Kgs. Lyngby, 1st June 2021
25th Annual Workshop of the National Reference Laboratories for Fish Diseases

Prevention and control of *F. psychrophilum* infections with bacteriophages

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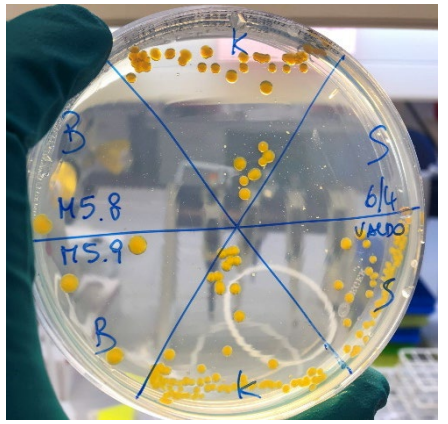
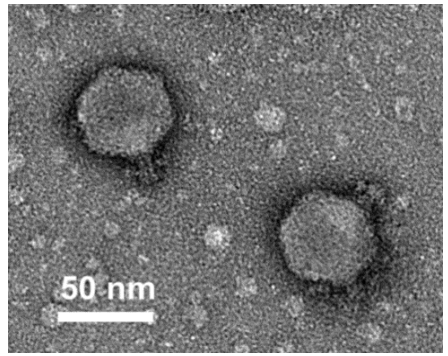




Donati V.L. et al (2021) Front. Microbiol. 12:628309. doi: 10.3389/fmicb.2021.628309.

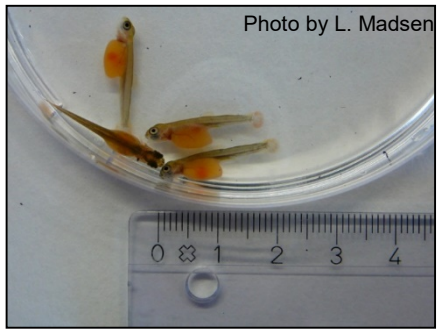
Background and outline of the presentation

- The shift to intensive farming, the introduction of recirculating aquaculture systems (RAS) and the administration of antibiotics has led to a rise in the occurrence of bacterial diseases and antimicrobial resistance (AMR)

➔ increased focus on **developing more sustainable solutions for disease control** as **phage therapy**

- Today we present the recent advances/findings on the use of phages to control and/or prevent the freshwater bacterial pathogen ***Flavobacterium psychrophilum*** in **rainbow trout** (*Oncorhynchus mykiss*, Walbaum).


Eyed eggs



Sac fry or Alevin

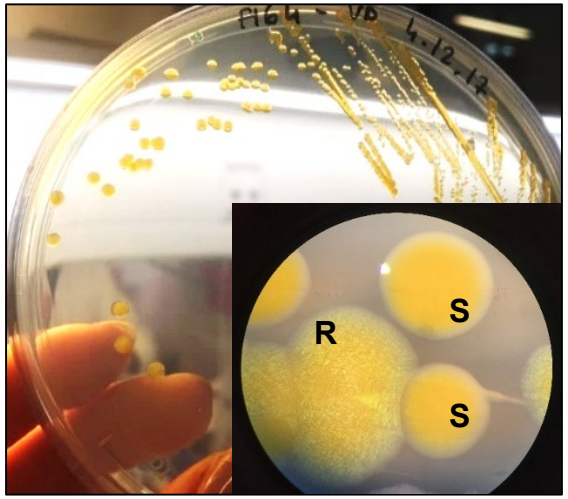
Fry

- **INTRODUCTION**
  - ***Flavobacterium psychrophilum***
  - ***Phage therapy in aquaculture***
- **STUDY CASE**
  - **I. Phage-mediated control of *F. psychrophilum* in aquaculture (rainbow trout fry)**
  - **II. Phages as *F. psychrophilum* control agents in rainbow trout eyed eggs**
- **OVERALL CONCLUSIONS AND FUTURE PERSPECTIVES**

INTRODUCTION

Flavobacterium psychrophilum

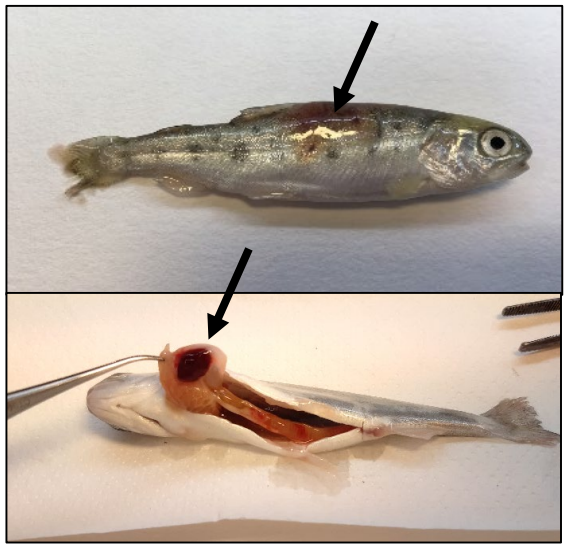
- **Gram negative rod-shaped bacterium**
- Isolated from diseased fish (skin lesions, spleen, kidney and brain)
- Bright convex, circular yellow colonies (flexirubin) with regular/sometimes spreading edges
- Temperature: 4-25°C (15°C)



Treatment

Antibiotics:

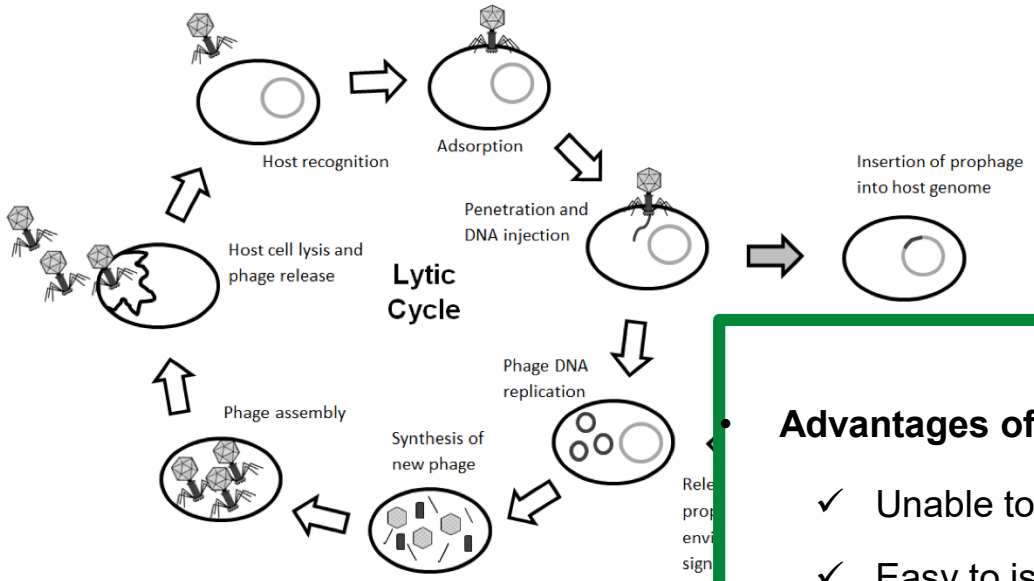
- Since 1996, only florfenicol in DK
- Resistance detected against oxolinic acid, oxytetracycline, amoxicillin and also sulphadiazine/trimethoprim



- Etiological agent of **Rainbow Trout Fry Syndrome** (**RTFS** – fry stage)
- 1st described in 1940s in USA. In the 1980s in DK. Worldwide.
- Most susceptible salmonid species: rainbow trout and coho salmon – degree of mortality and clinical signs depend on fish size.
- **Clinical signs of RTFS**: Dark skin coloration; Loss of appetite; Protrusion of the eyes; Anemia (pale gills and organs); Atrophy of kidney and intestine inflammation; Skin lesions; Splenomegaly.

