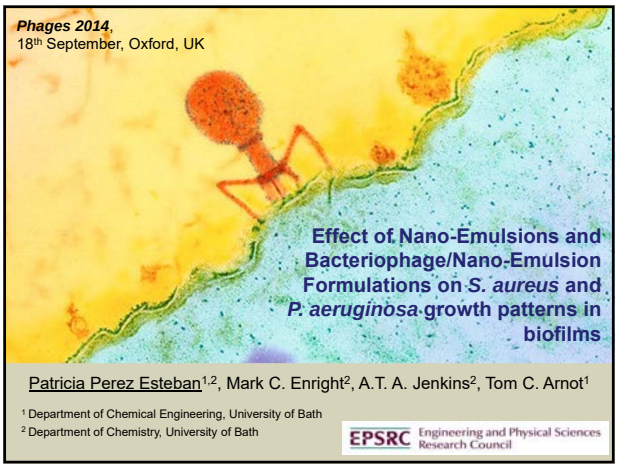

Phages 2014,
18th September, Oxford, UK

Effect of Nano-Emulsions and Bacteriophage/Nano-Emulsion Formulations on *S. aureus* and *P. aeruginosa* growth patterns in biofilms

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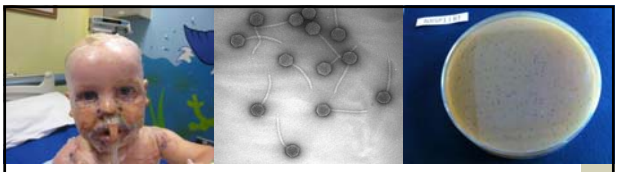
EPSRC Engineering and Physical Sciences Research Council



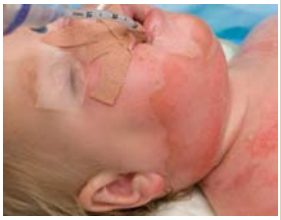
1. INTRODUCTION

- Description of the problem of burns and burn site infections
- Alternatives to antibiotics: Bacteriophages
- Available technologies and implementation of the emulsification technique as an effective storage/delivery medium

• Burns and burn infections[1]

Burns are experienced by thousands of people every year in the UK with high associated costs.

- Children under two years old are the most vulnerable to burn wound infections.
- Minor and moderate burns caused by **scalds** or **spillages** are the most common.
- The majority of cases are domestic injuries (79%).
- Complications caused by **Toxic Shock Syndrome**.



[1] "UK Burn Injury Data (1986-2007 inc.)," 2008. [Online]. Available: http://www.ibidb.org/. [Accessed: 02-Feb-2012].

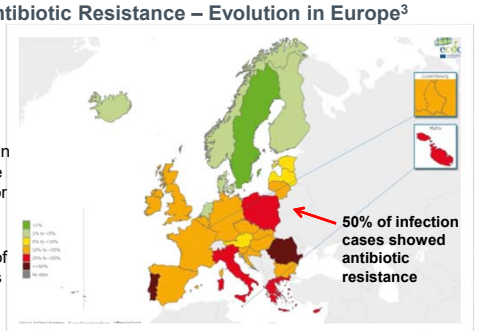
• Antibiotic Resistance – Evolution in Europe[2]

Methicillin Resistant *S. aureus* in Europe (2010)

[2] European Centre for Disease Prevention and Control, "Antimicrobial resistance surveillance in Europe 2010. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net).," 2011.

• Antibiotic Resistance – Evolution in Europe[3]

- Increasing bacterial antibiotic resistance in Europe due to misuse or abuse
- Necessity of alternatives



Methicillin Resistant *S. aureus* in Europe (2012)

[3] European Centre for Disease Prevention and Control, "Antimicrobial resistance surveillance in Europe 2012. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net).," 2013.

• Alternatives to antibiotics

Alternative therapies are emerging as a consequence of the widespread antibiotic resistance[4]:

- Improved antibiotics – difficult to make

Example: ***Only Linezolid*** *has been approved for the treatment of acute skin infections* ***since 2000****, although Tedizolid is currently being developed*

- Molecular Biology techniques to make bacteria more susceptible to antibiotics
- Activated antibacterial agents
- **Bacteriophages**

[4] P. W. Taylor, P. D. Stapleton, and J. Paul Luzio, "New ways to treat bacterial infections.," *Drug discovery today*, vol. 7, no. 21, pp. 1086-91, Nov. 2002.

