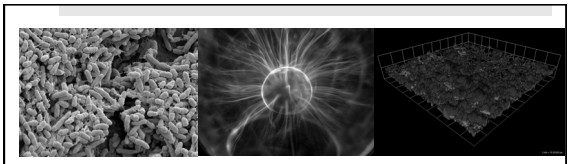
Antibacterial Efficacy of Atmospheric Pressure Non-Thermal Plasmas

Prof. Brendan Gilmore, Queen's University, Belfast, Ireland

The A. Denver Russell Memorial Teleclass Lecture for 2014



Antibacterial Efficacy of Atmospheric Pressure Non-Thermal Plasmas

A. Denver Russell Memorial Teleclass Lecture 2014

Brendan F. Gilmore
Queen's University Belfast

Hosted by Prof. Jean-Yves Maillard
University of Cardiff, Wales

www.webbertraining.com April 10, 2014

The Biofilm Theory

- First Proposed in 1978 in a publication in Scientific American "**How bacteria stick**" (Bill Costerton, 1934 – 2012)
- Earlier observations by van Leeuwenhoek (1684), Henrici (1933) and Zobell (1943)
- Studying bacteria in natural ecosystems, such as mountain streams
- Engineered systems
- Medical Microbiology

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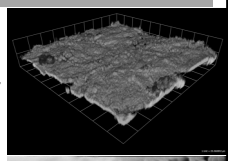
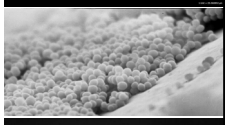
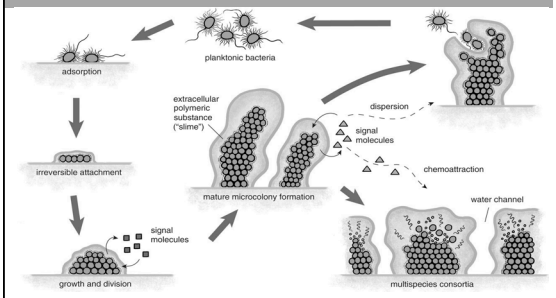
Biofilm & Infection Control - Overview

- Biofilms represent the predominant mode of growth of microorganisms in
  - natural ecosystems
  - & in chronic diseases of humans, animals, invertebrates and plants
- NIH estimate that 80% of human chronic infections involve microbial biofilms
- Once formed, biofilms are
  - up to 1,000 times more tolerant to antimicrobial challenge
  - Resist normal mechanical clearance and phagocytosis
- Biofilm-mediated, indwelling medical device-related infections account for 50% of all hospital acquired infections in the UK





Biofilm & Infection Control - Overview



Harrison, J.J., Turner, R.J., Marques, L. and Ceri, H., *Am. Sci.* 93: 507-515, 2005

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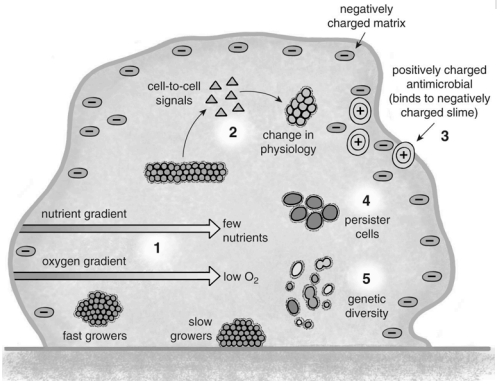

Harrison, J.J., Turner, R.J., Marques, L. and Ceri, H American Scientist 93: 507-515, 2005

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Bad Bugs, No Drugs: No ESKAPE!
An Update from the Infectious Diseases Society of America, January **2009**

"The Infectious Diseases Society of America (IDSA) continues to view with concern the lean pipeline for novel therapeutics to treat drug-resistant infections, especially those caused by gram-negative pathogens. Infections now occur that are resistant to all current antibacterial options."

ESKAPE = *Enterococcus, Staphylococcus, Klebsiella, Acinetobacter, Pseudomonas,* and *Enterobacter*

As Antibiotic Discovery Stagnates ... A Public Health Crisis Brews
Infectious Diseases Society of America, July **2004**

"Infectious diseases physicians are alarmed by the prospect that effective antibiotics may not be available to treat seriously ill patients in the near future. There simply aren't enough new drugs in the pharmaceutical pipeline to keep pace with drug resistant bacterial infections, so-called 'superbugs.'"

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Plasma – An Overview

- Plasma is the regarded as the fourth state of matter which is similar to the gaseous state but with certain degrees of ionisation and a higher energy content.
- Produced on laboratory scale by flowing gas through an electric field which drives the ionization, excitation and dissociation of gaseous molecules.
- This produces high densities of reactive oxygen and nitrogen species (RONS), charged particles (ions and electrons), radiation (from UV to IR), and electro-magnetic fields.

