm mass and destroys the biofilm extracellular matrix both rapidly and efficiently, Solution A is a uniquely effective means of biofilm kill and degradation on homogeneous and heterogeneous sessile biofilm organisms and biofilm structures."

Cytotoxic Effect of an Anti-Microbial Compound on cultured murine cells.

Report submitted to

John W.Beicrle,Ph.D.
Microbiologist

by

Jamuna Ramnath,Ph.D.
Cell Biologist/Molecular Biologist
&
Joseph R. Landolph, Ph.D.
Molecular Biologist/Genetic Toxicologist

Abstract of Report #3

Purpose

"To determine the cytotoxic activity of a proprietary anti-microbial compound [AMC] (*Formula A*) on cultured mammalian cells, we used (a) non-transformed... mouse embryo fibroblast cell line."

Conclusion

"Treatment of the (cultured mammalian cells) with AMC at .01% and lower final concentrations for 18 hours did not affect the growth and monolayer morphology of (the cultured mammalian) cells".

Note: This is an extremely sensitive cytotoxicity test using an extremely sensitive single cell-thickness mouse embryo fibroblast cell line. For our purposes this means that Formula A is non-toxic. This statement is not part of the above report and is for informational purposes only.

Confocal Introduction

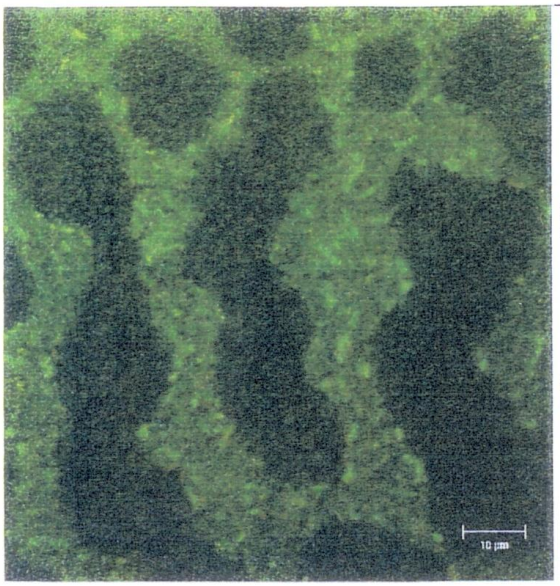
The Confocal Microscope has opened up the mysterious world of biofilms like nothing before. Biofilms have three-dimensional shape and are best observed on opaque surfaces. Previous to the development of the Confocal system the Scanning Electron Microscope (SEM) was the standard for biofilm research and observation. Using the SEM the biofilm samples necessarily had to be compressed and displayed on transparent material, usually glass slides. This resulted in one dimensional, dead samples.

With the Confocal system living biofilms can be observed in real time, virtually undisturbed, against opaque backgrounds. The result is a far more accurate depiction of the real world of biofilms allowing scientists and engineers to observe these living biofilm organisms in their complex and fascinating three-dimensional communities.

The value of the Confocal system in observing, quantifying, and understanding biofilm bacteria and their polysaccharide matrix is beyond question. At Montana State University this system has been employed for years at the Center for Biofilm Engineering. This is the system that was used to produce the images you see here.

The uppermost images on each page are the living biofilm bacteria communities in each sample (shown in green). The lower images show the complete bacterial kill and destruction of the biofilm mass that was the result of introducing Formula A into each sample (shown in red). These colors are indicators of the condition of the samples, green is living and red is dead. The radically changed shape of the biofilm masses between the upper and lower images is a graphic example of the destruction of the biofilm mass itself.

We are very pleased to be able to demonstrate the effectiveness of our formulation using the Confocal system at the Center for Biofilm Engineering under the supervision of Dr. J. William Costerton, Director. This is a small sample of typical biofilm bacteria and biofilm mass reactions after exposure to Formula A.