Phage therapy in aquaculture

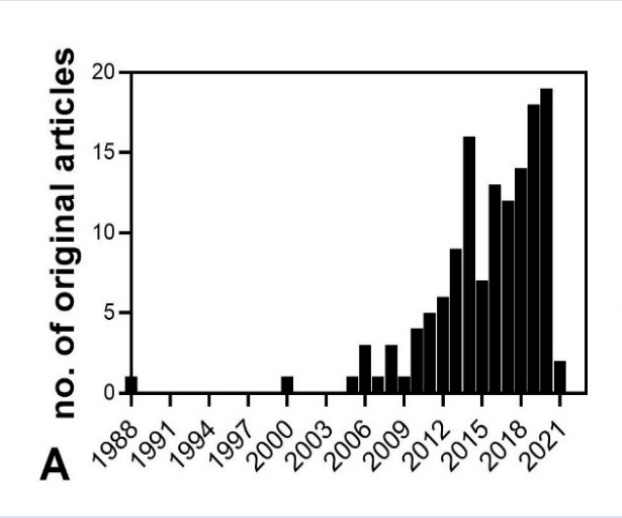
- **Virulent bacteriophages or phages**, natural enemies of bacteria and most abundant entities on our planet

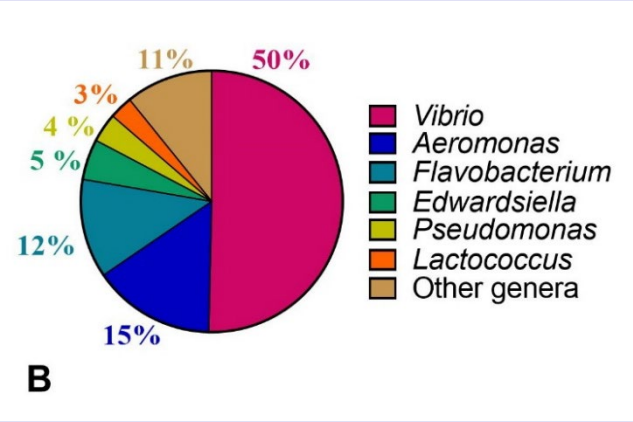


Doss, Janis; Culbertson, Kayla; Hahn, Delilah; Camacho, Joanna; Barekzi, Nazir. 2017. "A Review of Phage Therapy against Bacterial Pathogens of Aquatic and Terrestrial Organisms" *Viruses* 9, no. 3: 50. https://doi.org/10.3390/v9030050. © 2017 by the authors. Licensee MDPI, Basel, Switzerland. [Creative Commons Attribution (CC BY) license].

- **Advantages of virulent phages:**
  - ✓ Unable to replicate in eukaryotes;
  - ✓ Easy to isolate and propagate;
  - ✓ Able to kill either Gram-negative or Gram-positive bacteria;
  - ✓ Synergistic activity in cocktail preparations.

Overview of studies related to aquaculture and phage therapy





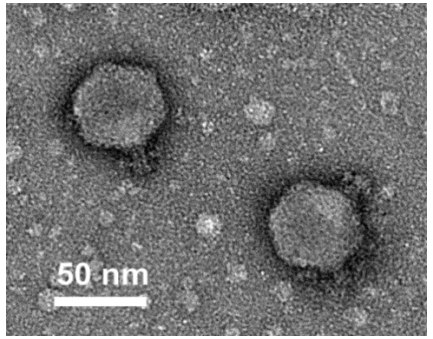
Donati V.L. (2021). Bacteriophage-based control of *Flavobacterium psychrophilum* in rainbow trout. Studies on phage-treatment of rainbow trout at fry and eyed egg stages and effects on gut microbial communities. [PhD Thesis]. [Kgs. Lyngby, DK]: Technical University of Denmark.

Phage therapy in aquaculture

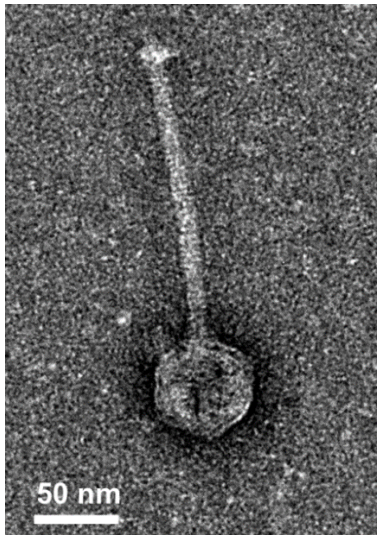
Previous experience with *F. psychrophilum* phages

In 2004-2006, 22 phages infecting *F. psychrophilum* were at first isolated from Danish rainbow trout farms (Stenholm et al. 2008).

Source: **water, water with feces or with presence of dead fish** - the Danish trout farms are important reservoirs, also in periods without RTFS.



Podoviridae



Siphoviridae

Donati V.L., Dalsgaard I., Sundell K., Castillo D., Er-Rafik M., Clark J., Wiklund T., Middelboe M. and Madsen L. (2021) Phage-Mediated Control of *Flavobacterium psychrophilum* in Aquaculture: In vivo Experiments to Compare Delivery Methods. Front. Microbiol. 12:628309. doi: 10.3389/fmicb.2021.628309. Copyright © 2021 Donati, Dalsgaard, Sundell, Castillo, Er-Rafik, Clark, Wiklund, Middelboe and Madsen [Creative Commons Attribution License (CC BY)].

Journal of Fish Diseases 2012, 35, 193–201 doi:10.1111/j.1365-2761.2011.01336.x

2012

Diversity of *Flavobacterium psychrophilum* and the potential use of its phages for protection against bacterial cold water disease in salmonids

D Castillo[1], G Higuera[1], M Villa[1], M Middelboe[2], I Dalsgaard[3], L Madsen[3] and R T Espejo[1]

1 Instituto de Nutrición y Tecnología de los Alimentos, Universidad de Chile, Santiago, Chile
2 Department of Biology, University of Copenhagen, Helsingør, Denmark
3 National Veterinary Institute, Technical University of Denmark, Kongens Lyngby, Denmark

Intraperitoneal (IP) injection of phages (10^9 PFU fish^{-1}) and bacteria (10^8 CFU fish^{-1}) in 20g fish ➔reduction in mortality of fish treated with phages.

