Biofilm model systems and quantification tools

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What are biofilms?

• Biofilms are *sessile* communities of

  – *prokaryotic* and/or *eukaryotic* cells,

  – *attached* to a substratum or interface or to each other,

  – embedded in a *matrix* composed, at least partially, of self-produced extracellular material,

  – exhibit an *altered phenotype* compared to planktonic cells

(Donlan and Costerton, 2002)
Biofilms
Biofilms are everywhere
Biofilm models

In vitro
- Closed
  - Batch
  - Static
- Open
  - Flow
  - Dynamic

Ex vivo
- Microcosm

In vivo
- Invertebrate
  - Non-mammalian
- vertebrate mammals
Batch systems vs flow systems

- Closed system
- Mixing is optional

- Cont. flow stirred tank reactor (CFSTR)
  - Perfect mixing
  - Steady state

- Plug flow reactor (PFR)
  - Mixing only in radial direction

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Coenye and Nelis, 2010 J. Microbiol Methods
In vitro batch systems

• Most commonly used
  • microtiter plate (MTP)

Step 1
• Adhesion step
• 0,5-4h

Step 2
• Remove PL cells
• Rinsing step

Step 3
• BF formation
• Fresh medium
• 20-48-....h
**In vitro batch systems**

- MTP: biofilms formed on the bottom and walls of the plate

  *B. cenocepacia* LMG 18828 (24h)
In vitro batch systems

- MTP: biofilms formed on glass bottom

*B. cepacia* LMG 16656 (WT and QS mutants) (24h; Live/Dead staining)

Udine et al., 2013, PloS ONE
**In vitro batch systems**

- MTP: biofilms formed on surface placed in MTP
  - Medical grade silicone
  - Orthopedic implant
  - Catheters
  - Contact lenses
  - Hydroxyapatite disks
  - Molars/teeth
  - Coated materials
  - ....

LPM, UGent
In vitro batch systems

- Minimum biofilm eradication concentration assay:
  - The Calgary device (Ceri et al, 1999)
**In vitro** batch systems

- Minimum biofilm eradication concentration assay:
  - The Calgary device (Ceri et al, 1999)
**In vitro batch systems**

- Minimum biofilm eradication concentration assay:
  - The Calgary device (Ceri et al, 1999)

![Diagram of the Calgary device](image-url)

Harrison et al., 2006
Biol. Proced Online
In vitro Flow systems
**In vitro** flow systems: CFSTR

- Centers for disease control (CDC) biofilm reactor

![Diagram of CFSTR reactor with labeled components: Media "IN", Polyethylene Lid, 1L vessel, Media "OUT", Magnetic stir, 8 Polypropylene coupon holders, Coupons, Air-vents, and "Water jacket".](image-url)
In vitro flow systems: CFSTR

- CDC biofilm reactor
In vitro flow systems: CFSTR

- *C. albicans* biofilm formed in CDC biofilm reactor
- stained with Concanavalin A bound to Alexa Fluor 647 (red labelled cells and red stained ECM)
In vitro flow systems: CFSTR

- CDC biofilm reactor
*In vitro* flow systems: CFSTR

- Other CFSTR systems

Rotating disk reactor

Constant depth film fermentor

Biofilm annular reactor

Pratten, J. 2007
In vitro flow systems: PFR

- Simple design of Flow cell set-up

In vitro flow systems: PFR

- Flow cells

Transmission FC  Capillary FC  Coupon evaluation FC
In vitro flow systems: PFR

- Flow cells: time-lapse view of biofilm treatment under flow conditions

Lorenz L, Buckingham-Meyer K, Pitts B, 2012, CBE, MSU
In vitro flow systems: PFR

- Microfluidic devices

Benoit et al., 2010
In vitro flow systems: PFR

- *P. fluorescence* biofilm development in microfluidic device
**In vitro** flow systems: PFR

- Modified Robbins device (MRD)

McBain A.J., 2009
**In vitro** flow systems: PFR

- Modified Robbins device (MRD)

set-up of the MRD BF model
LPM, UGent.
In vitro flow systems: PFR

- Modified Robbins device (MRD)
**In vitro** flow systems: PFR

- Modifications on the MRD design

Garcia et al., 2010. J. Microbiol. Meth.
In vitro flow systems: PFR

- Implemented in industrial environments:
In vitro flow systems: PFR

- (Colony-)Drip flow reactor (DFR)

Goeres et al., 2009, Nature Protocols
Method E2647-08 Annual Book of ASTM Standards
In vitro flow systems: PFR

- (Colony-)Drip flow reactor
In vitro flow systems: PFR

- Polymicrobial wound biofilm in a C-DFR

Simple in vitro flow systems

Virulence.

