Analysis of gfp-labelled bacteria in the gastrointestinal tract of germ-free sea bass larvae by confocal microscopy

A. Rekecki[1], R.A.Y.S.A. Gunasekara[1], K. Dierckens[2], H. Favoreel[3], N. Boon[4], P. Bossier[2], W. Van den Broeck[1]

[1]Department of Morphology, Faculty of Veterinary Medicine, Ghent University
[2]Laboratory of Aquaculture & Artemia Reference Center, Department of Animal Production, Faculty of Bioscience Engineering, Ghent University
[3]Department of Virology, Parasitology, and Immunology, Faculty of Veterinary Medicine, Ghent University
[4]Laboratory of Microbial Ecology and Technology (LabMET), Faculty of Bioscience Engineering, Ghent University
E-mail: Anamaria.Rekecki@Ugent.be

Introduction

1. Axenic sea bass system (Dierckens et al., 2009)

2. Histological and morphometrical comparison of axenic / conventional larvae (Rekecki et al., 2009)

Ax sea bass DAH6

3. Standardized challenge test with *Listonella anguillarum*

Introduction

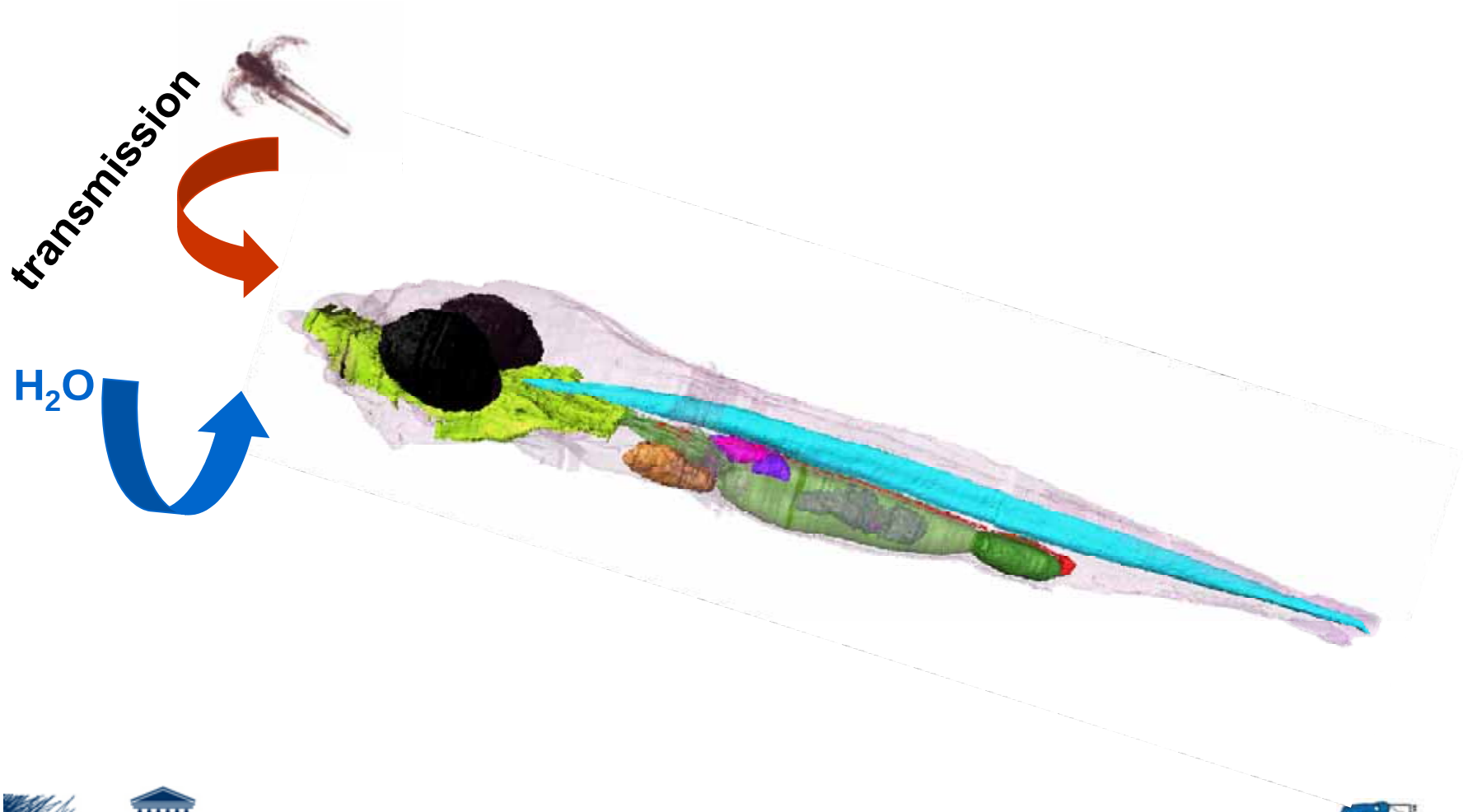
Localisation of the pathogen in the larval gut by using confocal microscopy

3. Standardized challenge test with *Listonella anguillarum*


Sea bass - DAH14

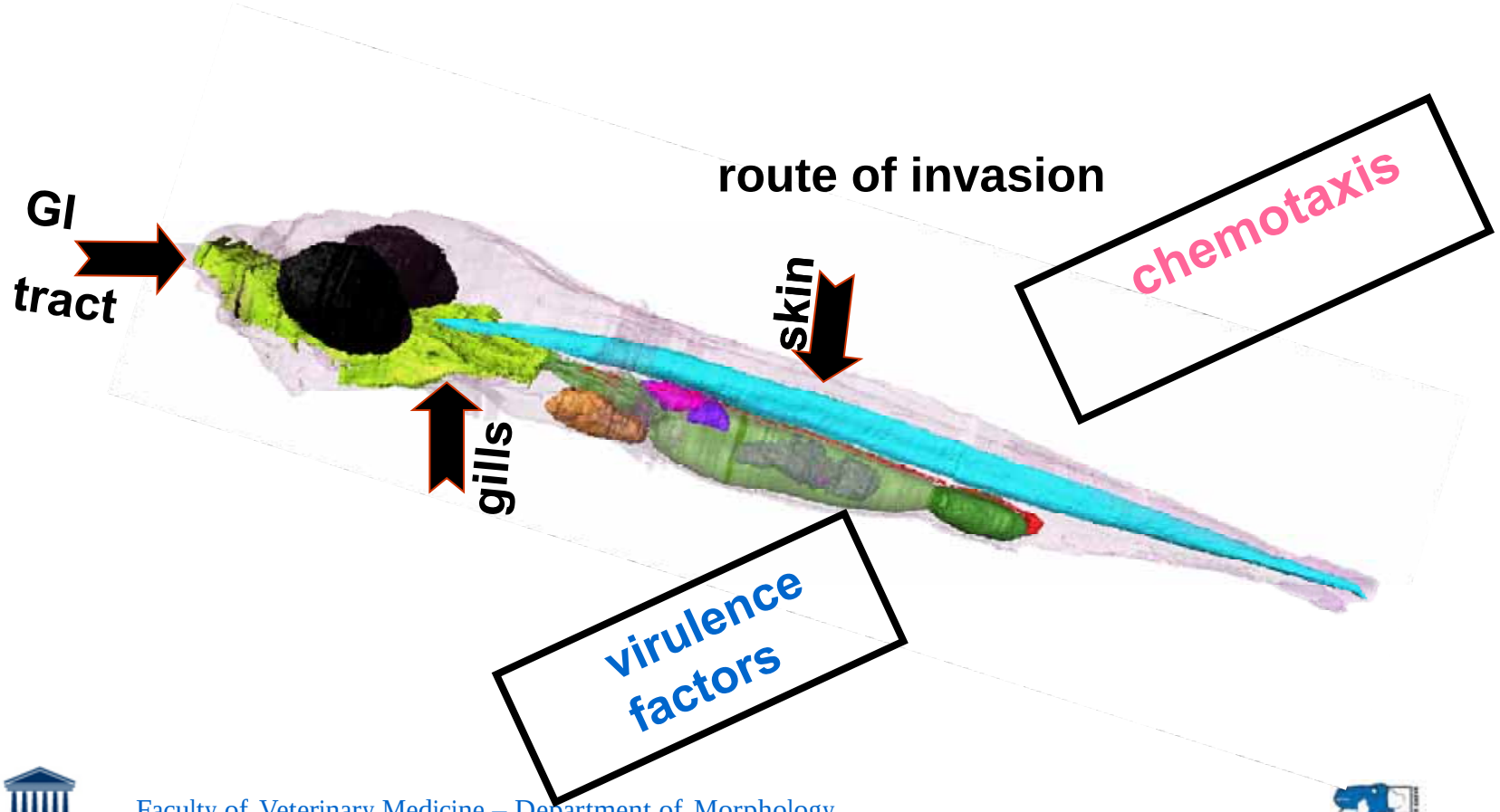
Introduction

Listonella anguillarum



Introduction

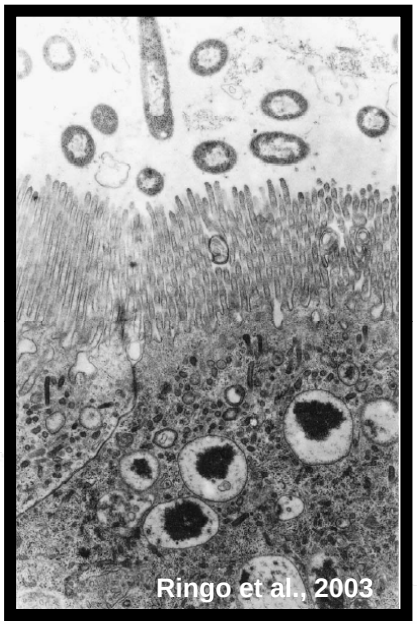
Listonella anguillarum



Introduction

Listonella anguillarum

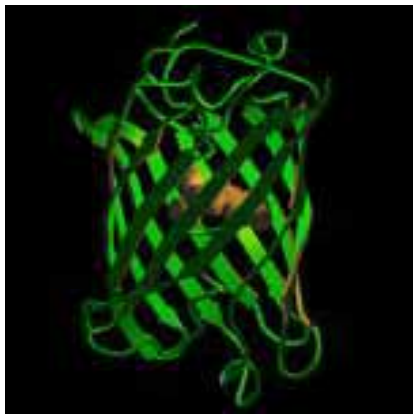
endocytosis



Introduction

Green Fluorescent Protein (Gfp)