AEM Journals.ASM.org 2013

Dispersal and Survival of *Flavobacterium psychrophilum* Phages *In Vivo* in Rainbow Trout and *In Vitro* under Laboratory Conditions: Implications for Their Use in Phage Therapy

Lone Madsen,[a] Sif K. Bertelsen,[a,b] Inger Dalsgaard,[a] Mathias Middelboe[b]
National Veterinary Institute, Technical University of Denmark, Frederiksberg C, Denmark[a]; Marine Biological Section, University of Copenhagen, Helsingør, Denmark[b]

Recovery of phages from the internal organs of rainbow trout fry after administration by IP injection (with and without the bacteria), by bath or via oral administration (oral intubation or by phage-coated feed – without bacterial infection).

AEM Journals.ASM.org 2014

Detection and Quantification of *Flavobacterium psychrophilum*-Specific Bacteriophages *In Vivo* in Rainbow Trout upon Oral Administration: Implications for Disease Control in Aquaculture

Rói Hammershaimb Christiansen,[a,b] Inger Dalsgaard,[a] Mathias Middelboe,[b] Anne H. Lauritsen,[c] Lone Madsen[a]
National Veterinary Institute, Technical University of Denmark, Frederiksberg, Denmark[a]; Marine Biological Section, University of Copenhagen, Elsinore, Denmark[b]; BioMar A/S, Brande, Denmark[c]

The results obtained by Christiansen et al. (2014) suggested that the oral delivery of phages by phage-coated feed was the most promising administration route since providing a constant concentration of phages in the fish (intestine and spleen).

Study case I

Phage-mediated control of *Flavobacterium psychrophilum* in aquaculture (rainbow trout fry)

https://doi.org/10.3389/fmicb.2021.628309

frontiers in Microbiology

ORIGINAL RESEARCH
published: 08 March 2021
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Phage-Mediated Control of *Flavobacterium psychrophilum* in Aquaculture: *In vivo* Experiments to Compare Delivery Methods

Valentina Laura Donati[1], Inger Dalsgaard[1], Krister Sundell[2], Daniel Castillo[3], Mériem Er-Rafik[4], Jason Clark[5], Tom Wiklund[2], Mathias Middelboe[3] and Lone Madsen[1]*

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Phage-based approaches have gained increasing interest as sustainable alternative strategies to antibiotic treatment or as prophylactic measures against disease outbreaks in aquaculture. The potential of three methods (oral, bath, and injection) for delivering a two-component phage mixture to rainbow trout fry for controlling *Flavobacterium psychrophilum* infections and reduce fish mortality was investigated using bacteriophages FpV4 and FPSV-D22. For the oral administration experiment, bacteriophages were applied on feed pellets by spraying (1.6×10^8 PFU g^{-1}) or by irreversible immobilization (8.3×10^7 PFU g^{-1}), using the corona discharge technology (Fixed Phage Ltd.). The fish showed normal growth for every group and no mortality was observed prior to infection as well as in control groups during the infection. Constant detection of phages in the intestine (~10^3 PFU mg^{-1}) and more sporadic occurrence in kidney, spleen, and brain was observed. When fish were exposed to *F. psychrophilum*, no significant effect on fish survival, nor a direct impact on the number of phages in the sampled organs, were detected. Similarly, no significant increase in fish survival was detected when phages were delivered by bath (1st and 2nd bath: ~10^6 PFU ml^{-1}; 3rd bath: ~10^5 PFU ml^{-1}). However, when phages FpV4 and FPSV-D22 (1.7×10^8 PFU $fish^{-1}$) were administered by intraperitoneal injection 3 days after the bacterial challenge, the final percent survival observed in the group injected with bacteriophages FpV4 and FPSV-D22 (80.0%) was significantly higher than in the control group (56.7%). The work demonstrates the delivery of phages to fish organs by oral administration, but also suggests that higher phage dosages than the tested ones may be needed on feed pellets to offer fish an adequate protection against *F. psychrophilum* infections.

Keywords: *Flavobacterium psychrophilum*, rainbow trout fry syndrome (RTFS), rainbow trout fry (*Oncorhynchus mykiss*), phage-therapy, bacteriophages

Short introduction and aim: Phage-mediated control of *Flavobacterium psychrophilum* in aquaculture

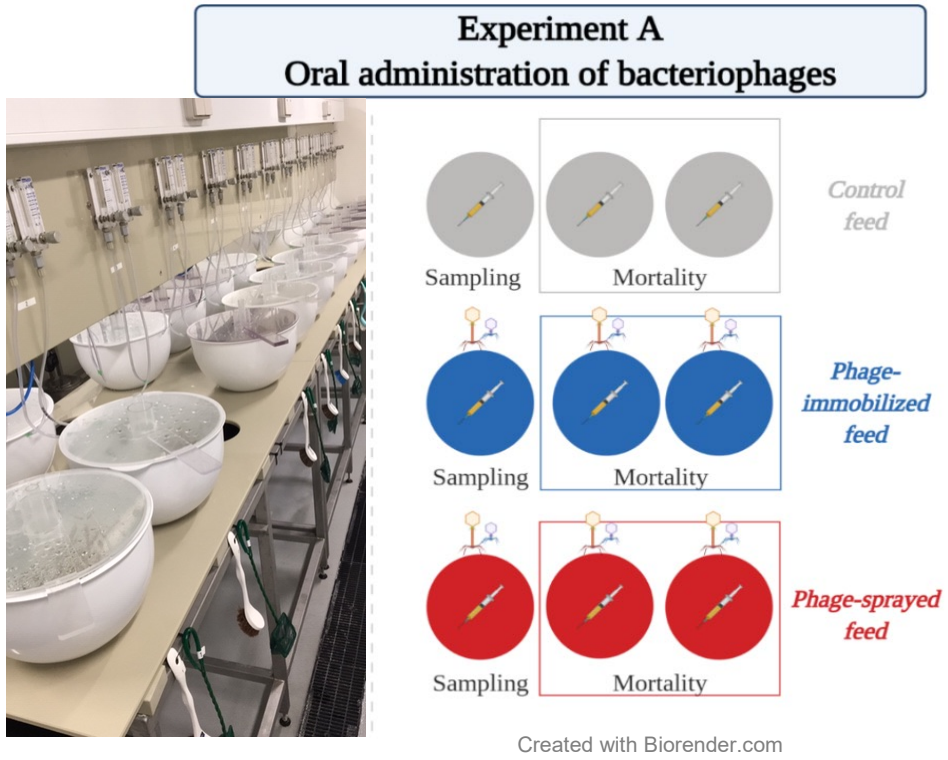
- The work includes **oral administration of bacteriophages** applied on feed pellets **by spraying or by irreversible immobilization**, using the corona discharge technology (Fixed Phage LtD)
- **Oral administration**: a strategy that could potentially be applied prophylactically in aquaculture facilities