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Non-Thermal Plasma

- Thermal plasmas have for many years been used in sterilization of medical equipment, packaging, implants
- Advantages include rapid bactericidal activity and access to narrow/confined recesses
- Recently atmospheric pressure, low temperature ('non-thermal' or 'cold') plasmas have been developed
- Typically less than 40°C at point of application
- Capable of delivering unique reactive dry chemistry at ambient temperatures to delicate surfaces – potentially viable tissues
- This has given rise to the emerging field of 'Plasma Medicine'

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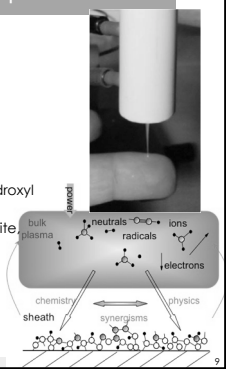
Plasma – reactive species

- Variable, tunable according to input gas admixture
- Base gas usually Helium or Argon
- With varying % air/nitrogen/oxygen
- Gives rise to:
  - Reactive Oxygen Species: ozone, atomic oxygen, single delta oxygen, peroxide, hydroxyl radicals
  - Reactive Nitrogen Species: nitric oxide, nitrite, nitrate, peroxynitrite
  - Neutral species



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Overview: Why APNTP?

- Provides highly reactive environment at ambient temperature and pressure.
- Tunability of plasma chemistry which makes it possible to optimise for different applications
- Low capital and operational cost.
- Personnel and environment friendly.
  - Utilisation of virtually non-toxic gases (He, Ar , O_2, N_2)
  - Absence of harmful residues.
- Multiple conformations (power input, electrode configurations, plasma geometry)

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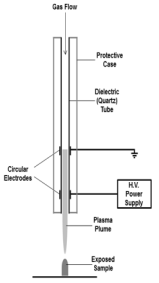
Plasma Source Configuration

- Non-thermal, non-equilibrium dielectric barrier discharge (DBD)-type plasma jet designed and manufactured in-house
- Quartz tube (6mm outer lumen diameter, 4mm lumen diameter)
- Two 2mm copper electrodes (2mm), separation distance 25mm
- High voltage pulse source operating at variable repetition of 20 kHz (and 40kHz for rate of biofilm kill comparison)
- Voltage amplitude of 6 kV applied to downstream electrode, positioned 5mm from end of the plasma tube
- Plasma operated with a gas mixture of 99-100% Helium, 0-1% Oxygen, flow rate 2 SLM into ambient air



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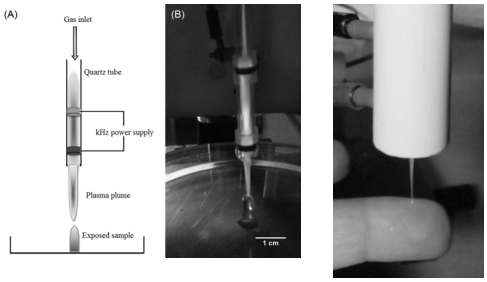
Overview: Plasma Jet



Schematic diagram (A) and photograph (B) of the plasma jet.

Alkawareek, M.Y., Algwari, Q.T., Laverty, G., Gorman, S.P., Graham, W.G., O'Connell, D. & Gilmore, B.F. 2012, *PLoS ONE* 7(8):e44289.

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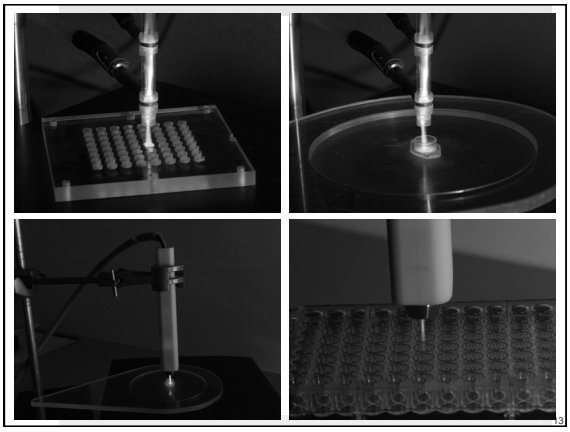
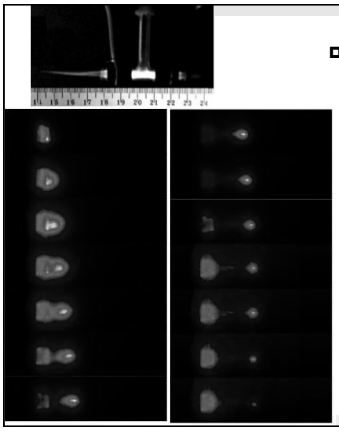
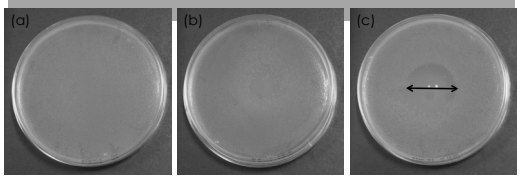




- time resolved ICCD images of the plasma jet emitted from the cylindrical dielectric barrier discharge jet illustrating individual plasma pulses on a nano-second timescale. The jet is operated in helium and the images are taken for a camera gate of 20 ns and delay of 40 ns between images.