- Fluorescent protein isolated from jellyfish *Aequoria victoria*



- Biomarker:
  - gene expression
  - protein localisation
  - protein-protein interaction in living cells
  - host-microbe interaction

Introduction

Confocal microscopy

- Overcome limitations of traditional wide-field fluorescence microscope
- Optical imaging technique
- Three-dimensional images
- Optical sectioning - X,Y and Z axis

LEICA TCS SP2 confocal microscope and an Argon 488nm excitation laser line (Leica Microsystems, GmbH, Heidelberg, Germany).

powerful approach to study bacteria *in situ* during colonisation of the host

The aim of this study:

visualisation of bacteria during early steps of infection

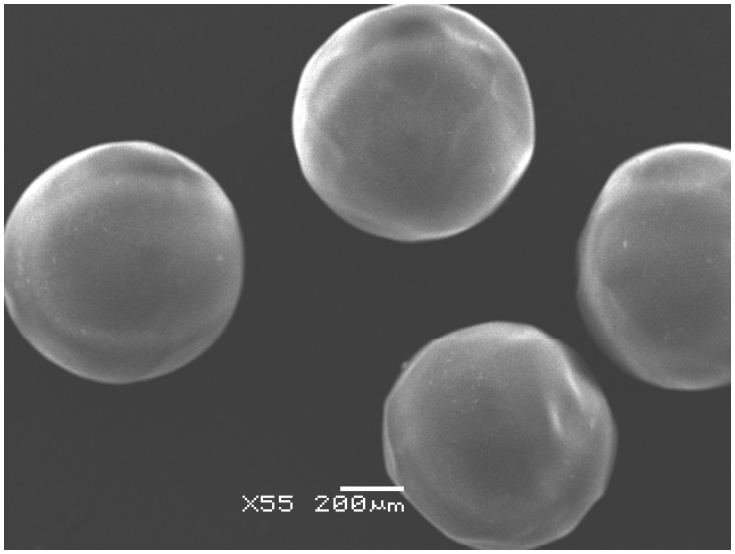
TEST: confocal imaging

1. Fluorescent beads (positive control)
2. Gfp-labelled *Escherichia coli* / *Comamonas testosteroni*
3. Gfp-labelled *Listonella anguillarum* HI610

UNIVERSITEIT GENT
Faculty of Veterinary Medicine – Department of Morphology
Faculty of Bioscience Engineering – Laboratory of Aquaculture & Artemia Reference Center

Axenic system

Disinfection procedure of eggs:



- Glutaraldehyde
  (broad spectrum chemosterilizer):
  100 mg/L for 3 minutes

- Incubation:
  in 10 mg/L rifampicin and 10 mg/L ampicillin
  filtered (0.2 µm), autoclaved seawater
  1500 eggs/L

Axenic system

Set up for larvae culture:

- Vials of 10 mL
- 12 fish larvae/replicate
- 10 mg/l rifampicin/vial
- Rotation 4rpm, longitudinal axis
- Feeding *Artemia* nauplii from DAH 7 (30 nauplii / vial) each second day
- Replicates are discarded after 1 sampling
- Axenity of eggs and larvae was tested by using 10% MB and 10%MA

Test 1: fluorescent green beads

- Sea bass eggs disinfected - standard protocol for axenic conditions (Dierckens et al., 2009)
- Incubation: 10^6 / mL DAH3 and DAH4 (1µm size)
- Sampling: on DAH4 and DAH5, after the first and second incubation

- Larvae were killed with an overdose of benzocain
- The external body surface was disinfected with benzalconium chloride
- Fixated with paraformaldehyde and stored at 4°C
- The whole larval body was individually mounted on glass slides
- Image series of larvae were acquired using a LEICA TCS SP2 confocal microscope and an Argon 488nm excitation laser line (Leica Microsystems, GmbH, Heidelberg, Germany).

Results

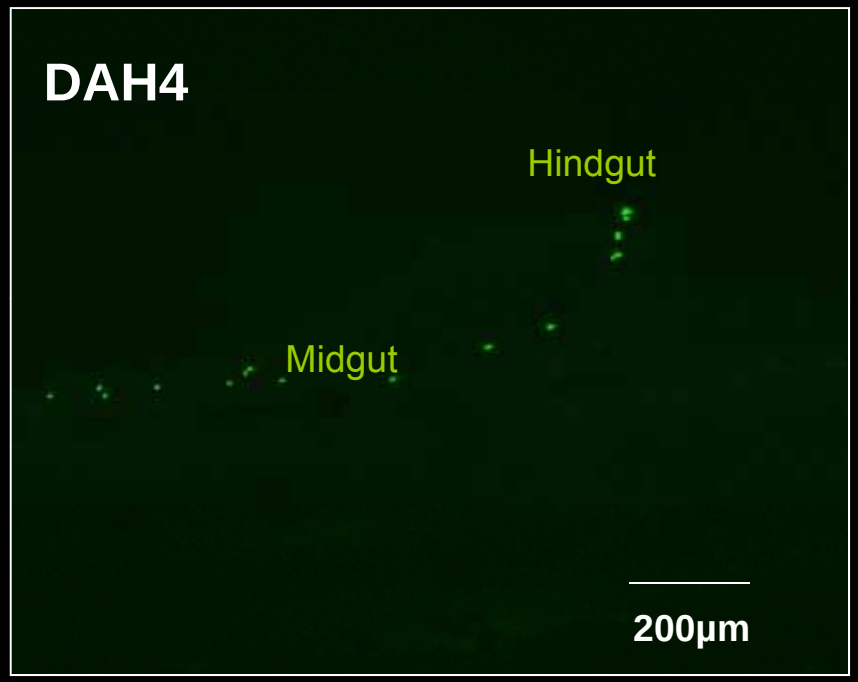


Fig.1a. Green beads in mid- and hindgut in DAH4 sea bass larva (confocal microscope),

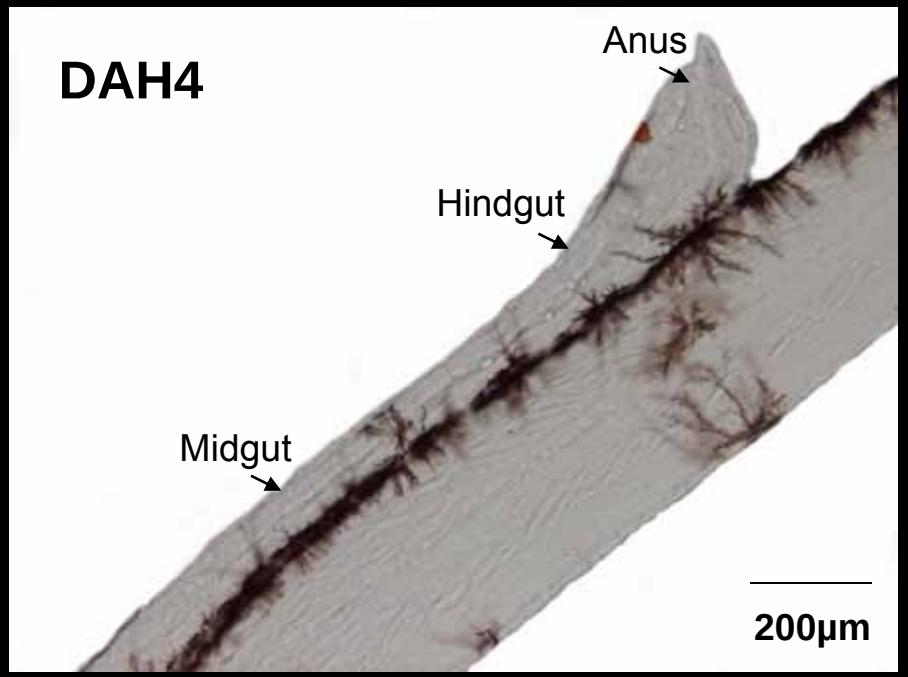


Fig.1b. Mid- and hindgut of larvae (LM)

UNIVERSITEIT GENT
Faculty of Veterinary Medicine – Department of Morphology
Faculty of Bioscience Engineering – Laboratory of Aquaculture & Artemia Reference Center