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MONTANA STATE UNIVERSITY
BOZEMAN

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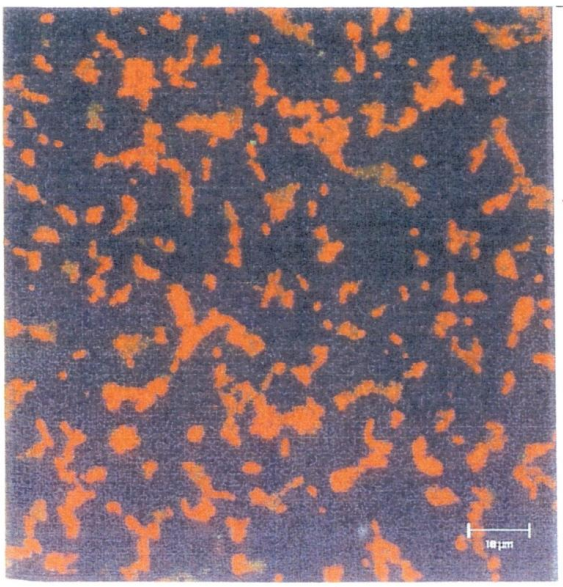
Klebsiella oxytoca – UTI - control



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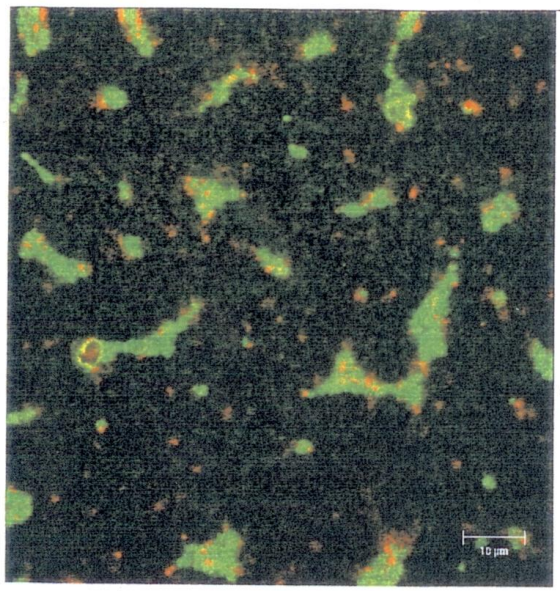
Klebsiella oxytoca – UTI – Compound A



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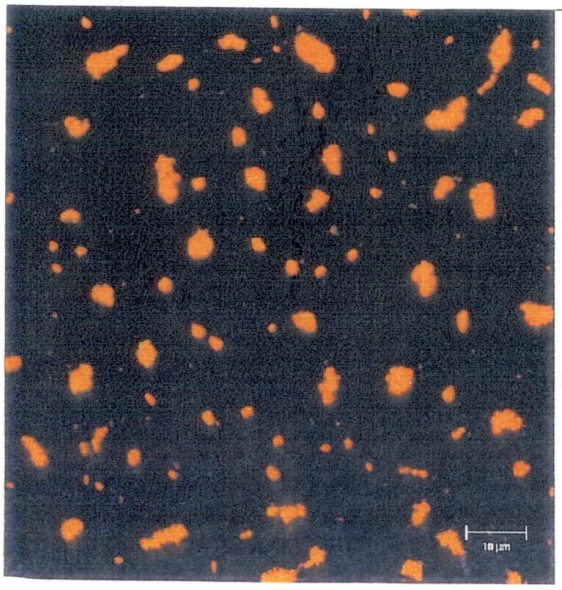
Objective	UV 40x1.25NA oil PL APO 1.25-0.75	

E. Faecalis – UTI – Control



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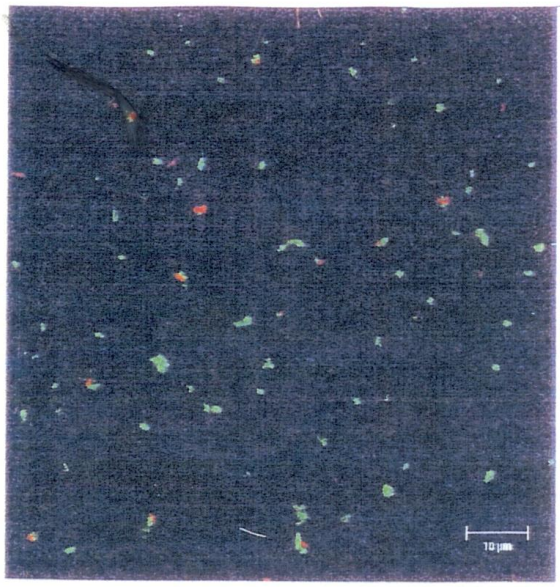
E. Faecalis – UTI – Compound A