Bacterial Growth Inhibition Zone



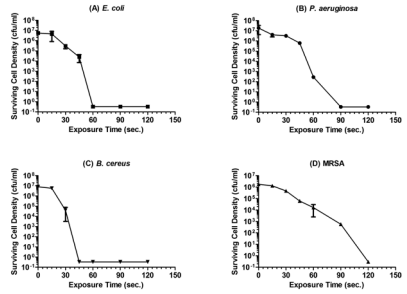
Photographs of *P. aeruginosa* seeded agar plates (9 cm in diameter) showing inhibition zones as a result of APNTP jet exposure for (a. 0 s), (b. 120 s), or (c. 240 s). [Alkawareek et al. 2012a]

Measured inhibition zone diameters of four bacterial species following plasma exposure.

Exposure time	Inhibition zone diameter (mm)			
(sec)	*B. cereus*	*S. aureus*	*E. coli*	*P. aeruginosa*
30	21	12	25	0
120	40	41	37	21
240	46	48	47	30

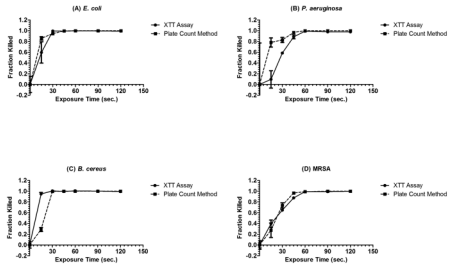
Alkawareek, M.Y. *et al.*, 2012, *FEMS Immunol. Med. Microbiol.* 65(2):381-4

Activity Against Planktonic Bacteria



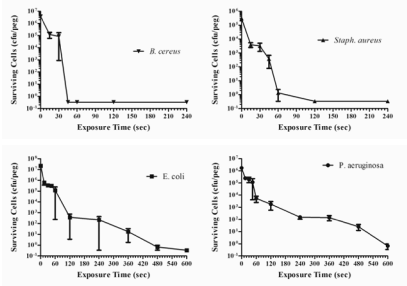
Survival curves for planktonic bacteria , suspended in PBS, of four bacterial species upon exposure to the 20 kHz atmospheric pressure plasma jet.

XTT Assay vs. Plate Count Method



Comparison between fraction cell killed values obtained using XTT assay and plate count method for the bacterial species in planktonic mode of growth.

Activity Against Bacterial Biofilms



Survival curves for 48-hour biofilms, grown on Calgary Biofilm Device, of four bacterial species upon exposure to the 20 kHz atmospheric pressure plasma jet.

Alkawareek, M.Y. *et al.*, 2012, *FEMS Immunol. Med. Microbiol.* 65(2):381-4

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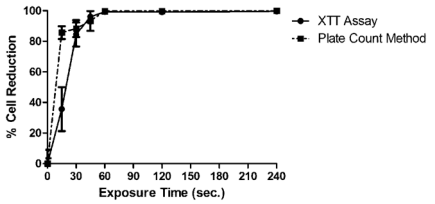
XTT Assay vs. Plate Count Method (*P. aeruginosa* Biofilm)



Comparison between % cell reduction values obtained using XTT assay and plate count method for *P. aeruginosa* biofilm

Alkawareek, M.Y., Algwari, Q.T., Laverty, G., Gorman, S.P., Graham, W.G., O'Connell, D. & Gilmore, B.F. 2012, *PLoS ONE* 7(8):e44289.

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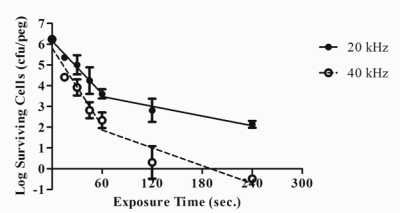
Effect of Frequency Variation



Log survival curves of *P. aeruginosa* biofilm cells upon exposure to a 20 kHz and a 40 kHz plasma jet.

Operating Frequency	D-Value (sec)	
	Phase 1	Phase 2
20 kHz	23.57	128.20
40 kHz	15.97	69.79

Alkawareek, M.Y., Algwari, Q.T., Laverty, G., Gorman, S.P., Graham, W.G., O'Connell, D. & Gilmore, B.F. 2012, *PLoS ONE* 7(8):e44289.