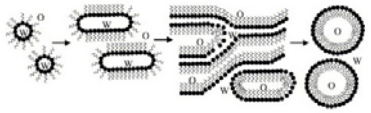
• Phage therapy as an alternative to antibiotics

- Bacteriophages have been used against skin and wound infections, with reported success rates of up to 90% against *S. aureus* [7].
- The advantages of bacteriophage therapy include their abundance and ecological 'friendliness'; they can be used as a 'phage-cocktail', they multiply exponentially, and they do not generate unwanted side-effects [8].
- There are challenges to implementing phage therapy *in vivo*, which may be partially addressed by modelling of population dynamics.

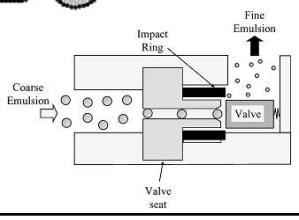
[7] Ahmad SI. 2002. Treatment of post-burns bacterial infections by bacteriophages, specifically ubiquitous *Pseudomonas* spp. notoriously resistant to antibiotics. *Medical Hypotheses* **58**:327–31.

[8] Hanlon GW. 2007. Bacteriophages: an appraisal of their role in the treatment of bacterial infections. *International Journal of Antimicrobial Agents* **30**:118–28.

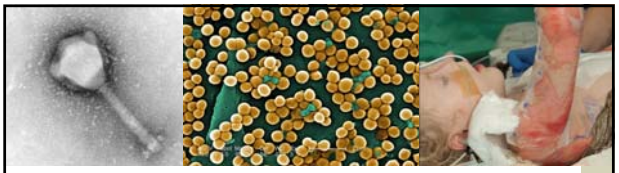
• Available Emulsification Technologies



PIT Emulsification

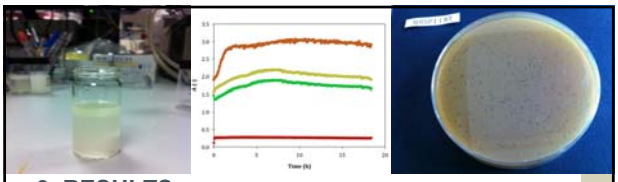


Homogenisation



2. PROJECT AIMS

- Delivery of phage or 'phage-cocktail' to the point of infection **without losing efficacy**, either **during delivery**, or **prior storage**.
- Use of **oil-in-water nano-emulsions** as a stabilising / delivery vehicle, due to their capacity to prevent virus precipitation, and to enhance transdermal penetration.
- Understanding the **mechanisms of interaction** in a mixture containing emulsion droplets, bacteriophage, and bacteria, and the relative effects of emulsion and phage on **bacterial growth**.



3. RESULTS

- Emulsification techniques
- Influence of oil-in-water Nanoemulsions on bacterial growth
- Influence of oil-in-water Nanoemulsions on bacteriophage lytic activity
- Product shelf-life
- Bacterial Biofilms

• Emulsification Techniques: PIT vs. Homogenisation

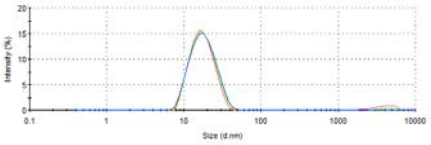
For the same formulation 80% SM Buffer (aq. phase), 15 % BrijO10 (surfactant), 5% Soybean oil (organic phase)

PIT Emulsification

Homogenisation after 45 min

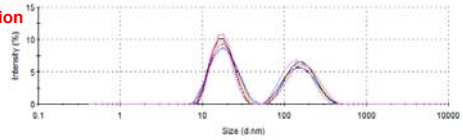
- **Emulsification Techniques: PIT vs. Homogenisation**

For the same formulation 80% SM Buffer (aq. phase), 15 % BrijO10 (surfactant), 5% Soybean oil (organic phase)