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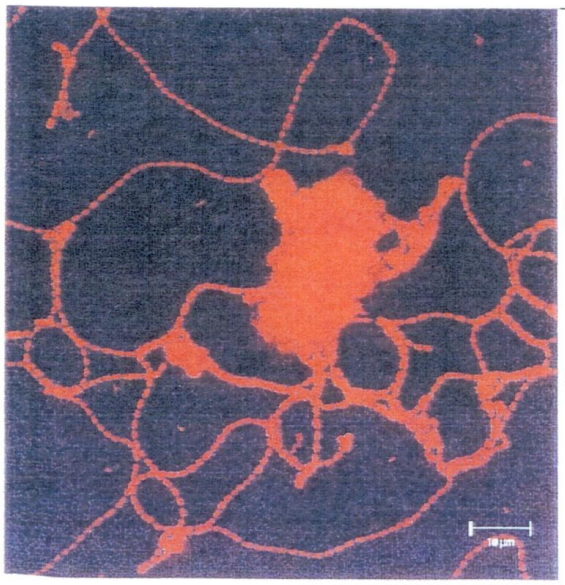
PMTTrans	100	V
Offset1	2	-
Offset2	2	-
Offset3	0	-
OffsetTrans	0	-
Pinhole dig	21	-
Scalebar	10 µm	
Z-Pos	-0.250	µ
Method	1 Live/Dead	
Objective	UV 40x 1.25NA oil PL APO 1.25-0.75	

E. clocae – Environemental Isolate – Dialysis Pathogen - Control



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MONTANA STATE UNIVERSITY
BOZEMAN

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PMTTrans	100	V
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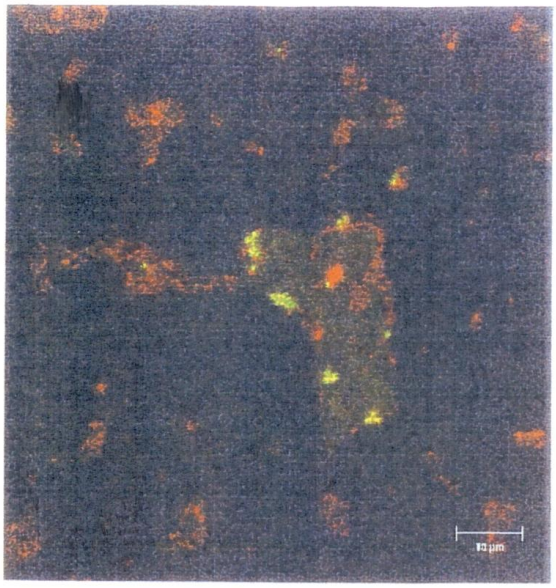
Offset3	0	-
OffsetTrans	0	-
Pinhole dig	28	-
Scalebar	10 µm	
Z-Pos	0.507	µ
Method	1 Live/Dead	
Objective	UV 40x 1.25NA oil PL APO 1.25-0.75	

E. clocae – Environemental Isolate – Dialysis Pathogen – Compound A



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OffsetTrans	0	-
Pinhole dig	35	-
Scalebar	10 µm	
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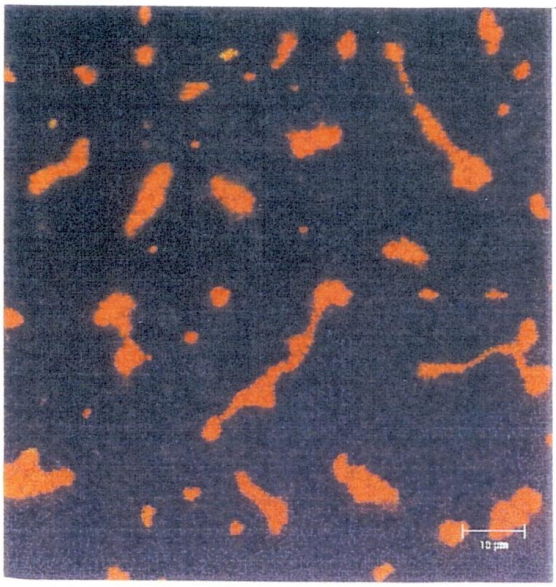
Method	1.Live/Dead	
Objective	UV 40x1.25NA oil PL APO 1.25-0.75	