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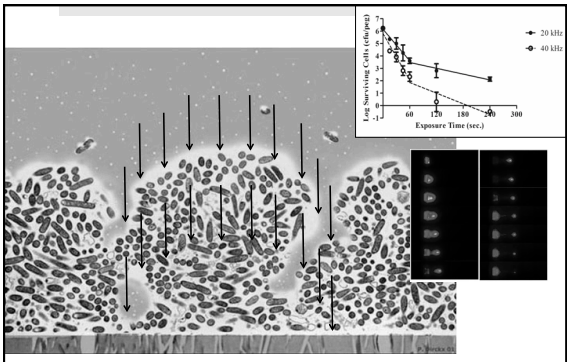

Image from Biofilms: The Hypertextbook © Cunningham, Rockford & Ross (Eds.) 2001 - 2010

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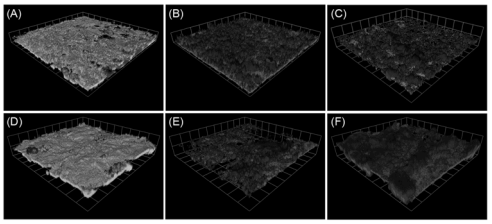
LIVE/DEAD Stain and Confocal Microscopy



3D rendered confocal laser scanning micrographs of *P. aeruginosa* biofilms exposed to the plasma jet for 0s (a and d), 60s (b and e), and 240s (c and f). Green color indicates surviving cells whereas red color indicates dead cells. Magnification power is 200x (a-c) and 600x (d-f).

Alkawareek, M.Y. *et al.*, 2012, *PLoS ONE* 7(8):e44289.

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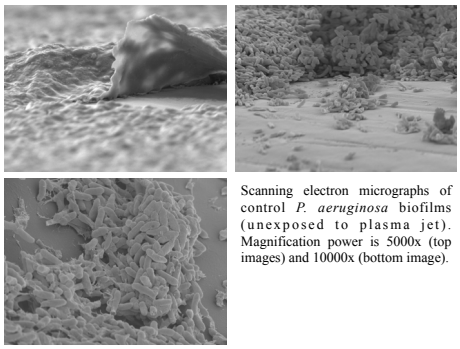

Scanning electron micrographs of control *P. aeruginosa* biofilms (unexposed to plasma jet). Magnification power is 5000x (top images) and 10000x (bottom image).

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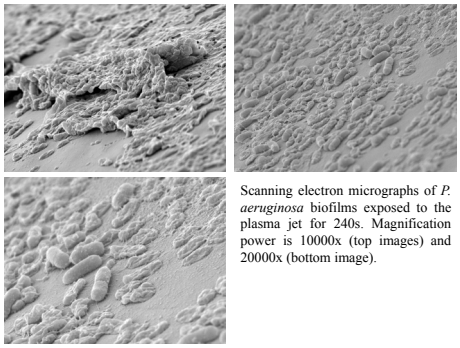

Scanning electron micrographs of *P. aeruginosa* biofilms exposed to the plasma jet for 240s. Magnification power is 10000x (top images) and 20000x (bottom image).

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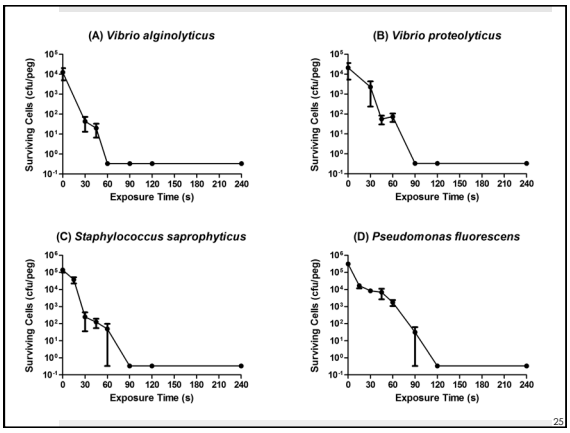

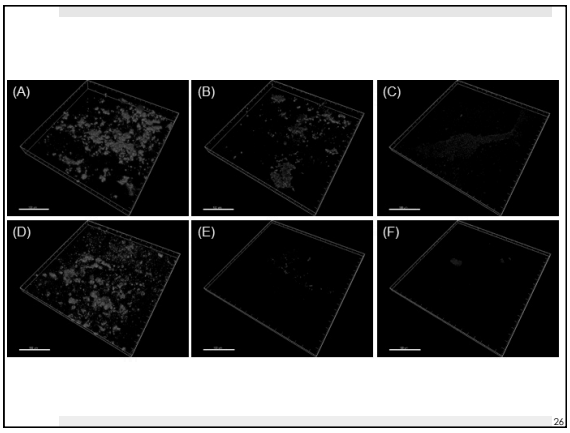

Cellular Targets & Mechanism

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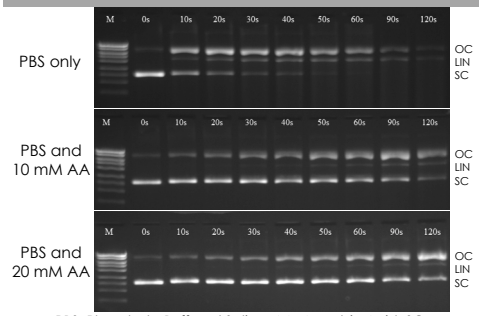
Effect on Plasmid DNA: Addition of AA