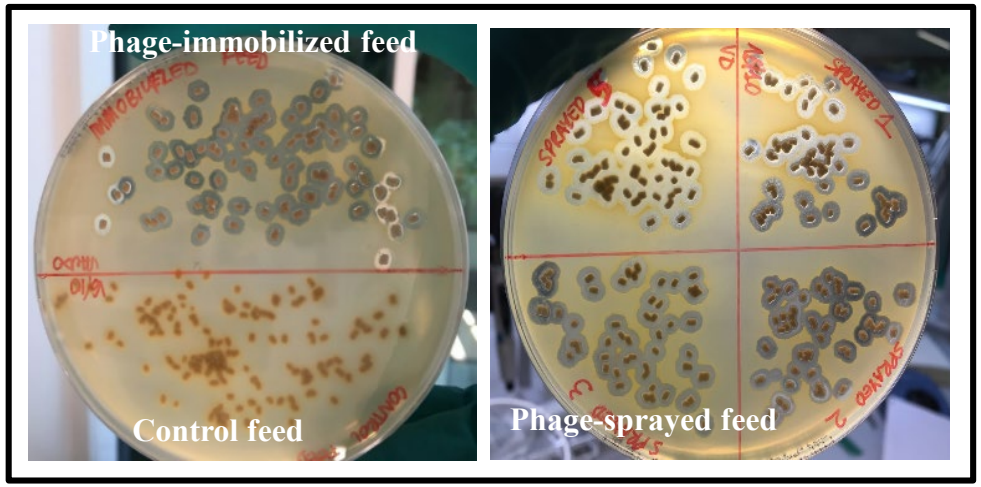
- **Aim:**
  a) Evaluate the effects of the **oral administration of phages on healthy and infected fish** comparing the two phage application methods on fish pellets (Experiment A)
     - Phage diffusion in internal organs
     - Fish health status/welfare during phage administration

  b) Assess the effects on **fish survival** of the **oral phage administration** during *F. psychrophilum* infections (Experiment A) in comparison to when phages are delivered by **repeated bath procedures** and by **intraperitoneal injection** (Experiments B and C).

Delivery of phages by phage-sprayed and phage-immobilized feed (Experiment A)

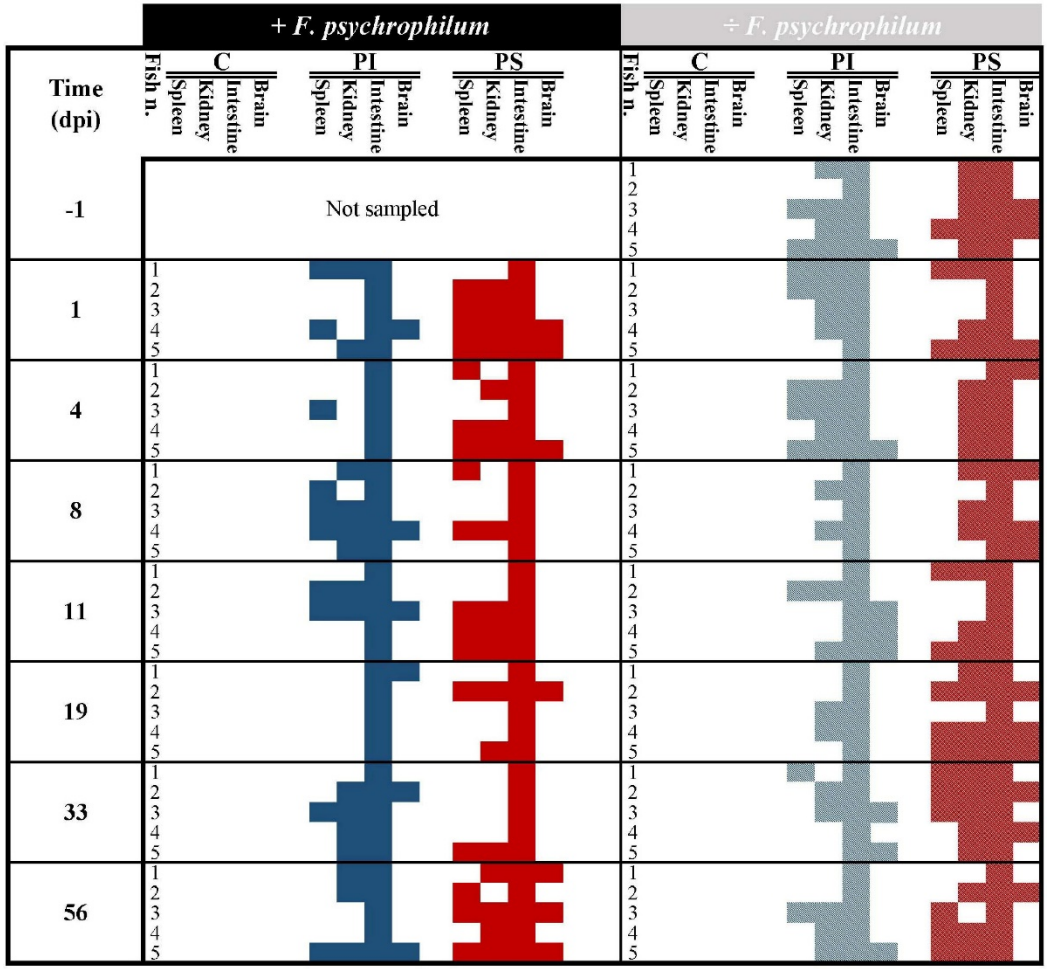
1. Production of phage-feed: application of FpV4 and FPSV-D22 (mixed 1:1) on feed pellets by
   - Fixed-Phage immobilization technique: $8.3*10^7$ PFU g^{-1}
   - Spraying: $1.6*10^8$ PFU g^{-1}



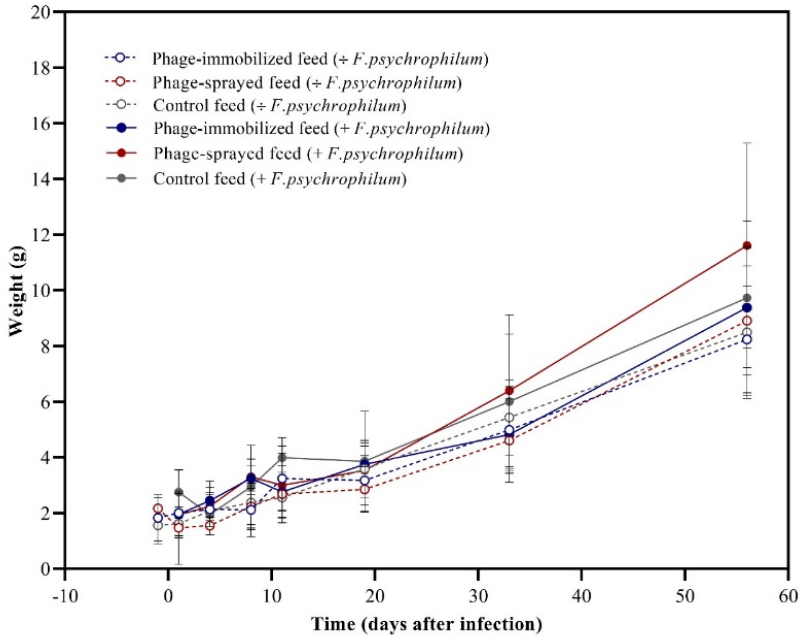


2. ~700 1.9 ± 0.7 g rainbow trout fry (~55/aquarium)
3. Phage-feed administration started 12 days before bacterial challenge
4. IP injection of *F. psychrophilum:* $1*10^4$ CFU $fish^{-1}$
5. Sampling of fish for bacteriological examination and phage detection up to 56 days p.i.
6. Evaluate fish health status: feed intake and swimming activity (behavioral observations); fin condition, presence of wounds and coloration (darkening) (external appearance); growth and abnormal mortality (production parameters)