C. albicans – Clinical Isolate – Control



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BOZEMAN

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OffsetTrans	0	-
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Scalebar	10 µm	
Z-Pos	-1.196	µ
Method	1.Live/Dead	
Objective	UV 40x1.25NA oil PL APO 1.25-0.75	

C. albicans – Clincal Isolate – Compound A

Graphs Introduction

John W. Beirle, PhD, Professor of Microbiology, School of Dental Medicine, University of Southern California produced this series of graphs. The planktonic microbes used are noted on each graphic presentation. These are representative of a much larger group of planktonic microbes that were tested against and in all cases we didn't find a resistant strain of microbes.

Dr. Beirle noted "The kill is usually so rapid at a 100 microliter addition that we can find 100% kill at less than 1 minute. We cannot run the assay any faster, but we feel that we are close to instantaneous kill."

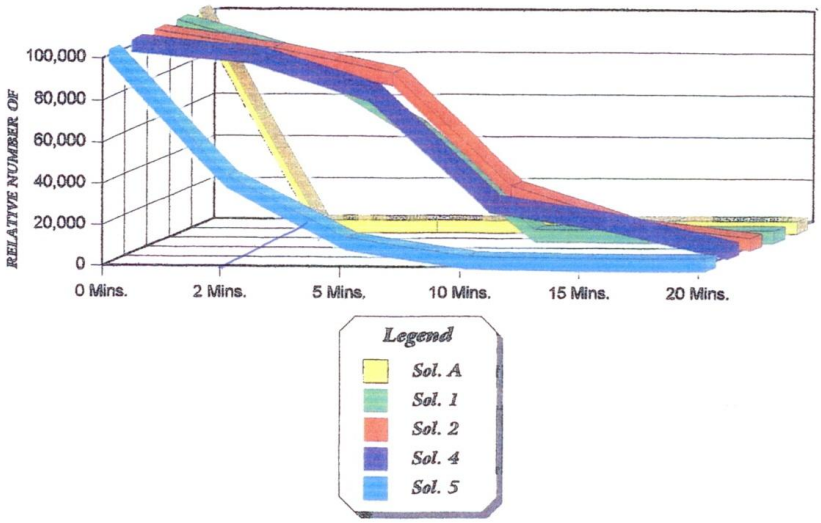
In all these graphs Formula A is represented by the yellow or gold color lines. Formula A is the active ingredient in Exxcl Oral Blue Oral Irrigant and Squeaky Clean Dental Cleanser, which you have been using for some time to effectively treat periodontitis, gingivitis, and the effects of xerostomia.

These graphs show Formula A's ability to very quickly and completely kill a variety of planktonic pathogenic organisms. In combination with the preceding Confocal images of Formula A's devastating effects on biofilm bacteria and their matrix material, Formula A's unique ability to completely kill planktonic as well as biofilm bacteria has been established beyond doubt. Formula A is the only compound, which is safe enough to use on humans that can provide anything near these results.

Note: Soultions 1,2,4, and 5 are other proprietary compounds, which were tested simultaneously with Formula A using the same subjects and process.

SUMMARY GRAPH

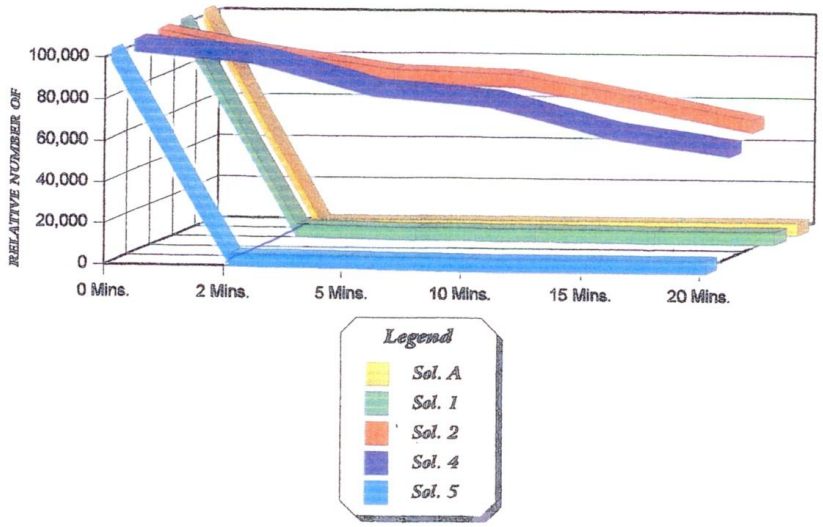
PSEUDOMONAS AERUGINOSA



3 min.