PBS: Phosphate Buffered Saline, AA: Ascorbic Acid, SC: Supercoiled, OC: Open Circular, LIN: Linear.

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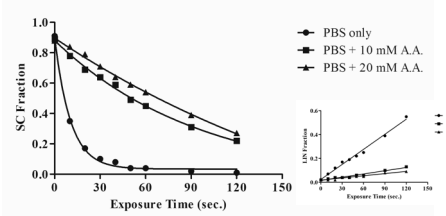
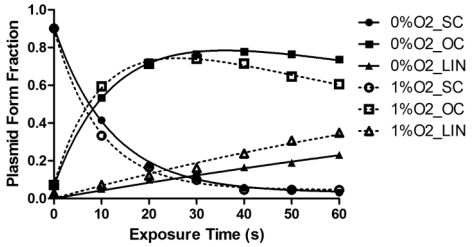
Effect on Plasmid DNA: Rate of SC Damage



Ascorbic Acid Conc. (mM)	Rate Constant (sec^{-1})	Half Life (sec)
0	0.0956	7.25
10	0.0114	60.79
20	0.0092	75.05

29

Effect on Plasmid DNA



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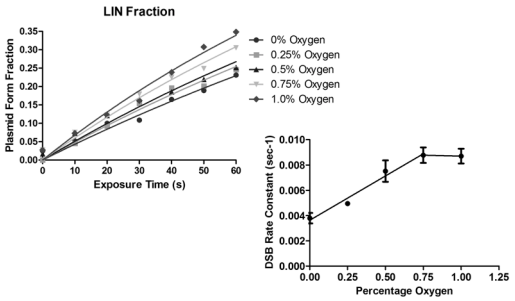

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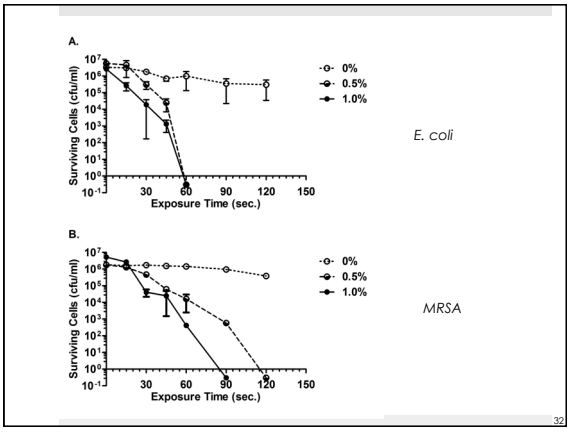

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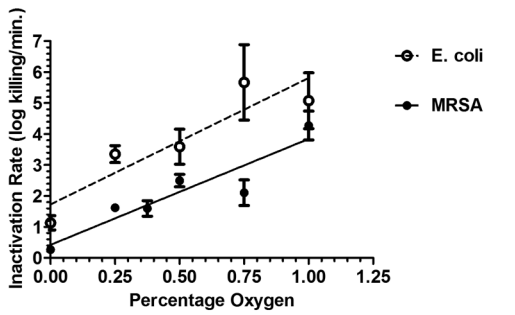
Influence of % O_2 on Inactivation Rate



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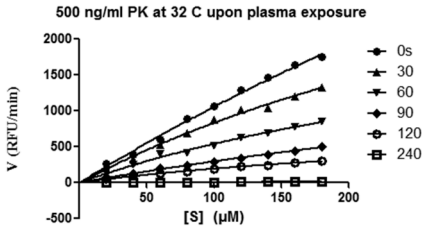
Effect on Proteinase K Activity: Michaelis–Menten Plot



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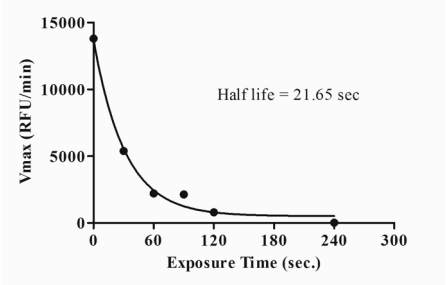
Effect on Proteinase K Activity: V_{max} Reduction



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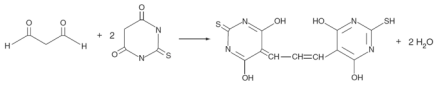
Lipid Peroxidation - TBARS Assay

- Malondialdehyde (MDA) is a product of the peroxidation of polyunsaturated fatty acids, usually caused by ROS.
- MDA reacts with two equivalents of thiobarbituric (TBA) acid to give a fluorescent red derivative that can be assayed colorimetrically or fluorometrically.
- MDA is a ROS itself and can form covalent adducts with proteins and purine deoxynucleosides (A&G) in DNA.