Phage diffusion in fish and fish health status

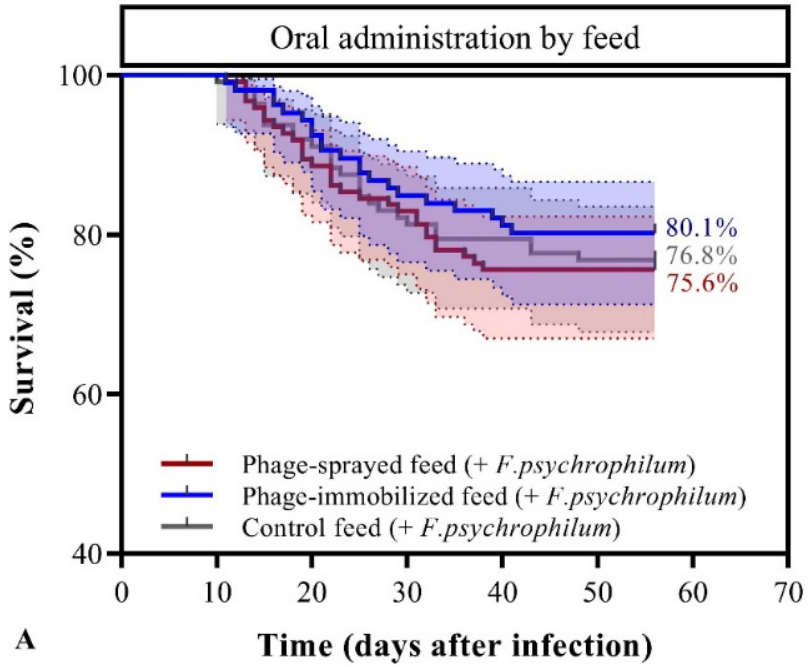


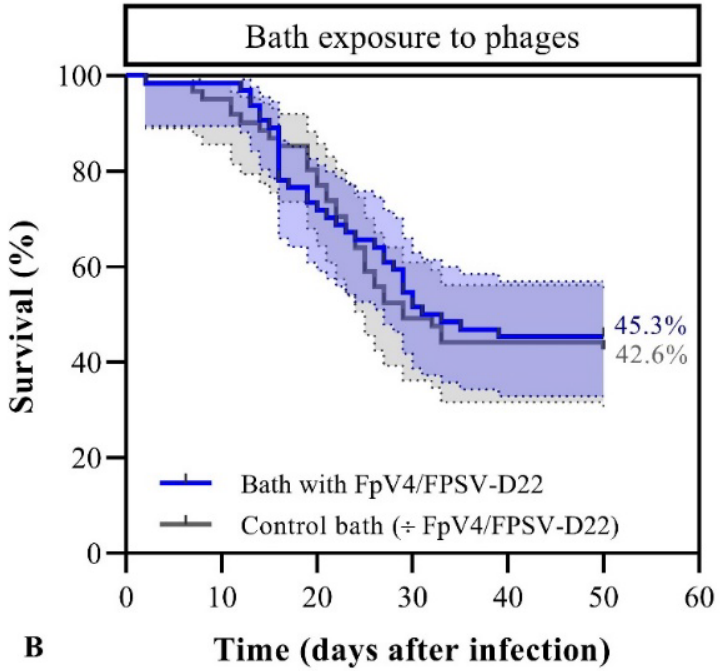
- Constant detection of phages in the intestine (100% of samples)
- More variable in kidney, spleen and brain

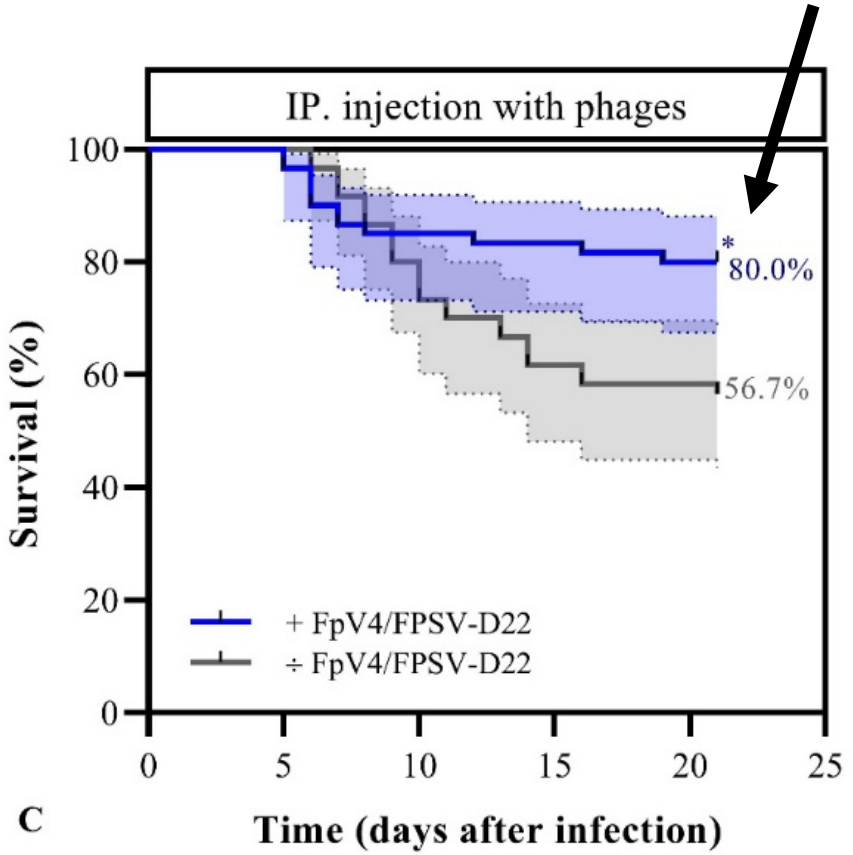


- **Positive growth** was detected for all groups and **no mortalities** were observed prior to infection.
- The addition of phages in either way **did not seem to change the taste of the feed** for the fish and the fish ate the amount of feed that they were offered (fish not challenged with the bacterium and prior to infection in all groups).
- **Other visual signs of improper health** as destroyed fins, lethargic swimming, color changes and skin ulceration were **not seen** prior to infection and in non-challenged groups.