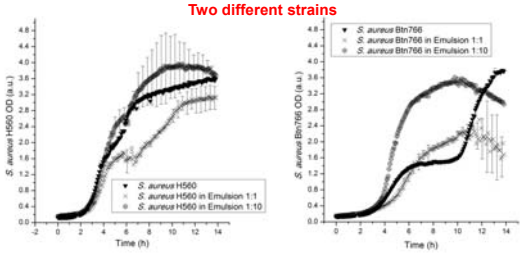
PIT Emulsification

Homogenisation after 45 min



- **Influence of Emulsion on Bacterial Growth**

Two different strains



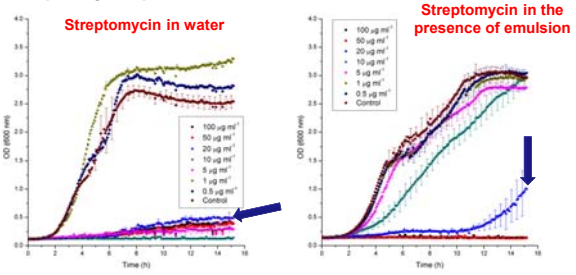
- Bacterial growth in more concentrated emulsion (1:1 dilution) appeared slower, showing a lag period.
- RSM Analysis showed that both growth rate and carrying capacity were influenced by the concentration of emulsion droplets.

P. P. Esteban et al. *Biotechnol. Prog.*, 30:932–944, 2014

- **Influence of Emulsion on Bacterial Growth: Testing Streptomycin penetration**

Streptomycin in water

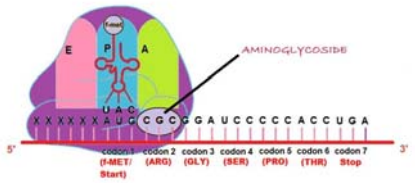
Streptomycin in the presence of emulsion



- Bacterial growth may be influenced by attachment of emulsion droplets to the **outer cell membrane**, depriving bacteria of nutrients.
- If so, penetration of antibiotics should also be affected.

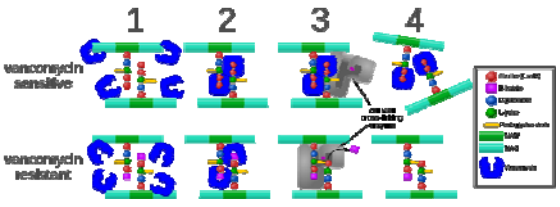
P. P. Esteban et al. *(In preparation)*

- **Influence of Emulsion on Bacterial Growth: Different Antibiotic Mechanisms - Streptomycin**



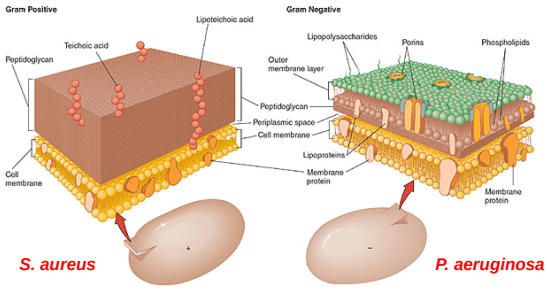
- Streptomycin needs to go through and reach the ribosomes of the cell.
- It binds irreversibly to the 30S ribosomal subunit.
- Codon misreading and protein inhibition.

- **Influence of Emulsion on Bacterial Growth: Different Antibiotic Mechanisms - Vancomycin**



- Vancomycin sits on the cell wall surface.
- It binds to the two D-ala residues.
- It inhibits cell wall synthesis.

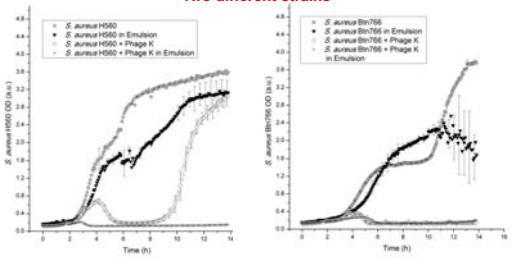
- **Influence of Emulsion on Bacterial Growth: Mechanism and/or Cell Wall Structure?**