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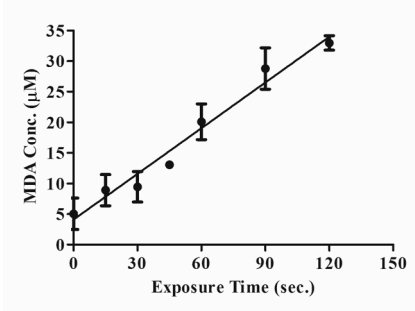
Lipid Peroxidation: MDA Concentration



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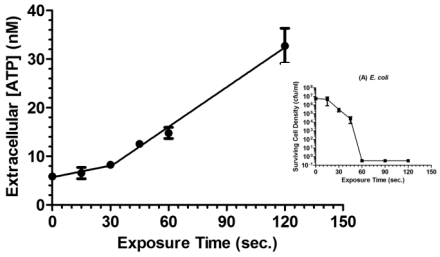
Leakage of Intracellular Components: Extracellular [ATP]



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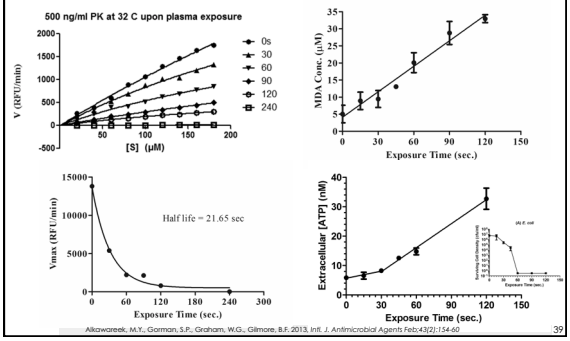
Cellular Targets of Non Thermal Plasmas



Alkawareek, M.Y., Gorman, S.P., Graham, W.G., Gilmore, B.F. 2013, Int. J. Antimicrobial Agents Feb;43(2):154-60

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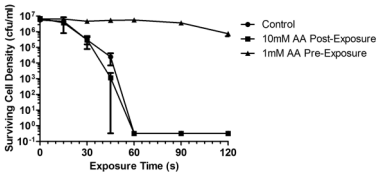
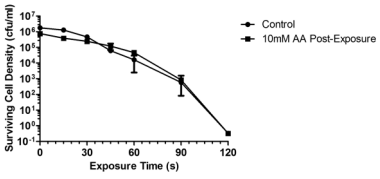
E coli + Ascorbic Acid



MRSA + Ascorbic Acid



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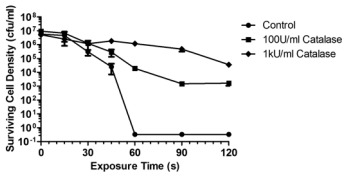
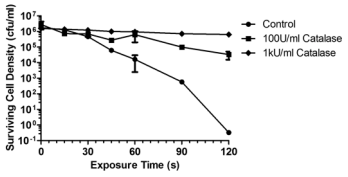
E coli + Catalase



MRSA + Catalase



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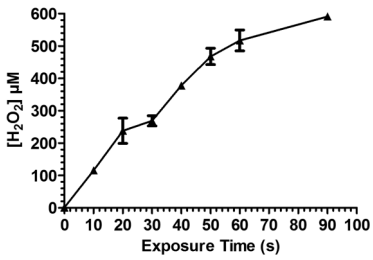
H_2O_2 Production in Plasma-Exposed PBS



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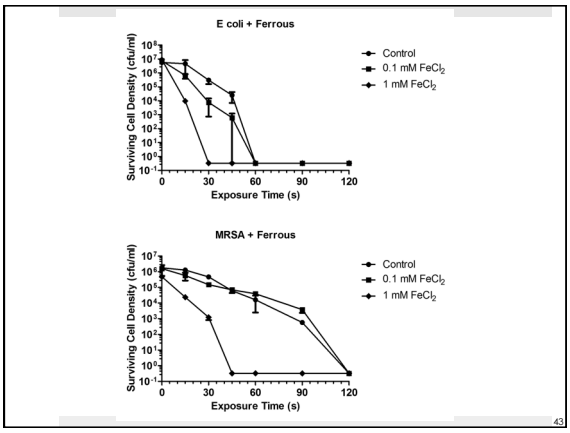