Survival of rainbow trout after *F. psychrophilum* challenge: comparison of phage delivery methods







To sum up

a) Evaluate the effects of the **oral administration of phages on healthy and infected fish** (Experiment A):

- Phage diffusion in internal organs:
  - **constant in the intestine, variable in kidney and spleen**
  - **a clear relation between the presence of the bacteria and the number of phages was not detected**
- Fish health status during phage administration:
  - **application of phages on feed pellets do not affect the fish health**

b) Assess the effects on **fish survival** of the **oral phage administration** during *F. psychrophilum* infections (Experiment A) in comparison to when phages are delivered by **repeated bath procedures** and by **intraperitoneal injection** (experiments B and C):

- **No beneficial effect observed in case of oral or bath phage delivery**
- **Significant increase in fish survival when phages were delivered by IP**

Study case II

Phages as *F. psychrophilum* control agents in rainbow trout eyed eggs

https://doi.org/10.3390/microorganisms9050971

microorganisms MDPI

Article

Interactions between Rainbow Trout Eyed Eggs and *Flavobacterium* spp. Using a Bath Challenge Model: Preliminary Evaluation of Bacteriophages as Pathogen Control Agents

Valentina L. Donati [1,*], Inger Dalsgaard [1], Anniina Runtuvuori-Salmela [2], Heidi Kunttu [2], Johanna Jørgensen [3], Daniel Castillo [3,†], Lotta-Riina Sundberg [2], Mathias Middelboe [3] and Lone Madsen [1]

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Abstract: The microbial community surrounding fish eyed eggs can harbor pathogenic bacteria. In this study we focused on rainbow trout (*Oncorhynchus mykiss*) eyed eggs and the potential of bacteriophages against the pathogenic bacteria *Flavobacterium psychrophilum* and *F. columnare*. An infection bath method was first established, and the effects of singular phages on fish eggs was assessed (survival of eyed eggs, interaction of phages with eyed eggs). Subsequently, bacteria-challenged eyed eggs were exposed to phages to evaluate their effects in controlling the bacterial population. Culture-based methods were used to enumerate the number of bacteria and/or phages associated with eyed eggs and in the surrounding environment. The results of the study showed that, with our infection model, it was possible to re-isolate *F. psychrophilum* associated with eyed eggs after the infection procedure, without affecting the survival of the eggs in the short term. However, this was not possible for *F. columnare*, as this bacterium grows at higher temperatures than the ones recommended for incubation of rainbow trout eyed eggs. Bacteriophages do not appear to negatively affect the survival of rainbow trout eyed eggs and they do not seem to strongly adhere to the surface of eyed eggs either. Finally, the results demonstrated a strong potential for short term (24 h) phage control of *F. psychrophilum*. However, further studies are needed to explore if phage control can be maintained for a longer period and to further elucidate the mechanisms of interactions between Flavobacteria and their phages in association with fish eggs.

Keywords: *Flavobacterium psychrophilum*; *Flavobacterium columnare*; rainbow trout; eyed eggs; phage-mediated control; bacteriophages

Short introduction and aim: Eyed eggs, *Flavobacterium psychrophilum* and phages

- The physical barrier of the thin chorion (*zona pellucida*) and the thicker inner membrane (*zona radiata*) of teleost eggs represents the first line of defense against bacterial and viral infections.

- The wide range of the bacteria that surrounds the eggs will contribute to the early establishment of the fish microbiota. Within these microbial communities, pathogenic bacteria such as *Cytophaga* spp., *Flavobacterium* spp., *Vibrio* spp., *Pseudomonas* spp., and *Aeromonas* spp. also exist, and may represent threats for the development and survival of the fish.

Aims of the study

a) To establish a bacterial challenge bath method (Section A)

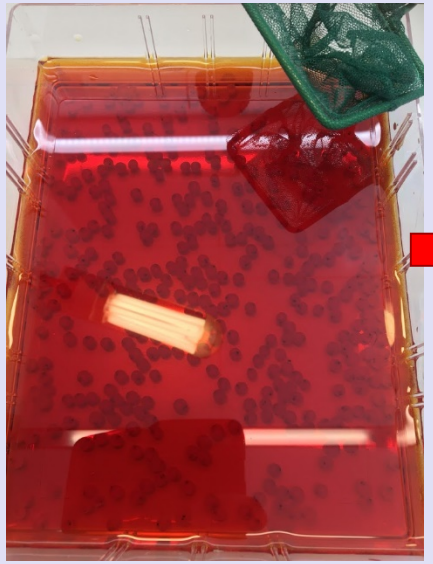
b) To evaluate the effects of phage addition on eyed eggs (Section B).

c) To expose rainbow trout eyed eggs to phages in order to assess their efficiency in eliminating the target bacterium (Section C)

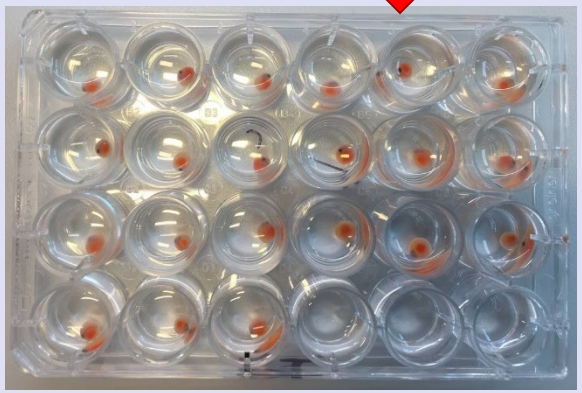
Section A: Set up of a bacterial challenge bath method

Disinfection:

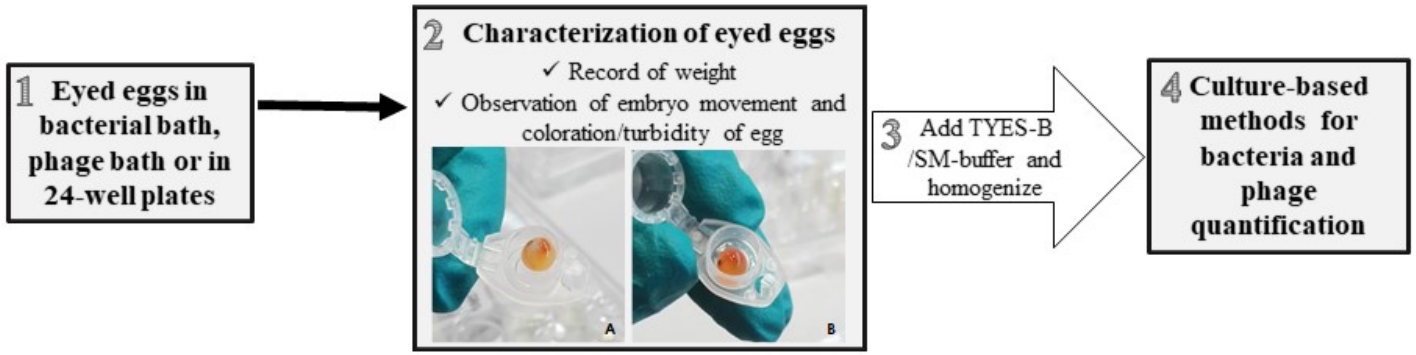
Iodophor-based solution



Bacterial bath: 2 hours at 10ºC



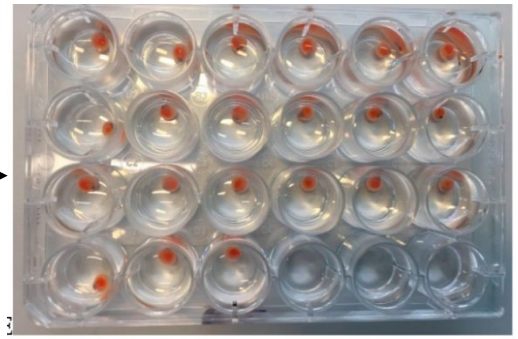
Transfer to 24-well plates with 2 ml Milli-Q water