Results

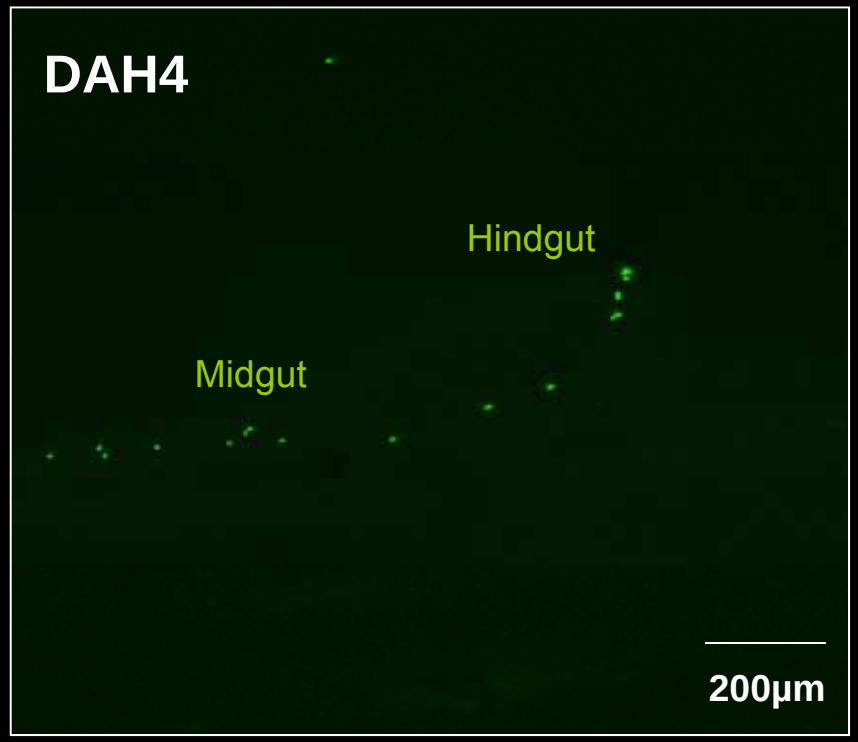


Fig.1a. Green beads in mid- and hindgut in DAH4 sea bass larva (confocal microscope),

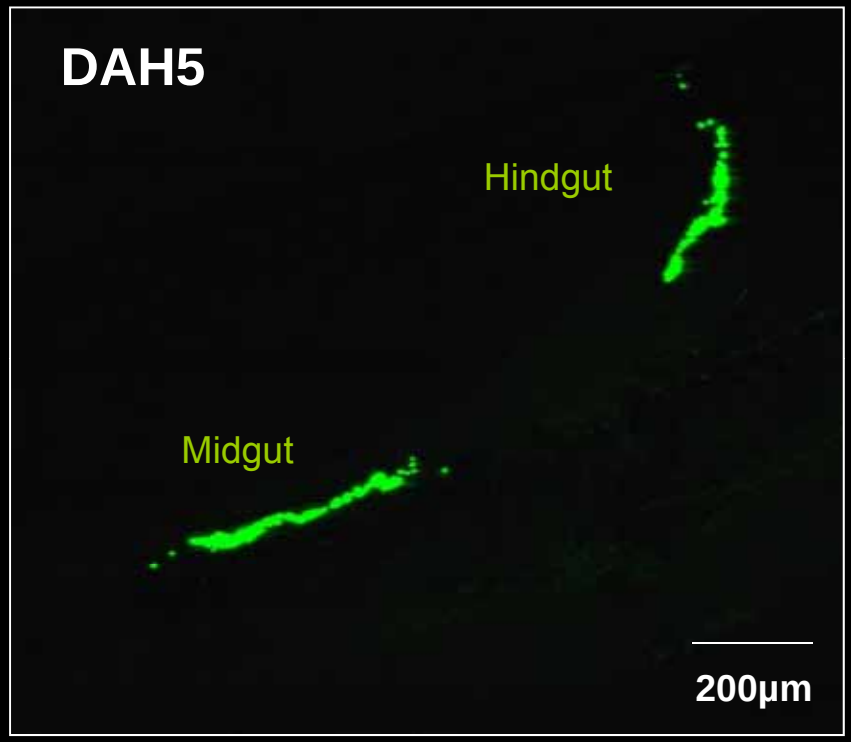


Fig.1b. Green beads visible in clusters in DAH5 larva.

UNIVERSITEIT GENT
Faculty of Veterinary Medicine – Department of Morphology
Faculty of Bioscience Engineering – Laboratory of Aquaculture & Artemia Reference Center

- After 24 hours of incubation - green beads in both the mid- and hindgut.

- After 48 hours from the first incubation (repeated incubations took place on DAH3 and DAH4), beads in the gastric region, mid- and hindgut of DAH5 larvae.

Test 2: gfp-labelled bacteria

- Sea bass eggs disinfected - standard protocol for axenic conditions (Dierckens et al., 2009)

- Challenge: gfp-labelled *Escherichia coli* (10^8 CFU/mL)
  gfp-labelled *Comamonas testosteroni* (10^8 CFU/mL)

- Sampling: on DAH4 and DAH5, after the first and second incubation

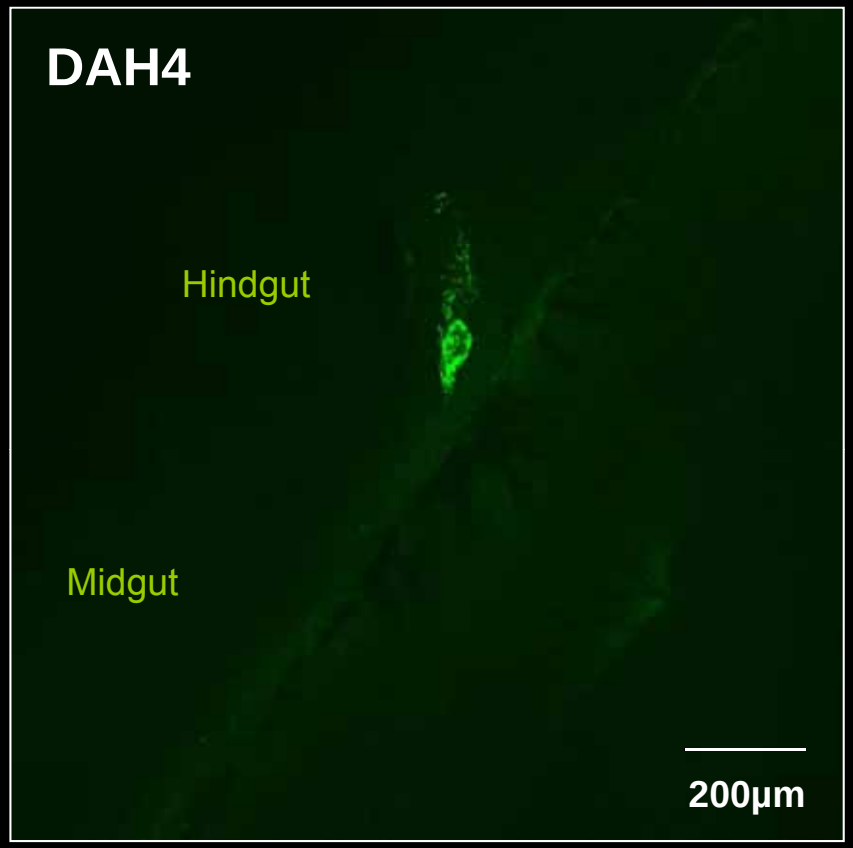


Fig.1a. *E. coli* in hindgut of DAH4 larva

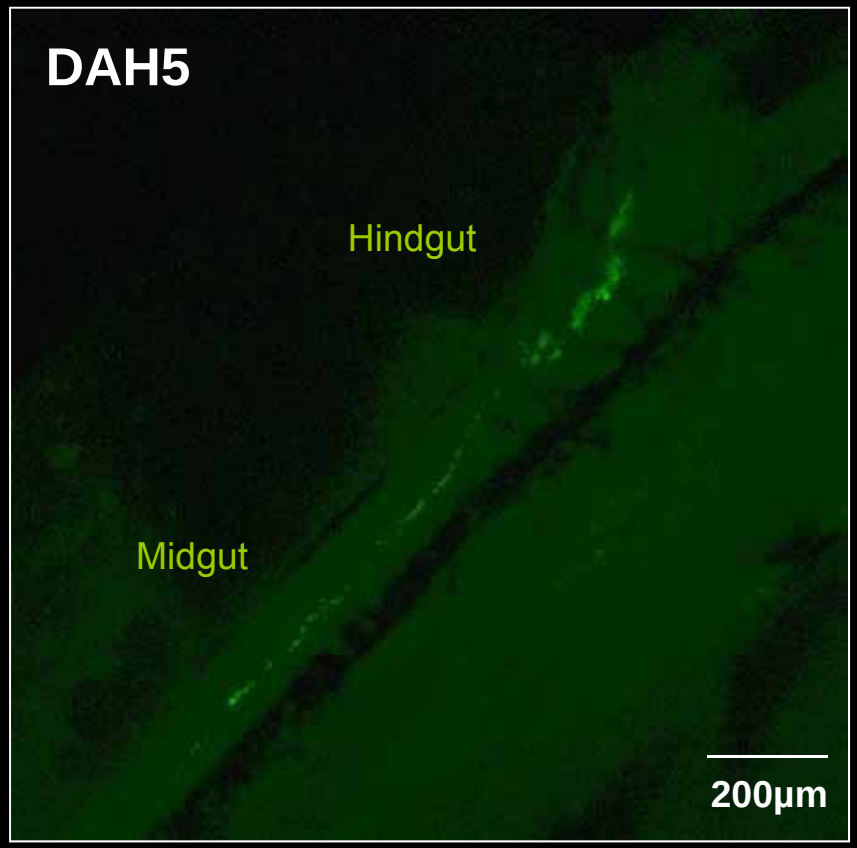


Fig.1b. *E. coli* in mid- and hindgut of DAH5 larva

UNIVERSITEIT GENT
Faculty of Veterinary Medicine – Department of Morphology
Faculty of Bioscience Engineering – Laboratory of Aquaculture & Artemia Reference Center