Biofilms Hypertextbook
P. Stoodley and J. Lennox
Ex vivo/Microcosms:
Specific Biofilm models
Microcosms: Wound Biofilm model

- Lubbock chronic wound pathogenic biofilm model (LCWPB)
  - Rapid (24h)
  - Multispecies (Sa, Pa, Ef)
  - Macro/Microscopically resembles *in vivo* wound biofilms

Sun et al., 2008. Wound Repair Regen.
Microcosms: Wound Biofilm model

• Collagen matrix with simulated wound fluid
  • *P. aeruginosa*
  • *S. aureus*

• Microscopically resembles *in vivo* wound biofilms

Werthen et al., 2010. APMIS.
Ex vivo biofilm models

- Reconstituted Human Epithelia (RHE)
- Human sinusal epithelial, skin, molars,…
- Microvascular endothelial cells (HMEC-1 Cells)
- HeLa cells
- CF-derived bronchial cells
- U2OS osteosarcoma cells
- …..
Overview

MTP/Batch:
- Less labor intensive
- No specialized equipment
- Relatively cheap
- multiplexing
- ≠ in vivo situation?

Flow systems:
- More labor intensive
- Require specialized equipment/skills
- more expensive
- Limitation/run
- Fluid Flow ≠ in vivo situation?

Microcosm/cell-culture-based model
In vivo (non-)mammalian Biofilm models
In vivo non-mammalian biofilm models

- *Tetrahymena pyriformis*
- *Acanthamoeba sp.*
- *Dictyostelium discoideum* (Slime mould)
- *Lemna minor* (Duckweed)
- *Medicago sativa* (Wounded alfalfa)
- *Arabidopsis thaliana* (Thale cress)
- *Nematospora cyrtonemoides* (Leach)
- *Pseudovelles redivivus* (Sow paste nematode)

- *Caenorhabditis elegans* (Round worm)
- *Galleria mellonella* (Wax moth caterpillar)
- *Drosophila melanogaster* (Fruit fly)
- *Danio rerio* (Zebrafish)