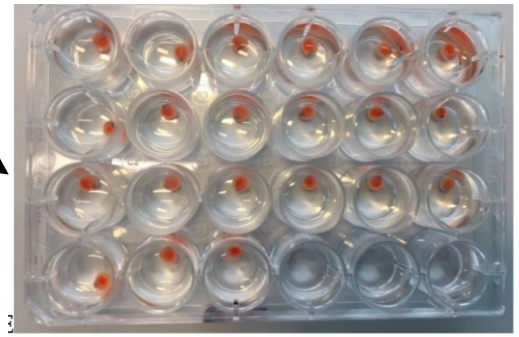


Section B: Interactions of phages with rainbow trout eyed eggs

Bath exposure

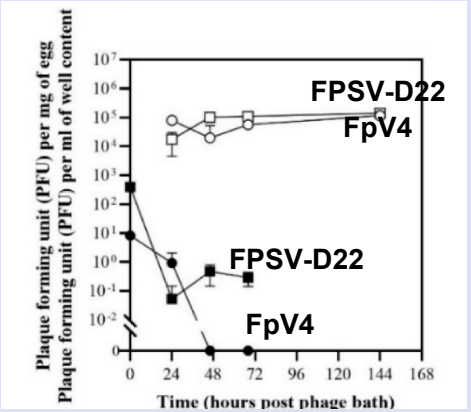
Constant exposure





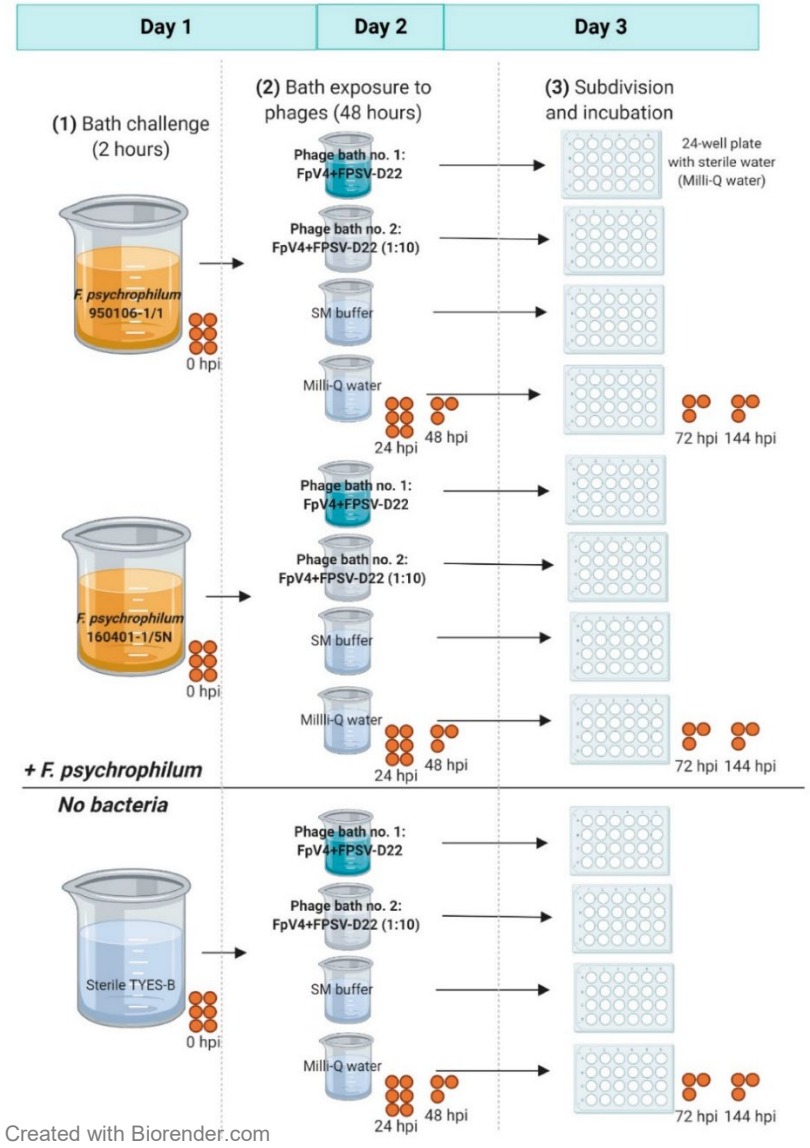
Results in brief:

- ✓ Phages do not seem to affect negatively the eggs' survival
- ✓ TYES-B medium decreases eggs survival
- ✓ Phage-egg membrane interactions: the number of phages in connection with eggs decreases over time in bath exp.



Section C: Phages as control agents

Exp. I, Section C: Phage bath of *F. psychrophilum* challenged eyed eggs



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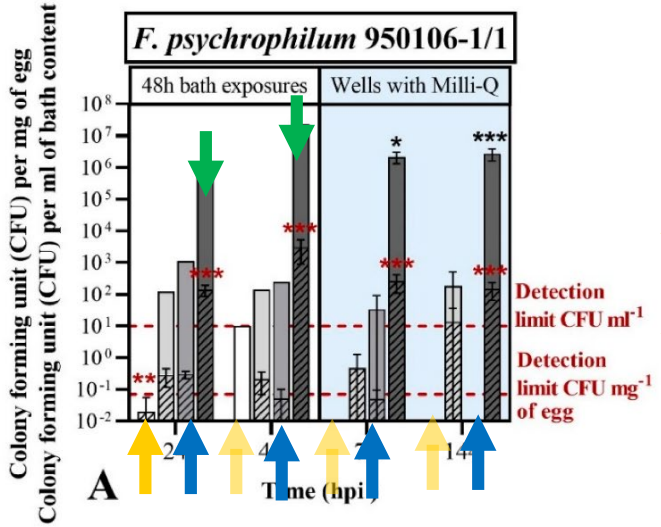