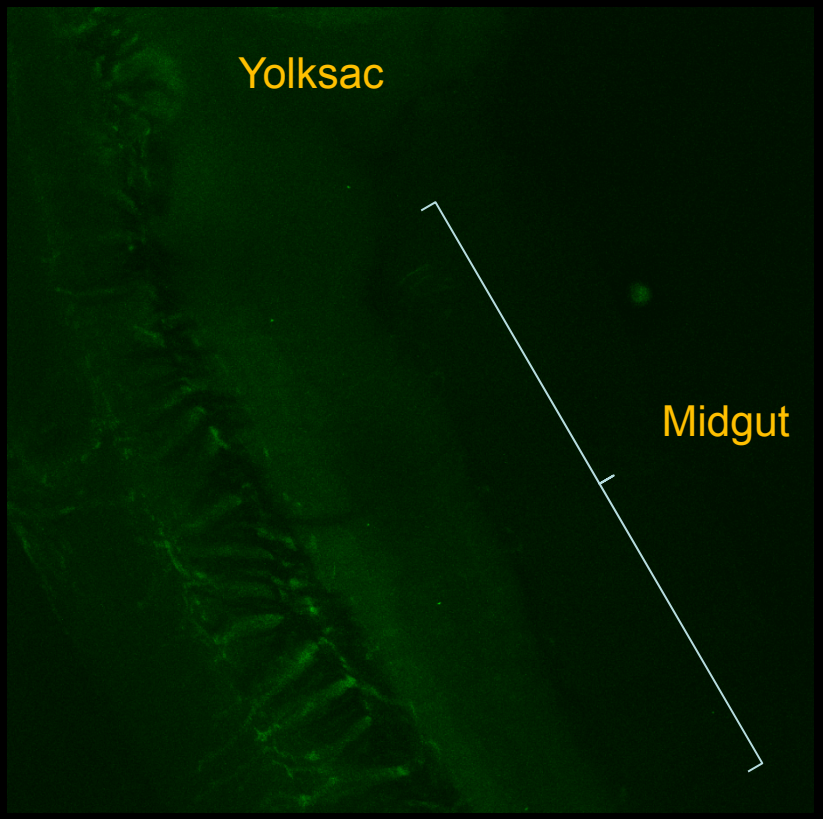
Test 3a: gfp-labelled *Listonella anguillarum* HI610

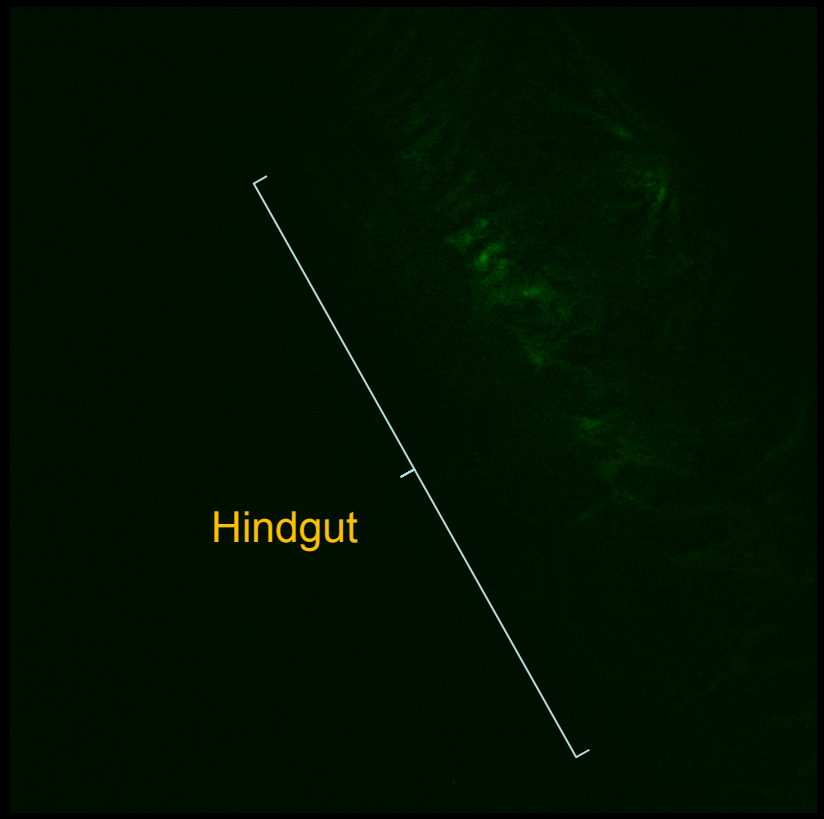
- Sea bass eggs disinfected - standard protocol for axenic conditions (Dierckens et al., 2009)