Lebeaux et al, 2013 Pathogens
## In vivo non-mammalian biofilm models

<table>
<thead>
<tr>
<th>Organism</th>
<th>Size</th>
<th>Generation time</th>
<th>Temp. (°C)</th>
<th>Immune system</th>
<th>Follow-up of host infection</th>
<th>Relevant Model</th>
<th>Human Pathogen studied</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Terahyema pyriformis</em></td>
<td>20 × 40 μm</td>
<td>7 hours</td>
<td>22–26</td>
<td>Unknown</td>
<td>Real-time through bacterial fluorescent markers</td>
<td>Biofilm grazing, Virulence and toxicity</td>
<td>Klebsiella pneumonia, Legionella pneumophila, Vibrio cholerae</td>
<td>[412]</td>
</tr>
<tr>
<td><em>Acanthamoeba sp.</em></td>
<td>13 to 35 μm</td>
<td>6–10 hours</td>
<td>19–25</td>
<td>Macrophage analog</td>
<td>--</td>
<td>Biofilm grazing, Phagocytosis, Intracellular survival</td>
<td>L. pneumophila, Cryptococcus neoformans, Candida albicans, Methicillin-resistant Staphylococcus aureus (MRSA), V. cholerae</td>
<td>[414]</td>
</tr>
<tr>
<td><em>Dictyostelium discoideum</em></td>
<td>10–20 μm</td>
<td>4–12 hours</td>
<td>19–25</td>
<td>Macrophage analog</td>
<td>Real-time through bacterial fluorescent markers</td>
<td>Biofilm grazing, Phagocytosis, Intracellular survival</td>
<td>Pseudomonas aeruginosa, L. pneumophila, Listeria monocytogenes (intracellular pathogens)</td>
<td>[415]</td>
</tr>
<tr>
<td><em>Lema minor</em> (Duckweed)</td>
<td>2.5 mm × 1.5–3.5 mm</td>
<td>1 week</td>
<td>28</td>
<td>Unknown</td>
<td>X</td>
<td>Biofilm formation and virulence</td>
<td>S. aureus, P. aeruginosa, Salmonella spp., Shigella spp., Yersinia spp.</td>
<td>[34]</td>
</tr>
<tr>
<td><em>Medicago sativa</em> (Wounded alfalfa)</td>
<td>Seedlings</td>
<td>3 months</td>
<td>30</td>
<td>Unknown</td>
<td>X</td>
<td>Chronic bacterial lung infections</td>
<td>P. aeruginosa, Burkholderia cepacia</td>
<td>[416]</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em> (Tale cress)</td>
<td>1 to 20–25 cm</td>
<td>3 weeks</td>
<td>20–25</td>
<td>Analog pathways to MAPK</td>
<td>X</td>
<td>Biofilm formation and virulence</td>
<td>Pseudomonas spp., S. aureus</td>
<td>[417]</td>
</tr>
<tr>
<td><em>Hirudo sp</em> (Leech)</td>
<td>15–40 mm (adult)</td>
<td>10–35</td>
<td>Unknown</td>
<td>X</td>
<td>Biofilm competition and gut colonization</td>
<td>Aeromonas spp.</td>
<td>[418]</td>
<td></td>
</tr>
<tr>
<td><em>Pseudagrellus ovinus</em> (Samu pasta nematode)</td>
<td>1 mm × 50 μm</td>
<td>3–5 days</td>
<td>37</td>
<td>Innate immunity (Toll-like receptor, MAPK)</td>
<td>--</td>
<td>Biofilm formation, Virulence and gut colonization</td>
<td>P. aeruginosa, Salmonella enterica, and S. aureus</td>
<td>[419]</td>
</tr>
<tr>
<td><em>Caenorhabditis elegans</em> (Round worm)</td>
<td>1 mm × 100 μm</td>
<td>4–7 days</td>
<td>22–27</td>
<td>Innate immunity (Toll-like receptor, MAPK)</td>
<td>Real-time through bacterial fluorescent markers</td>
<td>Biofilm formation, Virulence and gut colonization</td>
<td>Microbacterium nematophilum, Escherichia coli, Shigella flexneri, V. cholerae, Shewanella spp., Listeria spp., S. aureus, Staphylococcus sp.</td>
<td>[420]</td>
</tr>
<tr>
<td><em>Galleria mellonella</em> (Wax moth caterpilar)</td>
<td>3 cm in length</td>
<td>30</td>
<td>30</td>
<td>Innate immunity (Toll-like receptor, MAPK, NFκB)</td>
<td>--</td>
<td>Biofilm formation and virulence</td>
<td>Pseudomonas spp., Proteus mirabilis, E. coli, Bacillus cereus, Bacillus thuringiensis, C. albicans, C. neoformans</td>
<td>[421]</td>
</tr>
<tr>
<td><em>Drosophila melanogaster</em> (Fruit fly)</td>
<td>3 mm</td>
<td>10 days</td>
<td>12–30</td>
<td>Innate immunity (Toll-like receptor, Imad, MAPK, NFκB)</td>
<td>Real-time through fluorescent markers, LacZ fusions available</td>
<td>Biofilm formation, Virulence and gut colonization</td>
<td>Wolbachia spp., Serratia marcescens, Erwinia spp., Pseudomonas entomophila, C. neoformans, Francisella novicida, L. monocytogenes, V. cholerae, C. albicans</td>
<td>[422]</td>
</tr>
<tr>
<td><em>Drosophila viridis</em> (Zebrashish)</td>
<td>3–5 mm (larvae) 6–6.7 cm (adult)</td>
<td>3–4 months</td>
<td>23–28</td>
<td>Adaptive and innate</td>
<td>Real-time through fluorescent markers both on host and bacteria</td>
<td>Biofilm formation, Virulence and gut colonization</td>
<td>Mycobacterium marinum, Oodinium, Microsporidia, E. coli, Pseudomonas spp., Salmonella spp, Vibrio spp.</td>
<td>[423]</td>
</tr>
</tbody>
</table>

1 As insects, they could also be used to model gut colonization and commensal-pathogen interaction. However, to the best of our knowledge, it has not yet been used with this objective. X: not possible; --: not described.