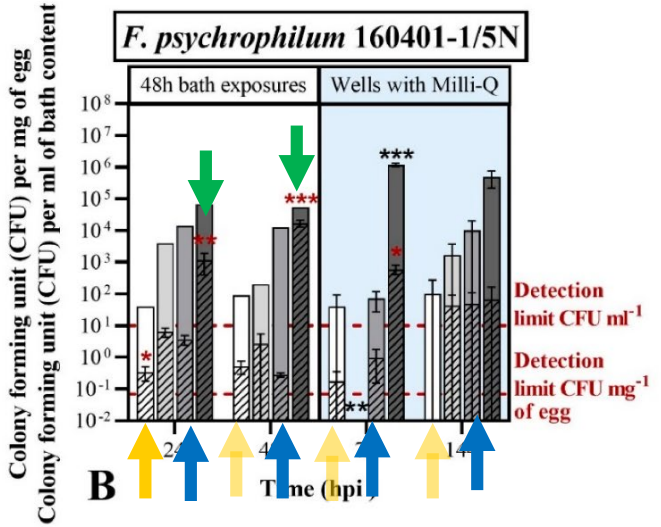

F. psychrophilum in connection with eyed eggs and bath/well content

- 24hpi: decrease if F.p in eggs/bath no.1 compared to SM bath
- Only temporary. Control lost after 24 hpi.
- Fp growth increasingly negatively affected by SM buffer
- 24 and 48 hpi: 160401-1/5N 10-fold higher conc. than 950106/1/1 in connection with eggs

Bacteria in the corresponding bath/wells
- Phage bath no. 1
- Phage bath no. 2
- Control bath (SM buffer)
- Control bath (Milli-Q water)

Bacteria in connection with eyed eggs
- Phage bath no. 1
- Phage bath no. 2
- Control bath (SM buffer)
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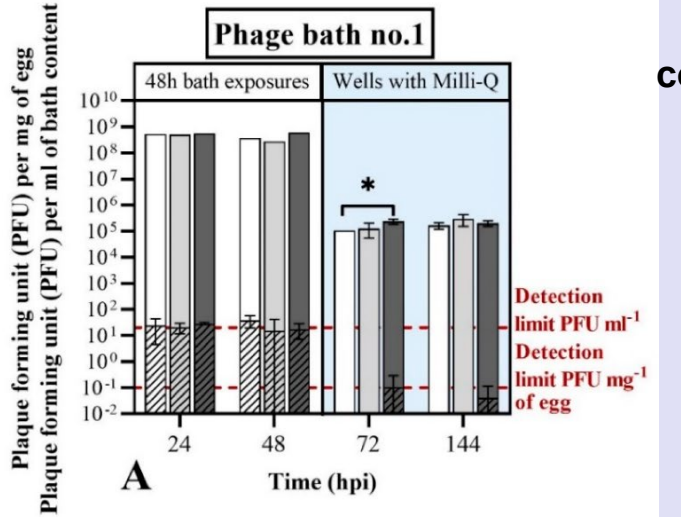

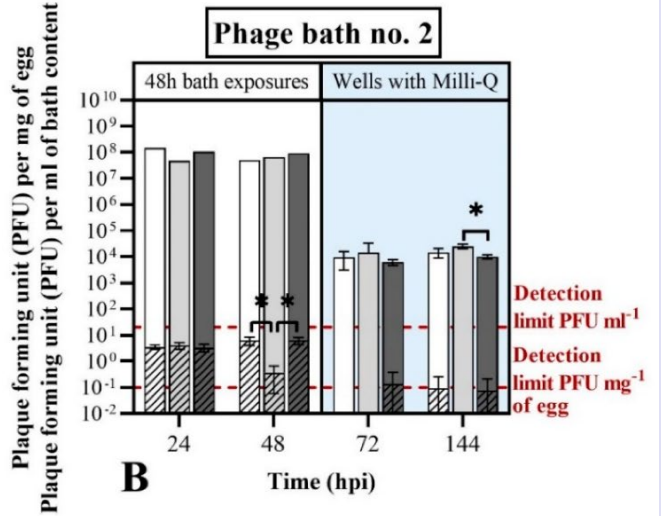

FpV4/FPSV-D22 in connection with eyed eggs and bath/well content

Phages constant in connection with the eggs during the bath – lost after transfer to wells even if their concentration was maintained in the wells over time

Phages in the corresponding bath/wells
- + *F. psychrophilum* 950106-1/1
- + *F. psychrophilum* 160401-1/5N
- - *F. psychrophilum*

Phages in connection with eyed eggs
- + *F. psychrophilum* 950106-1/1
- + *F. psychrophilum* 160401-1/5N
- - *F. psychrophilum*

To sum up

a) With our infection model, it was possible to **re-isolate *F. psychrophilum* associated with eyed eggs,** without affecting the survival of the eggs in the short term (aim a).

- The used experimental set up allowed the study of bacteria/phage interactions with eyed eggs at a small scale under controlled conditions
- The experimental set up might also be applied for other pathogenic bacteria.

b) **Phages did not appear to negatively affect the survival of rainbow trout eyed eggs nor to strongly adhere** to the surface of eyed eggs (aim b).

c) Demonstrated **a strong potential for short term (24h) phage control of *F. psychrophilum*** (aim c).

OVERALL CONCLUSIONS AND FUTURE PERSPECTIVES

- Phage therapy: valuable alternative to antibiotic use →The results obtained support the potential of using phages for *F. psychrophilum* prevention and control but also reveal challenges that should be evaluated in the future studies.

- **Study case I [*Donati V.L. et al. (2021) Phage-Mediated Control of Flavobacterium psychrophilum in Aquaculture: In vivo Experiments to Compare Delivery Methods. Front. Microbiol. 12:628309. doi: 10.3389/fmicb.2021.628309*]:**
  - Oral delivery of phages applied on feed pellets by Fixed Phage Ltd technology: an effective method of delivering phages to the intestine
  - The main reason for the lack of a beneficial effect on fish survival: the inefficient phage delivery to the fish organs
  - The hypothesis that delivering higher phage dosages at the infection site could positively increase the fish recovery/survival is supported by the significant increase in fish survival after intraperitoneal administration
  - ➢ Creating **stable highly concentrated phage solutions**.
  - ➢ An alternative option: **combination of phages with other antimicrobial agents or dietary supplements** e.g. antibiotics, probiotics

- **Study case II [*Donati V.L. et al. (2021) Interactions between Rainbow Trout Eyed Eggs and Flavobacterium spp. Using a Bath Challenge Model: Preliminary Evaluation of Bacteriophages as Pathogen Control Agents. Microorganisms 9:971. doi: 10.3390/ microorganisms9050971*]:**
  - Rainbow trout eyed eggs are not negatively affected by *Flavobacterium spp.* phages
  - ➢ Next step: **better understand the mechanisms of interactions bacteria/phages/rainbow trout eyed eggs** (e.g. by microscopy-based techniques) and to further explore **if phage control can be maintained beyond 24 h**.

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Donati V.L. (2021) Bacteriophage-based control of *Flavobacterium psychrophilum* in rainbow trout. Studies on phage-treatment of rainbow trout at fry and eyed egg stages and effects on gut microbial communities. [PhD Thesis]. [Kgs. Lyngby, DK]: Technical University of Denmark.

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Thank you for your attention ☺