- **Influence of Emulsion on Bacteriophage K Lytic Activity**

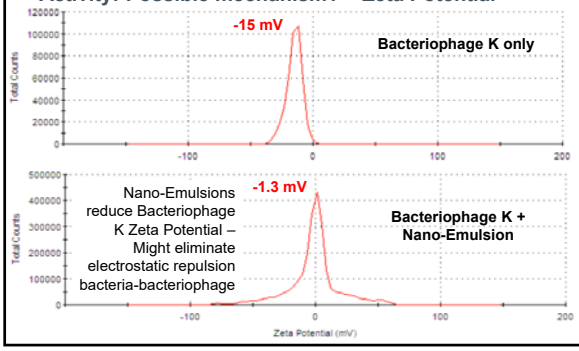
P. P. Esteban et al. *Biotechnol. Prog.*, 30:932–944, 2014

Two different strains



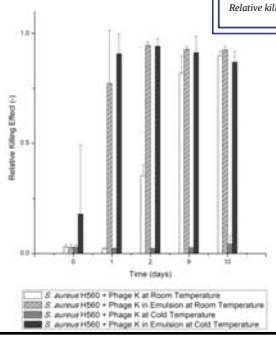
- Bacterial concentration was dramatically reduced after the first 5 hours of treatment with bacteriophage-emulsion formulation, when compared with bacterial growth in emulsion. Re-growth is also avoided.

- **Influence of Emulsion on Bacteriophage K Lytic Activity: Possible mechanism? – Zeta Potential**



- **Shelf-life of Bacteriophage K-Emulsion Preparations**

$$\text{Relative killing effect} = \frac{OD_{570}(control) - OD_{570}(within\ phage\ preparation)}{OD_{570}(control)}$$

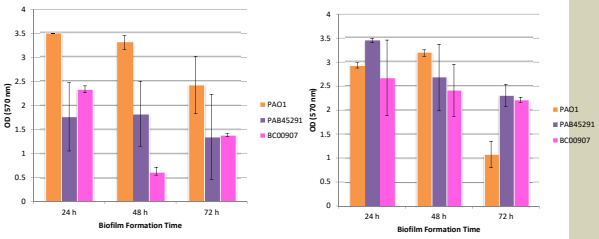


- Relative killing effect = 1 ➔Total killing of bacteria
- Relative killing effect = 0 ➔No killing effect

Bacteriophage-emulsion formulations, both at room (~20ºC) and cold (4ºC) temperature show enhanced antibacterial activity.

P. P. Esteban et al. *Biotechnol. Prog.*, 30:932–944, 2014

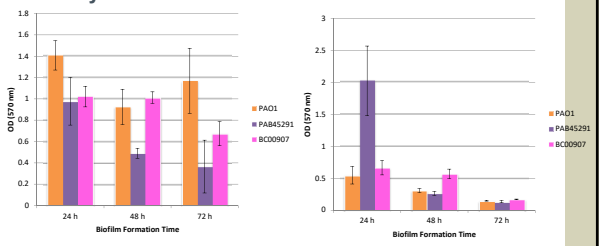
- **Growth of Bacterial Biofilms – Crystal Violet Assay**



Growth without Emulsion **Growth in Emulsion**

P. P. Esteban et al. *(In preparation)*

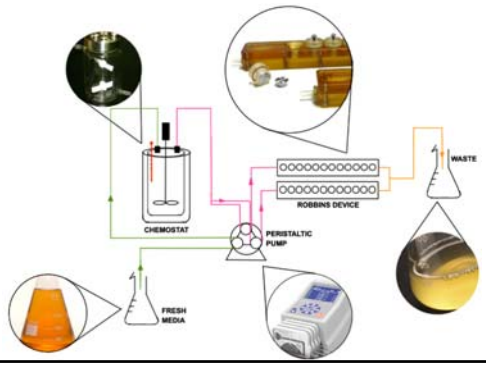
- **Eradication of Bacterial Biofilms – Crystal Violet Assay**



Eradication with Phage cocktail in Buffer **Eradication with Phage cocktail in Emulsion**

P. P. Esteban et al. *(In preparation)*

- **Bacterial Biofilms – Modified Robbins Device**



4. MODELLING STRATEGIES

- Modelling of **bacterial growth** – Test of existing models.
- Influence of the **ratio emulsion droplets : bacterial cells** on growth parameters.
- Proposal of a **modified logistic growth model** in the presence of emulsion droplets.
- **Infectivity models**: general principles and difficulties.