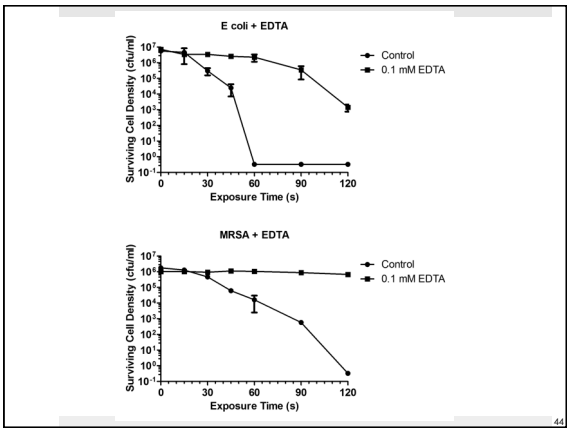

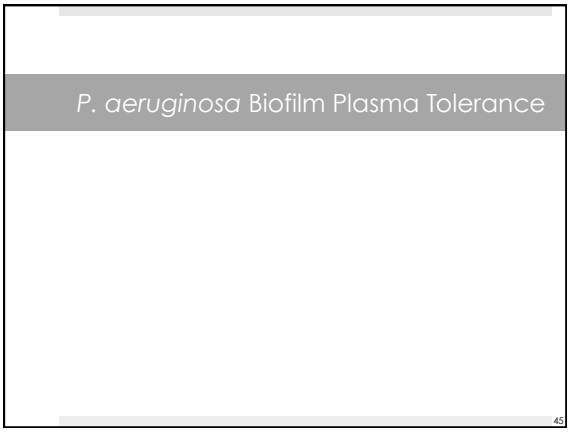

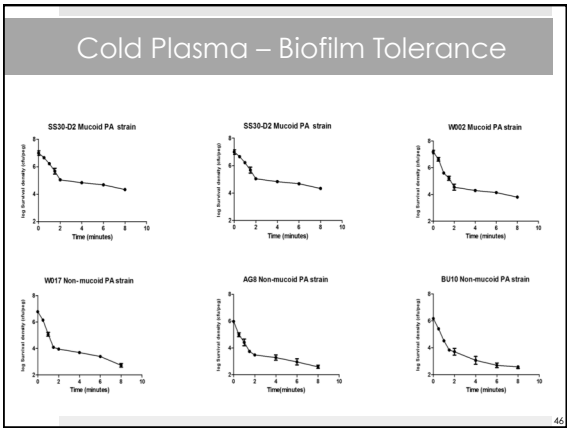

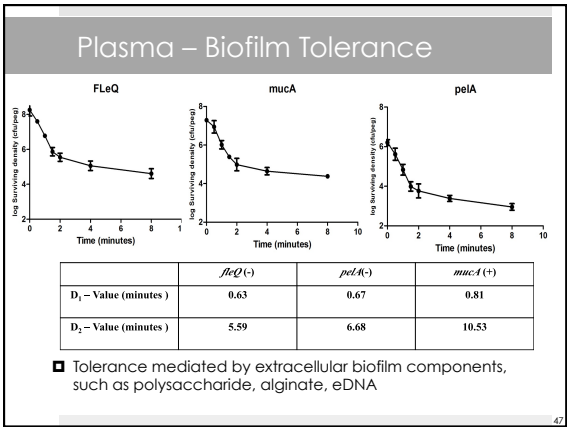

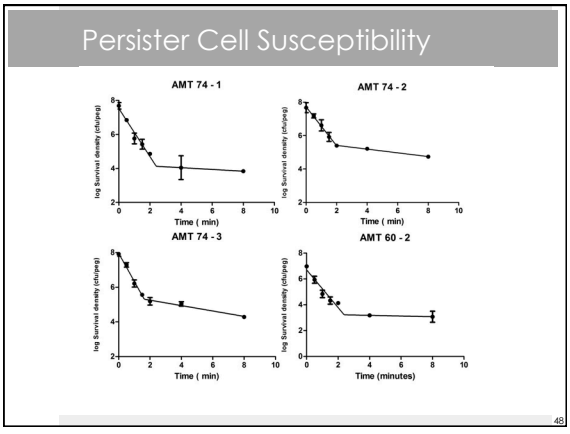

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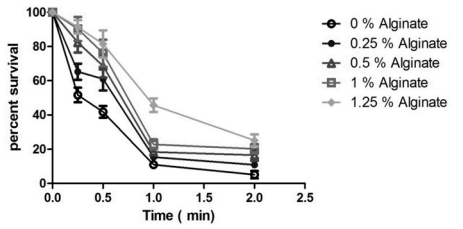
Effect of Different Alginate % survival of *P. aeruginosa*