- Challenge on DAH4: 10^3 CFU/mL
  10^5 CFU/mL
  10^7 CFU/mL

- Sampling on DAH5 and DAH6: 24hrs and 48hrs after infection

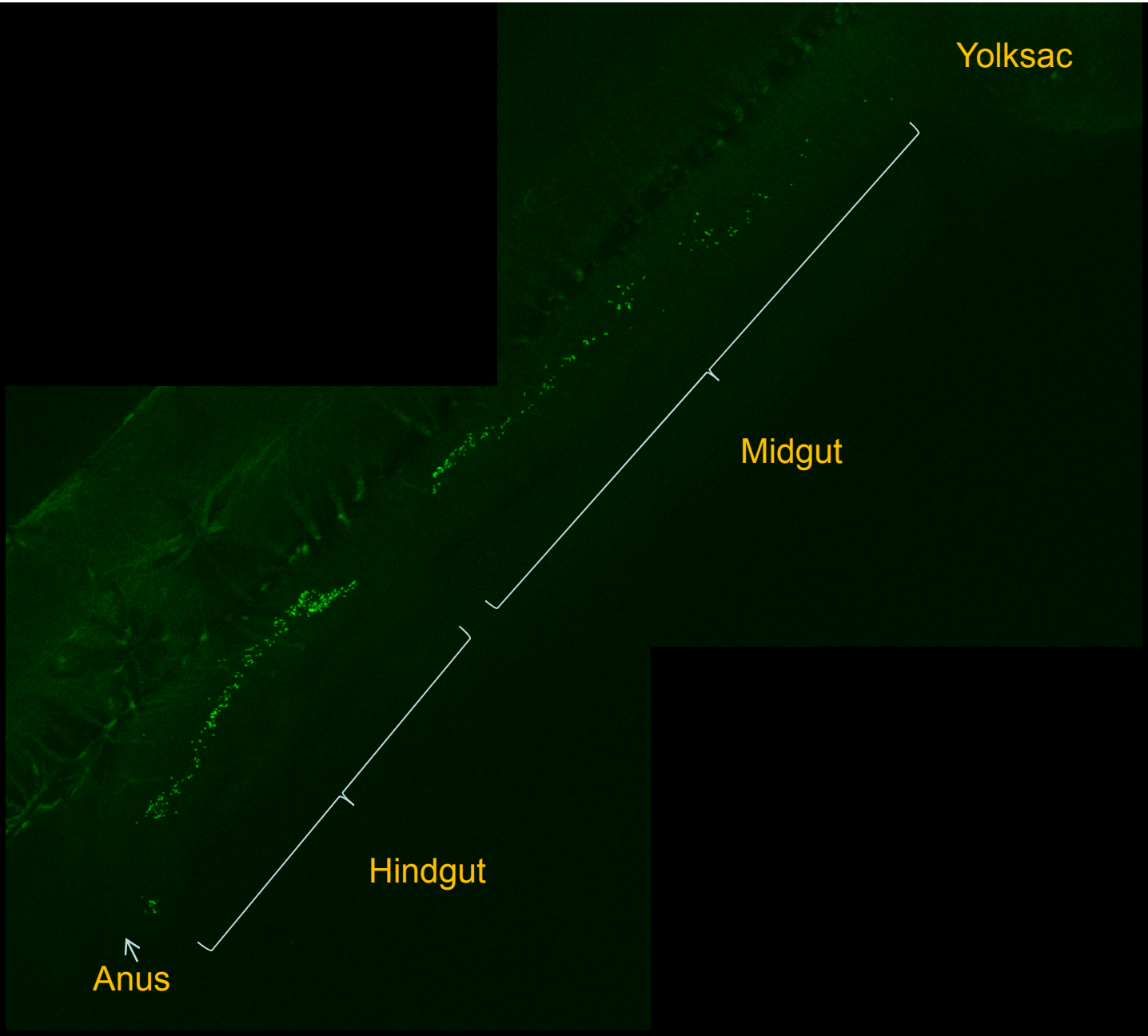
10^3 CFU/mL after 48hrs of infection





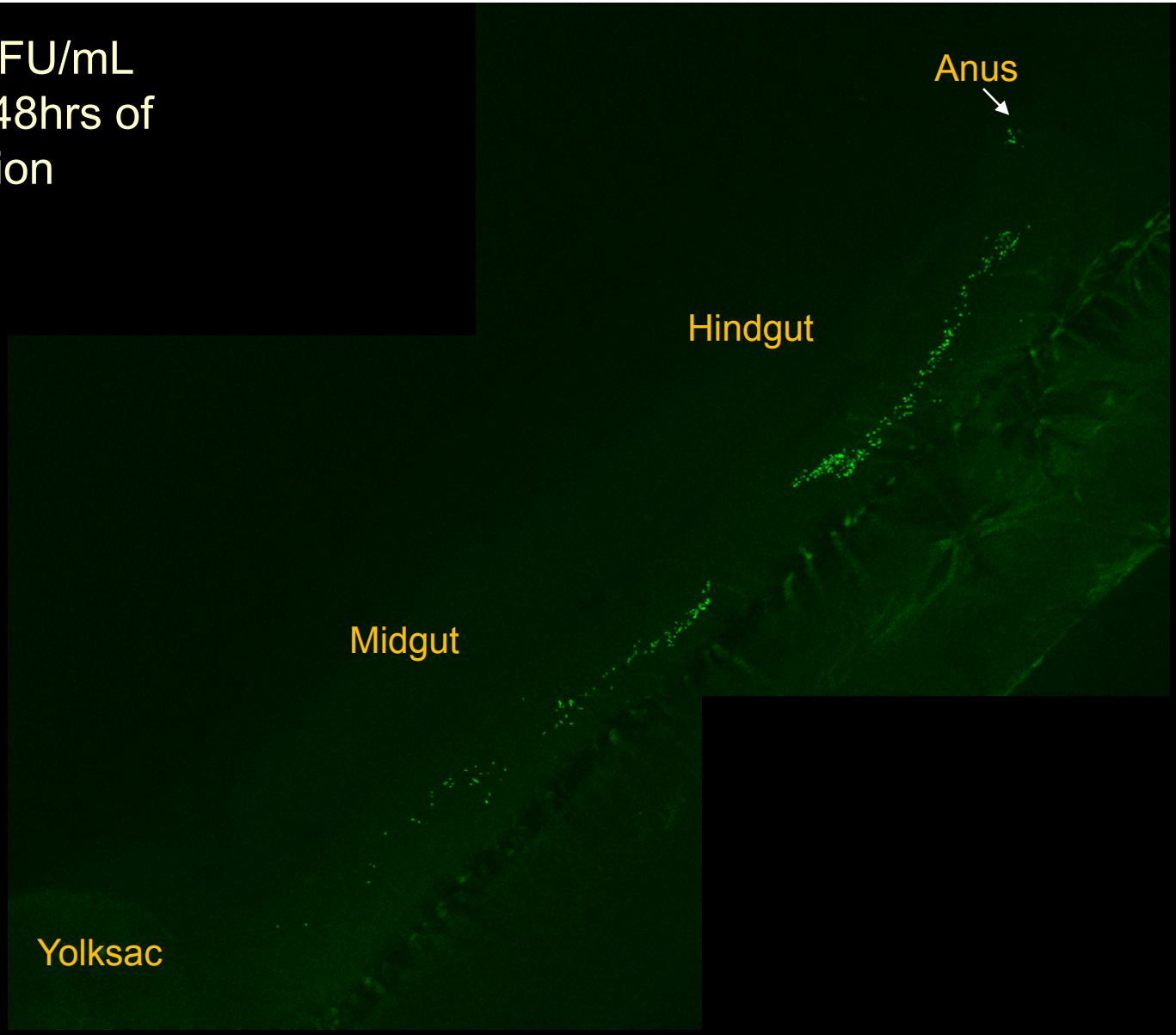
UNIVERSITEIT GENT
Faculty of Veterinary Medicine – Department of Morphology
Faculty of Bioscience Engineering – Laboratory of Aquaculture & Artemia Reference Center

10^5 CFU/mL after 48hrs of infection



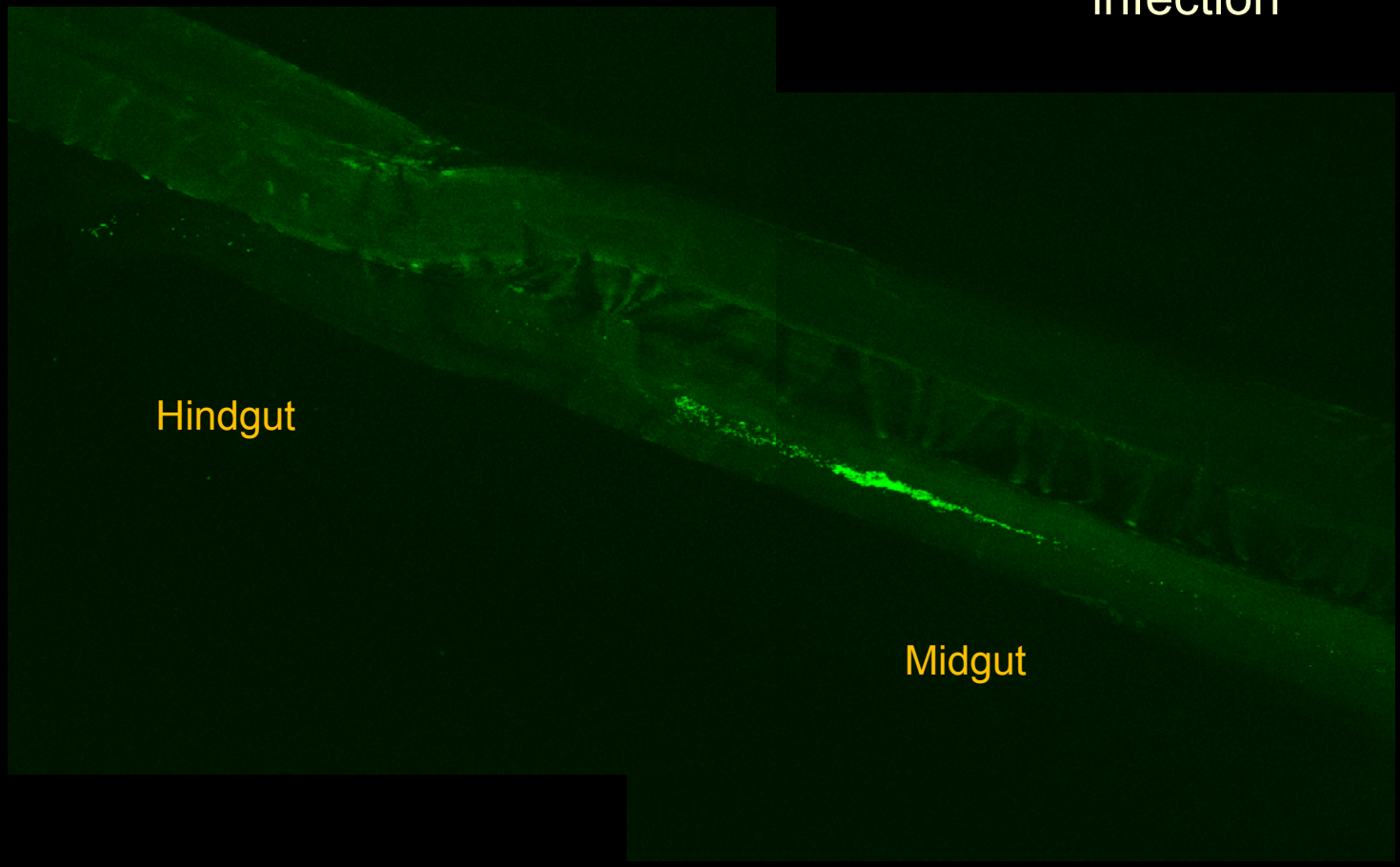
UNIVERSITEIT GENT
Faculty of Veterinary Medicine – Department of Morphology
Faculty of Bioscience Engineering – Laboratory of Aquaculture & Artemia Reference Center

10^5 CFU/mL after 48hrs of infection

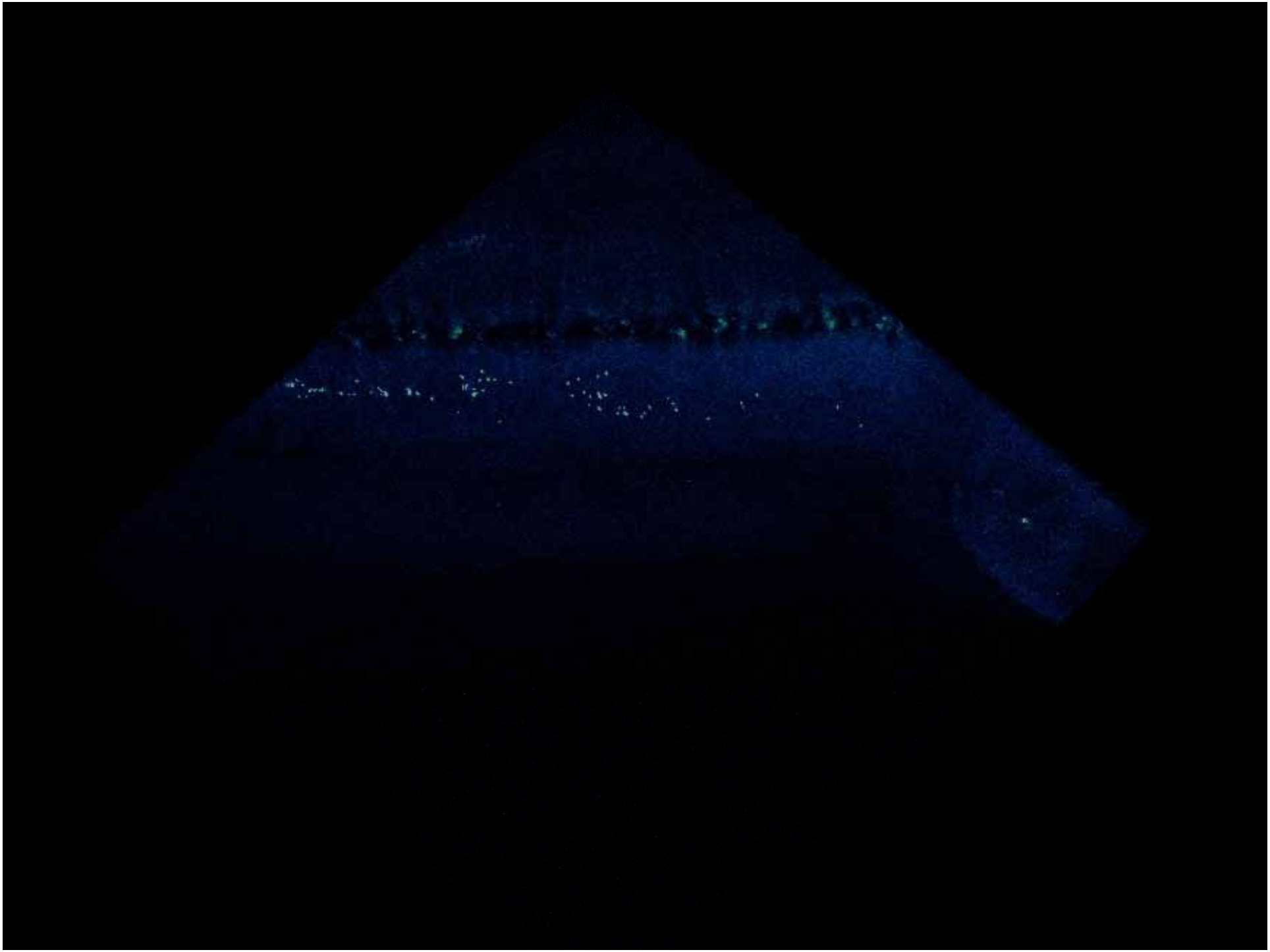


UNIVERSITEIT GENT
Faculty of Veterinary Medicine – Department of Morphology
Faculty of Bioscience Engineering – Laboratory of Aquaculture & Artemia Reference Center