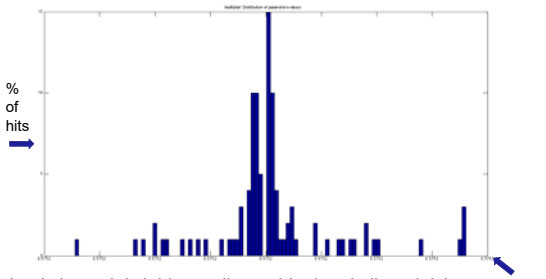
• Modelling bacterial growth

Model	Formulation	Parameters
Logistic	$\frac{dS(t)}{dt} = aS(t)\left(1-\frac{S(t)}{K}\right)$	a=Growth rate (time^{-1}) K=Carrying capacity (concentration)
Gompertz	$\frac{dS(t)}{dt} = aS(t)\log\left(\frac{K}{S(t)}\right)$	a=Growth rate (time^{-1}) K=Carrying capacity (concentration)
Richards	$\frac{dS(t)}{dt} = aS(t)\left(1-\frac{S(t)}{K}\right)^{\eta}$	a=Growth rate (time^{-1}) K=Carrying capacity (concentration) η =Parameter
Hyperbolastic H1	$\frac{dS(t)}{dt} = \frac{1}{K}S(t)(K-S(t))\left(Ka+\frac{\theta}{\sqrt{1+t^2}}\right)$ If θ =0 - equivalent to the Logistic model.	a=Intrinsic growth rate (time^{-1} concentration^{-1}) K=Carrying capacity (concentration) θ =Parameter

• Modelling bacterial growth - METHOD

- In-built parameter estimation model of Matlab (lsqnonlin) not powerful enough for stiff systems of DEs.
- Self-made parameter estimation algorithm using Matlab.
- Multistart run for 100 random initial guesses for all growth models.
- Determination of parameters, % of hits using different initial guesses, and value of total residual after fitting.
- Preferred model – Simplest model.

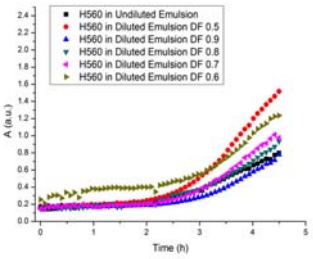
• Modelling bacterial growth - RESULTS



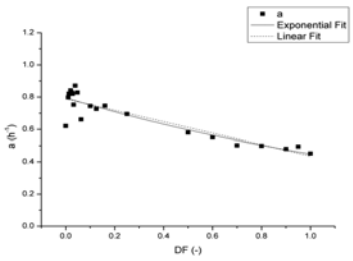
- Logistic model yields smaller residual and all models' parameters lead towards logistic model

• Influence of the ratio emulsion droplets : bacterial cells on growth parameters.

- First moments of growth curve can be approximated to an **exponential** – Fitting growth rate
- Use different initial dilution factors of emulsion (vary amount of droplets per bacteria)



• Proposal of a modified logistic growth model in the presence of emulsion droplets.



$$\frac{dS(t)}{dt} = aS(t)\left(1-\frac{S(t)}{K}\right)$$

New growth model

$$\frac{dS(t)}{dt} = a_0 e^{-DF} S(t)\left(1-\frac{S(t)}{K}\right)$$

- Growth rate is dependent on the droplet : bacteria ratio.
- What about carrying capacity?

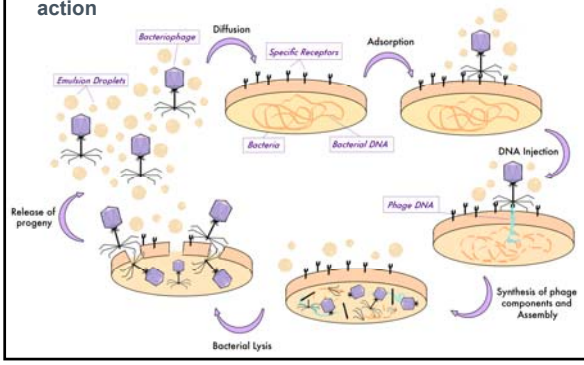
- **Infectivity models**

- **Steps from a microscopic point of view:**

1. Diffusion or transport from the bulk of the solution to bacterial surface.
2. Recognition and adsorption due to specific receptors on bacterial outer membrane.
3. Injection of bacteriophage genetic material.
4. Bacteriophage self-replication.