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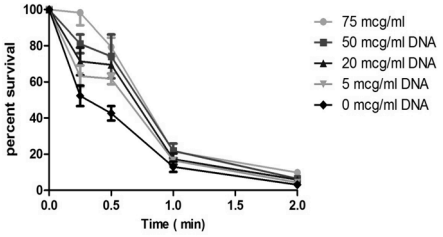
Effect of eDNA on *P. aeruginosa* survival



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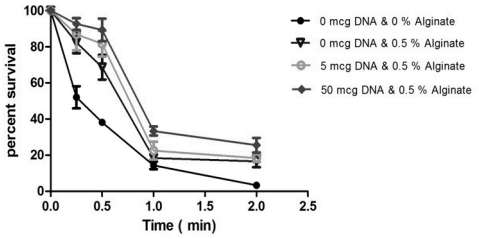
Combined effect of DNA and Alginate on *P. aeruginosa* survival



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Combined effect of Alginate and eDNA on log_{10} reduction of *P. aeruginosa* viability

Exposure Time (minutes)	log_{10} Reduction in PA01 Viability			
	0mcg/ml DNA & 0% Alginate	0 mcg/ml DNA & 0.5 % Alginate	5 mcg/ml DNA & 0.5 % Alginate	50 mcg/ml DNA & 0.5 % Alginate
0.25	0.29±0.09	0.08±0.06	0.07±0.08	0.03±0.03
0.5	0.42±0.02	0.15±0.08	0.09±0.07	0.05±0.05
1	0.85±0.1	0.73 ±0.7	0.67±0.17	0.48±0.06
2	1.49±0.1	0.8±0.17	0.74±0.09	0.60±0.12

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The Daily Telegraph

telegraph.co.uk

Norovirus closes hundreds of wards

Number of cases reaches 750,000 as experts fear this year's winter vomiting bug outbreak could prove the worst on record

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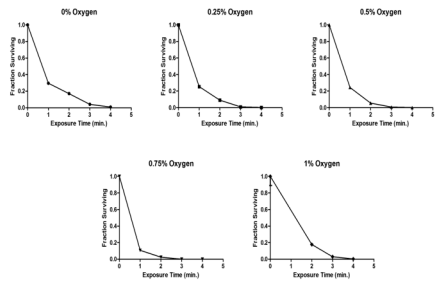
MS2 Phage Inactivation



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Summary

- Rapid bactericidal effect, more than one target, more than reactive one species involved
- >4 log reduction in 48 hr *P. aeruginosa* biofilm in 4 minutes
- Biphasic biofilm kill curve may indicate a 'shielding effect' from surface layers of biofilm or;
- Sequestering of active species by cellular component of sacrificial outer layer of biofilm
- Plasma interaction with liquid has implications for planktonic kill rate – rate of propagation of reactive species
- Multiple cellular targets (interactions with lipid membrane, protein, DNA)
- Effect of biofilm subpopulations (persisters) must be considered in chronic or longterm infections
- Biofilm components are critical mediators of bacterial biofilm tolerance to non-thermal plasma treatments

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Future Directions (QUB Plasma Medicine Group)

- Invest Northern Ireland funded Proof of Concept Grant
- Development of a portable system based on the device described for hospital control of biofilms, planktonic bacteria and viral pathogens
- Ward Testing – infection control (2014)
- Safety and Biocompatibility testing
- Phase I safety trials
- Trials in animals (and eventually patients) topical wounds

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Acknowledgements

Queen's University Belfast

Brendan Gilmore
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Robert P Ryan (Dundee)

THE UNIVERSITY of York

Deborah O'Connell
Timo Gans

society for applied sfam microbiology

Department of Health, Social Services and Public Safety

Invest Northern Ireland
Building Locally Competing Globally

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Coming Soon

April 17	**CHLORHEXIDINE PATIENT BATHING AS A MEANS TO PREVENT HEALTHCARE ASSOCIATED INFECTIONS** *Prof. Mark Rupp, University of Nebraska Medical Center*
April 24	*(Free Teleclass)* **ARE WE TOO CLEAN FOR OUR OWN GOOD? THE HYGIENE HYPOTHESIS AND ITS IMPLICATIONS FOR HYGIENE, LIFESTYLE, AND PUBLIC HEALTH** *Dr. Sally Bloomfield, London School of Hygiene and Tropical Medicine*
May 5	*(Free ... WHO Teleclass – Europe)* **SPECIAL LECTURE FOR 5 MAY, 2014** *Prof. Didier Pittet, World Health Organization*
May 8	**VENTILATOR-ASSOCIATED EVENTS: A PATIENT SAFETY OPPORTUNITY** *Dr. Michael Klompas, Harvard Medical School*

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