Lebeaux et al, 2013 Pathogens
In vivo non-mammalian biofilm models

Caenorhabditis elegans

Brackman G., 2013, unpublished data
**In vivo non-mammalian biofilm models**

*Caenorhabditis elegans*

# In vivo vertebrate biofilm models

<table>
<thead>
<tr>
<th>Animal</th>
<th>Biofilm infection model</th>
<th>Micro-organism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice/Rat</td>
<td>Sub-cutaneous foreign body model systems</td>
<td>C. albicans</td>
<td>Kucharikova et al., 2010</td>
</tr>
<tr>
<td></td>
<td>Sub-cutaneous foreign body model systems</td>
<td>S. aureus</td>
<td>Engelsman et al., 2003</td>
</tr>
<tr>
<td></td>
<td>Respiratory tract infection model</td>
<td>B. cenocepacia</td>
<td>Brackman et al., 2011</td>
</tr>
<tr>
<td></td>
<td>Intraperitoneal foreign-body infection model</td>
<td>P. aeruginosa</td>
<td>Christensen et al., 2012</td>
</tr>
<tr>
<td></td>
<td>CVC infection model</td>
<td>S. aureus</td>
<td>Beenken et al., 2012</td>
</tr>
<tr>
<td></td>
<td>Oropharyngal candidiase</td>
<td>Candida spp.</td>
<td>Fanning et al., 2012</td>
</tr>
<tr>
<td></td>
<td>Urethral stent infection model</td>
<td>Staphylococcus spp.</td>
<td>Cirioni et al., 2011</td>
</tr>
<tr>
<td></td>
<td>Skin/wound infection model</td>
<td>different species</td>
<td>Dalton et al., 2011</td>
</tr>
<tr>
<td></td>
<td>Denture model</td>
<td>C. albicans</td>
<td>Nett et al., 2010</td>
</tr>
<tr>
<td></td>
<td>Otitis infection model</td>
<td>S. pneumoniae</td>
<td>Yadav et al., 2012</td>
</tr>
<tr>
<td>Cats</td>
<td>foreign-body infection model</td>
<td>different species</td>
<td>Leung et al., 2000</td>
</tr>
<tr>
<td>Chinchilla</td>
<td>Otitis media infection model</td>
<td>P. aeruginosa</td>
<td>Byrd et al., 2011</td>
</tr>
<tr>
<td>Dog</td>
<td>Wound infection model</td>
<td>S. aureus</td>
<td>Kiran et al., 2008</td>
</tr>
<tr>
<td>Gerbils</td>
<td>Otitis media infection model</td>
<td>H. influenza</td>
<td>Swords et al., 2004</td>
</tr>
<tr>
<td>Goat</td>
<td>Osteomyelitis infection model</td>
<td>S. aureus</td>
<td>Tran et al., 2013</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>Sub-cutaneous foreign body model systems</td>
<td>Staphylococcus spp.</td>
<td>Fluckiger et al., 2005</td>
</tr>
<tr>
<td>Monkeys</td>
<td>Otitis media infection model</td>
<td>P. aeruginosa</td>
<td>Dohar et al., 2005</td>
</tr>
<tr>
<td>Pigs</td>
<td>Wound infection model</td>
<td>S. aureus</td>
<td>Davies et al., 2008</td>
</tr>
<tr>
<td>Pony</td>
<td>Sub-cutaneous foreign body model systems</td>
<td>S. aureus</td>
<td>Voermans et al., 2006</td>
</tr>
<tr>
<td>Rabbits</td>
<td>Wound infection model</td>
<td>S. aureus</td>
<td>Gurjala et al., 2011</td>
</tr>
<tr>
<td></td>
<td>Catheter infection model</td>
<td>Fungal spp.</td>
<td>Chandra et al., 2011</td>
</tr>
<tr>
<td></td>
<td>Urethral infection</td>
<td>E. coli</td>
<td>Hachem et al., 2009</td>
</tr>
<tr>
<td>Sheep</td>
<td>Implant infection model</td>
<td>S. aureus</td>
<td>Gimeno et al, 2013</td>
</tr>
<tr>
<td></td>
<td>Sinusitis infection model</td>
<td>S. aureus</td>
<td>Singhal et al., 2011</td>
</tr>
</tbody>
</table>