10^7 CFU/mL after 48hrs of infection



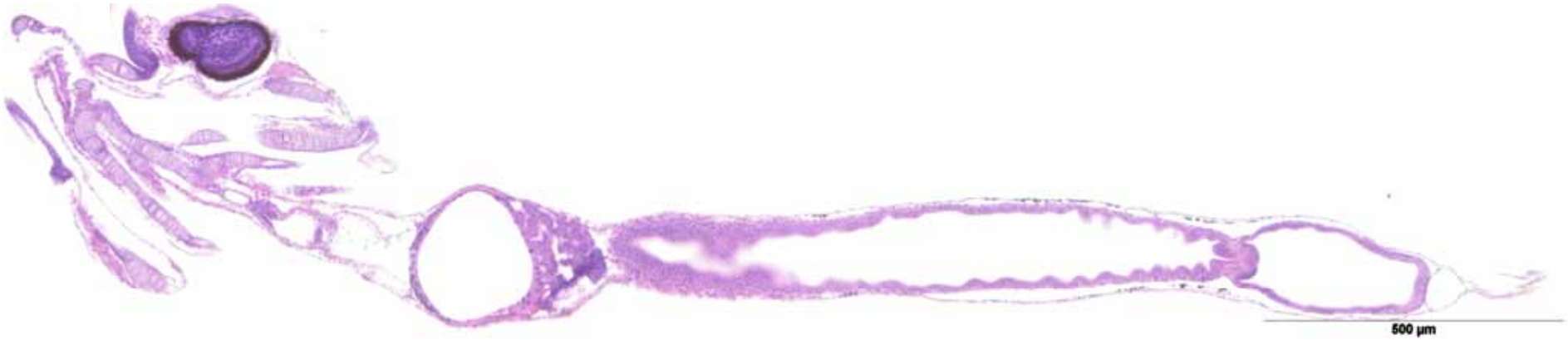
UNIVERSITEIT GENT
Faculty of Veterinary Medicine – Department of Morphology
Faculty of Bioscience Engineering – Laboratory of Aquaculture & Artemia Reference Center



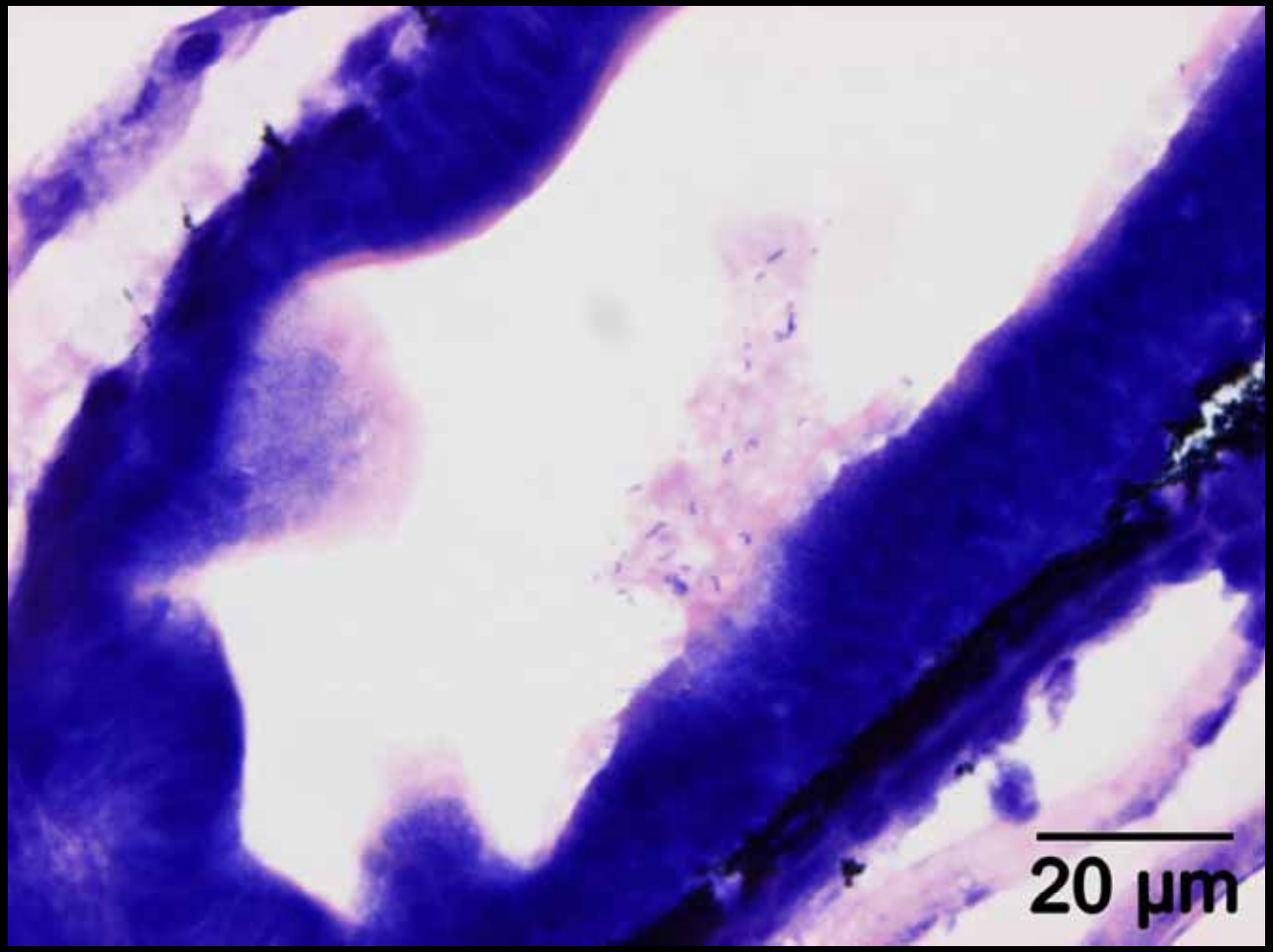
Test 3b: gfp-labelled *Listonella anguillarum* HI610

- Sea bass eggs disinfected - standard protocol for axenic conditions (Dierckens et al., 2009)
- Challenge on DAH4: 10^7 CFU/mL
- Sampling on DAH5: 6hrs after infection