- **General mass-action law.**

- **Alternatives to antibiotics: Bacteriophages' mode of action**

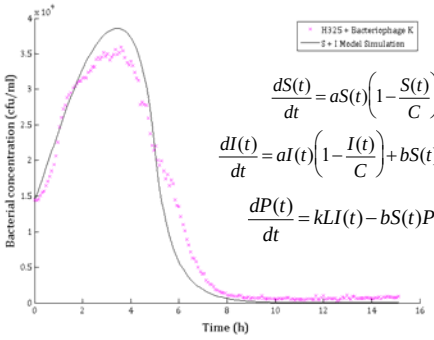


- **Infectivity models**

- **General system of ODEs:**

$$\begin{cases} \frac{dS}{dt} = \{Rate\ of\ appearance\ of\ bacteria\ by\ growth\} - \{Rate\ of\ disappearance\ of\ bacteria\ by\ infection\} \\ \frac{dI}{dt} = \{Rate\ of\ appearance\ of\ bacteria\} + \{Bacterial\ Infection\ Rate\} - \\ -\{Bacterial\ \ Inactivation\ Rate\} - \{Bacterial\ Lysis\ Rate\} \\ \frac{dP}{dt} = \{Phage\ Inflow\ Rate\} - \{Phage\ Inactivation\ Rate\} - \{Phage\ Adsorption\ Rate\} + \\ +\{Phage\ Release\ of\ Progenie\ Rate\} \end{cases}$$

- **Infectivity models - Example**



$$\frac{dS(t)}{dt} = aS(t)\left(1 - \frac{S(t)}{C}\right) - bS(t)P(t)$$

$$\frac{dI(t)}{dt} = aI(t)\left(1 - \frac{I(t)}{C}\right) + bS(t)P(t) - kI(t)$$

$$\frac{dP(t)}{dt} = kLI(t) - bS(t)P(t) - mP(t)$$

- **Infectivity models - Difficulties**

- System of ODEs very non-linear.
- Parameter estimation is not trivial.
- Outcome highly dependent on initial guesses of parameters.

- **Having a working and reliable parameter estimation method would help elucidating the mechanisms.**

- Possible improvement: experimental determination of some parameters and use them as initial guesses/fixed parameters.

5. CONCLUSIONS

- We present a novel approach for the **efficient storage and delivery** of Bacteriophage K for the treatment of *Staphylococcus aureus* infections.
- More **concentrated** oil-in-water nano-emulsions had a **bigger effect** on bacterial growth.
- The nano-emulsion-bacteriophage preparations show **enhanced and stable antimicrobial activity**, with reduced fluctuations of infectivity over time, when compared to a simple phage suspension.
- This work demonstrates the **potential for a responsive wound dressing preparation**.

6. ONGOING WORK

- Investigation of the influence of outer cell wall properties on emulsion formulations performance in terms of growth and phage infectivity – *Pseudomonas aureuginosa (***Gram negative bacteria)**
- Experimental determination of some of the infectivity parameters in order to achieve better fitting for the modelling strategies.
- Investigation of more **realistic wound environments**, where the presence of biofilms is determinant and critical – Use of our formulations in *S. aureus* and *P. aureuginosa* biofilms.

7. FUTURE WORK

- We are exploring the **biological mechanisms** within the system and evaluating more **favourable formulations** in terms of biocompatibility and cost.
- We are evaluating more comprehensive approaches to **modelling** the bacteriophage / emulsion / bacterial interactions.
- We are moving towards a **more realistic wound environment**.

8. ACKNOWLEDGEMENTS

- This project is funded by the Engineering and Physical Sciences Research Council (EPSRC).
- I thank my supervisors Dr Tom Arnot and Dr Toby Jenkins for their support and advice.
- We thank North Bristol NHS Frenchay Hospital for their support.