SUMMARY GRAPH

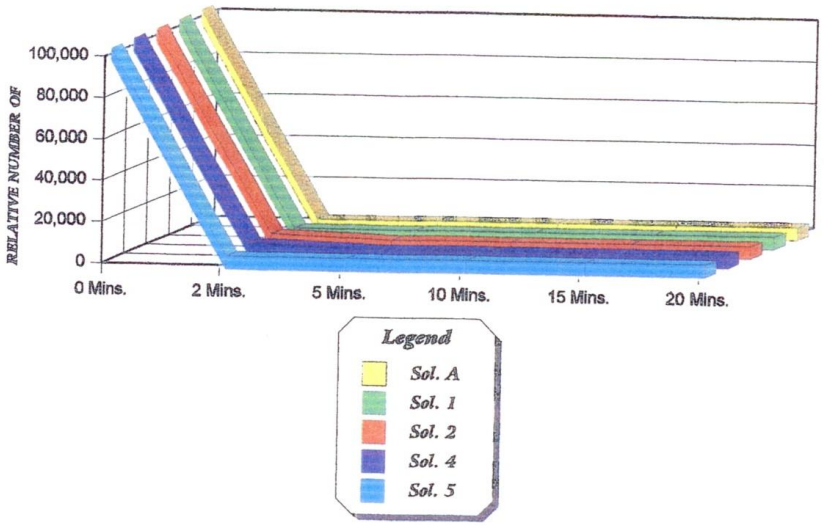
CANDIDA ALBICANS



2 min.

SUMMARY GRAPH

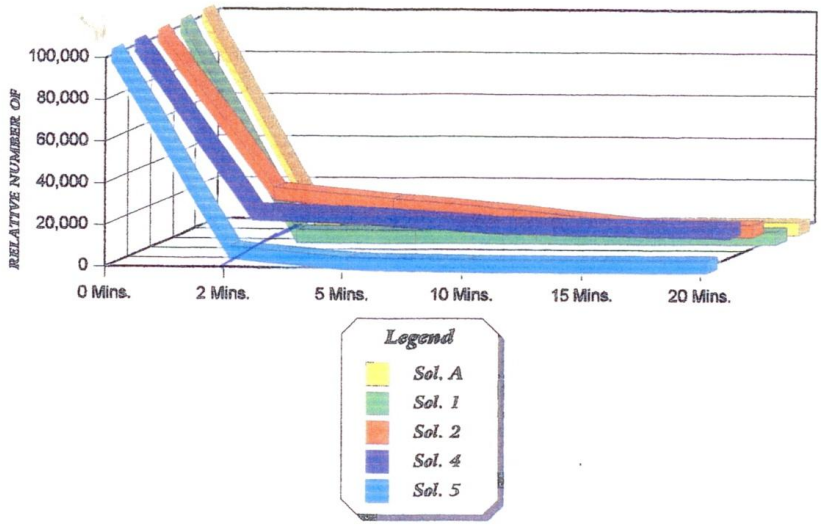
E. COLI



2 min 30 sec

SUMMARY GRAPH

STEAROTHERMOPHILUS



2 min 15 sec.

1/02 Fiant

MICROORGANISMS TESTED AGAINST FORMULA A

Bacteria – Fungi – Protozoa – Spores – Virus

Bacteria

K.oxytoca
S. aureus
P. vulgaris
B. subtilis
K. pneumoniae
S. sonnie
S. maracens
B. strep
C. trachomatis
S. epidermidis
E. coli
P. aeruginosa
E. feccalis
P. mirabilis
M. polymorpha
H. vaginicola
S. viridans
E. clocae

Fungi

C. albicans
T. mentagrophytes
S. cerevisiae

Protozoa

T. vaginalis
E. cerevisiae

Virus

Herpes simplex

Spores

Bacillus Stearothermophilus (two strains)

All subjects were killed on contact with Formula A except the spores, which took less than 10 minutes at ambient temperature to expire after exposure to Formula A