Brackman G., 2013, Unpublished data
In vivo vertebrate biofilm models

Rat sub-cutaneous foreign body model

Van Wijngaarden et al. 1999, AAC
Ricicova et al., 2010, Microbiology
Invertebrate vs vertebrate

Relatively cheap
High throughput
Ethical concerns?
Natural pathology?

Natural pathology?
Limited
Larger= higher costs
Why is it important?

Susceptibility (Sa)

Gene expression (Ca)

Importance of selecting a model that closely fits the research question in a certain research frame

Smith et al. 2013
Infection and Immunity.

Based on Nailis et al. 2010
BMC Microbiology.
Quantification tools

Direct quantification
- Plating
- Counting the colonies

Indirect quantification
- Staining
- Labelling
- Intensity $\approx$ # cells
Direct quantification

- Absolute cell numbers
- Mixed BF: Possible to use selective conditions
- Isolates are available for further research

- Time consuming & labor-intensive
- Time to results is organism dependent (>18h)
- VBNC are not accounted for
- Artificial situation might hinder growth
Great plate count anomaly

- Often observed in biofilms (although systematic studies are lacking!)
  - If you plate pieces of the biofilm:
    - no recovery
  - If you first disperse the biofilm:
    - recovery is much better
- Importance of dispersing the biofilm
  - vortexing and sonication vs scraping

"THE GREAT PLATE COUNT ANOMALY"
~100 times > cells than colonies, 99% unculturable
Indirect quantification
Staining

• Crystal violet (living & dead cells, part of the matrix)

• DMMB (matrix in some organisms; particularly useful for staphylococci)

• Calcofluor white (EPS stain, N-acetylglucosamine)

• SYTO9 (living & dead cells, DNA in the matrix)

• FDA, XTT/MTT/..., CellTiter Blue/Resazurin/Alamar Blue (metabolically active cells)

... and many more!
Resazurin staining

Viable Cell

Reduction Reactions

\[ \text{Resazurin} \rightarrow \text{Resorufin} \]

\(
\begin{array}{c}
\text{Na}^+ - O
\end{array}
\)

\[ \text{Resorufin} \]

Emits fluorescence at 590nm

\[ \rightarrow \]

\[ \text{hydroresorufin} \]

\[ \begin{array}{c}
\text{HO-} \\
\text{O} \\
\text{N} \\
\text{H} \\
\end{array}\]
Indirect quantification

Luminescent (LuxCDABE) S. aureus

Plaut et al., 2013 Plos ONE
Indirect quantification

- Fast, high-throughput
- Equipment
- What are you measuring?
- Optimization is required

CTB fluorescence signal of a *S. aureus* Mu50 biofilm after treatment

Brackman et al., 2013, Unpublished data.
Indirect quantification

- Detection limit for quantification
- Mixed biofilms?

Correlation between *S. aureus* pMV158gfp fluorescence signal and total CFU harvested from the biofilms.

Brackman et al., 2013, J. Appl Microbiol.
Combined method: PMA-qPCR

- *Streptococcus spp.*
- *P. gingivalis*
- *A. actinomycetemcomitans*
- *F. nucleatum*
- *V. parvula, P. intermedia*
- *L. monocytogenes*
- .....
Take home messages

• Model systems are essential to study microbial biofilms

• In combination with various quantification approaches they allow to mimic *in vivo* situations *in vitro*

• Importance of selecting a Biofilm model/quantification method that closely fits the research question in a certain research frame

• Selection based on
  • Research question, Resources, level of the research, Preferences, ...

• Caution extrapolating results from one model/method to another
Thank you for your attention

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