Light microscopy



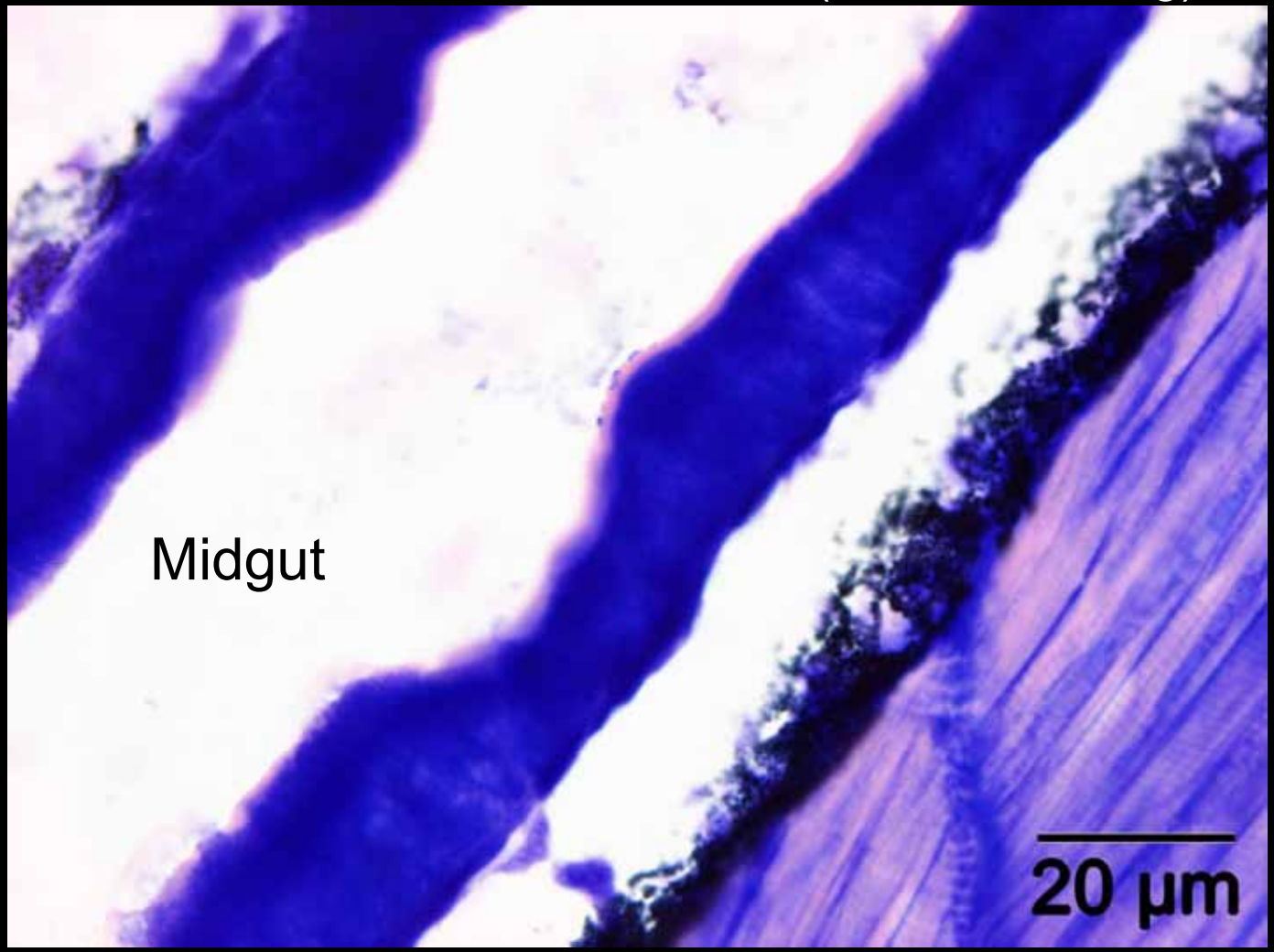
Axenic sea bass DAH6



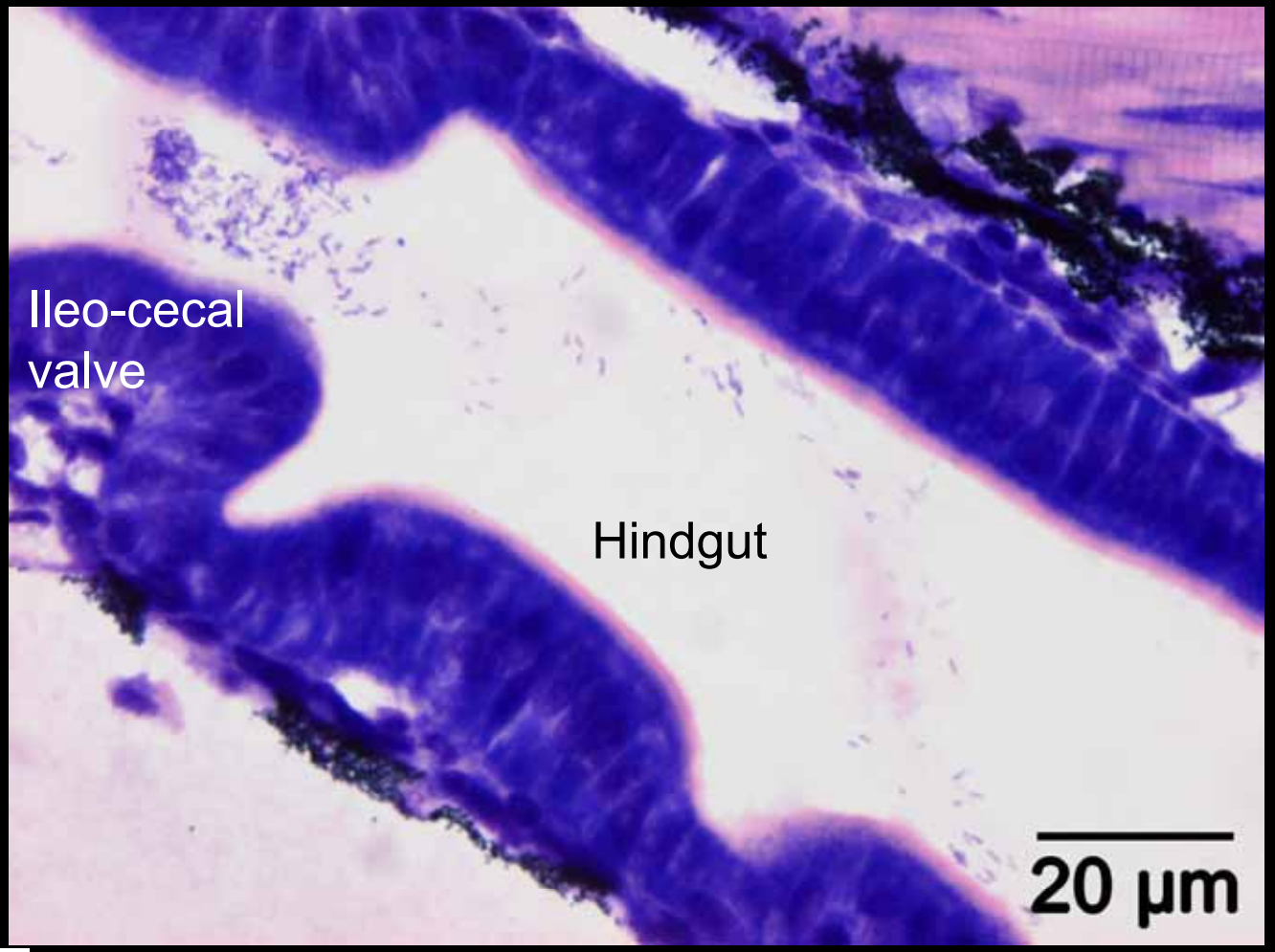
UNIVERSITEIT GENT

Faculty of Veterinary Medicine – Department of Morphology
Faculty of Bioscience Engineering – Laboratory of Aquaculture & Artemia Reference Center

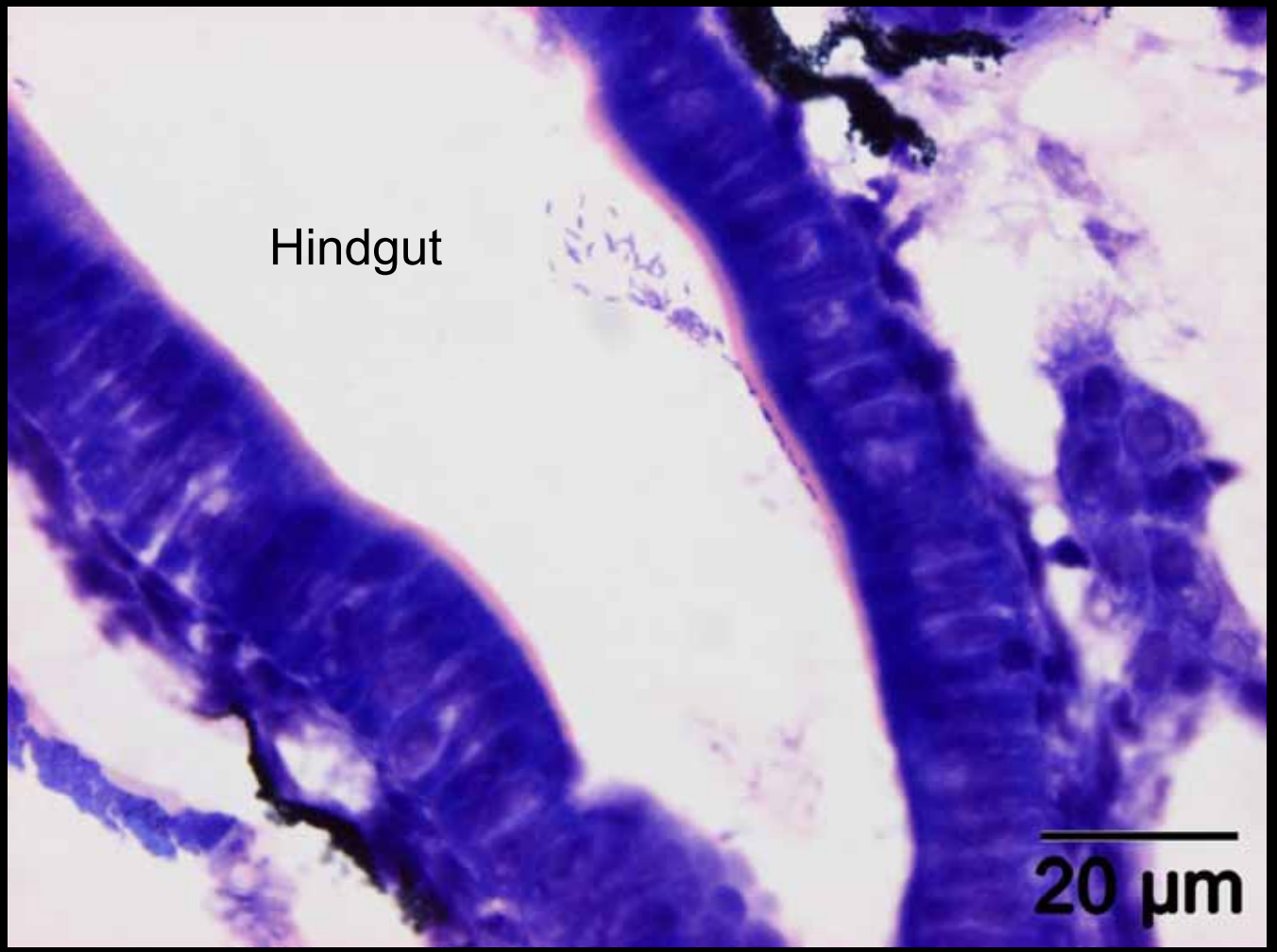
5x10^7 CFU/mL after 6hrs of infection (Giemsa staining)



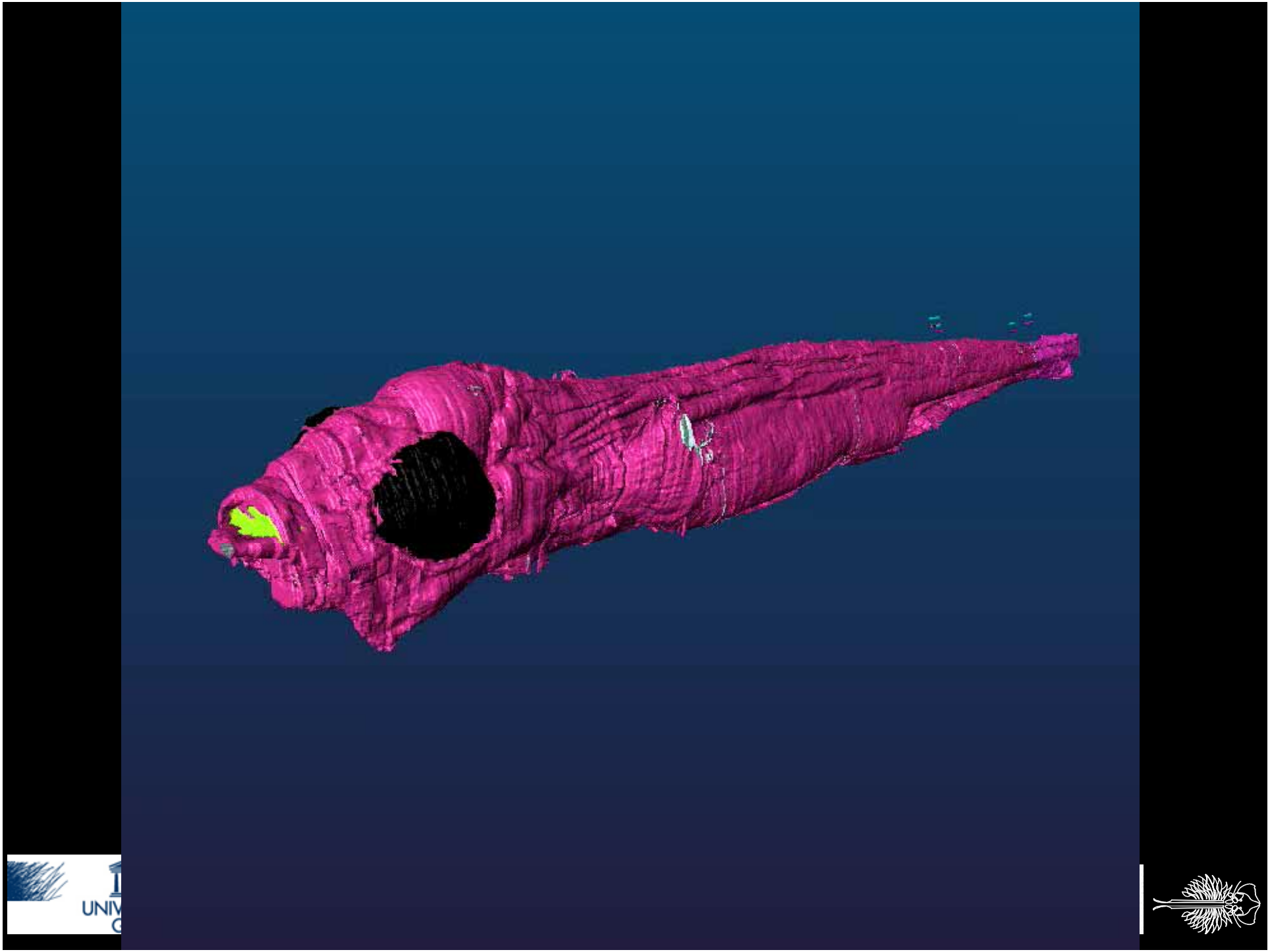
UNIVERSITEIT GENT
Faculty of Veterinary Medicine – Department of Morphology
Faculty of Bioscience Engineering – Laboratory of Aquaculture & Artemia Reference Center



UNIVERSITEIT GENT
Faculty of Veterinary Medicine – Department of Morphology
Faculty of Bioscience Engineering – Laboratory of Aquaculture & Artemia Reference Center



UNIVERSITEIT GENT
Faculty of Veterinary Medicine – Department of Morphology
Faculty of Bioscience Engineering – Laboratory of Aquaculture & Artemia Reference Center



Discussion

- Literature
  - Histopathological changes in the GI tract of fish larvae difficult to observe
  - Findings on endocytosis of bacteria
  - In recent studies no conclusions concerning portal of entry

- Our findings
  - Close contact to brush border (putative adhesion) in both mid- and hindgut enterocytes (LM)
  - No bacteria were detected outside the digestive tract

Conclusion

- Gfp labelled *L.anguillarum* can be detected and tracked in the GI tract of sea bass larvae

- Adjusted challenge tests needed (longer infection time with higher concentration of bacteria)

Future research

- TEM study on bacterial adhesion / translocation in epithelial cells
- Qualitative analysis + semiquantitative analysis by the use of Image J software – volume determination
- Comparison pathogenic colonisation / adhesion in the gut in both monognotobiotic and xenic systems
- Rfp labelling of bacteria with probiotic characteristics/track interaction of probiotics and pathogen

Acknowledgements

Dr. Pieter Cornillie

Lobke de Bels

Christian Puttevils

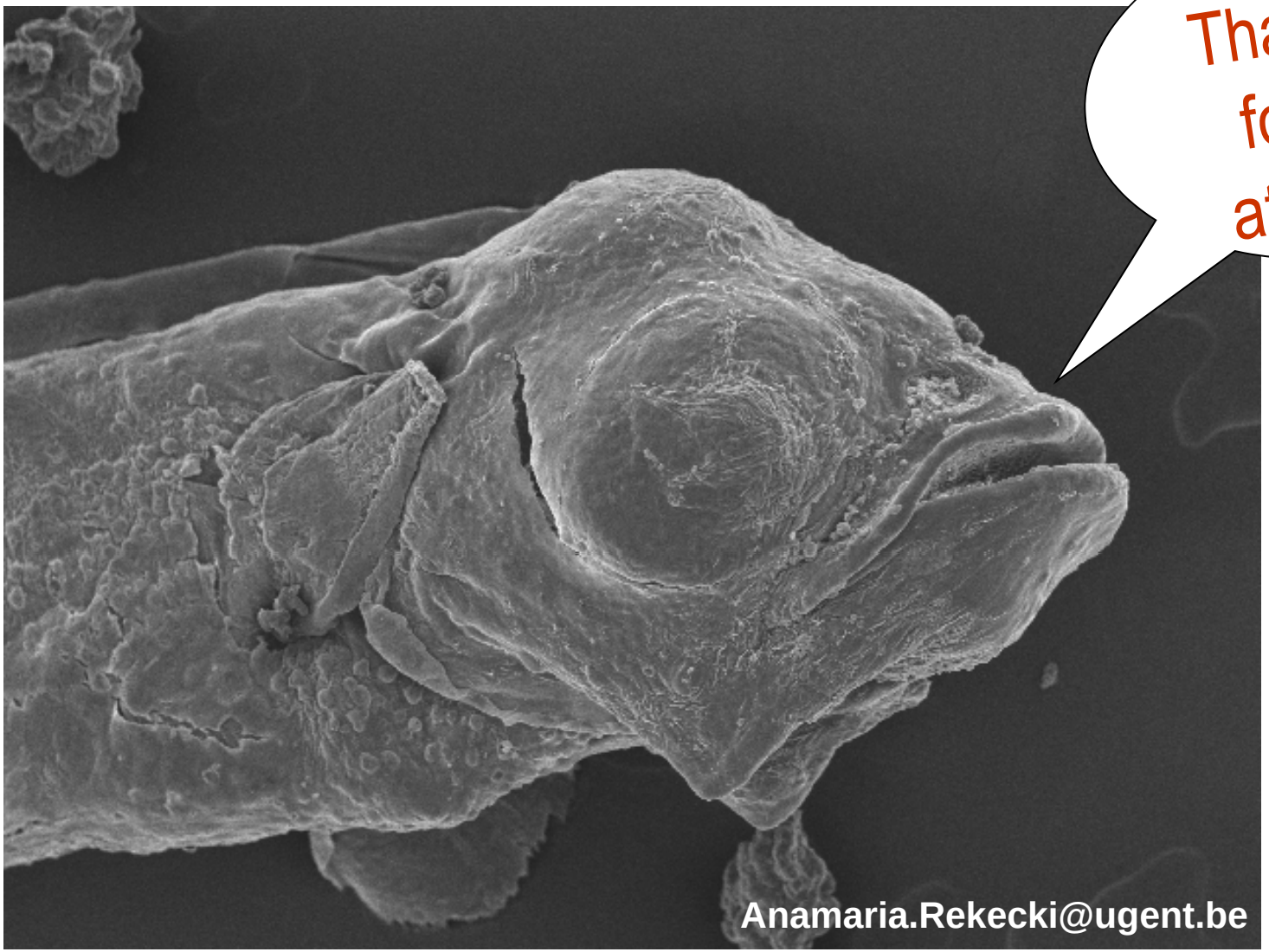
Liliane Standaert

Brigitte van Moffaert

Tom Baelemans

Jorg Desmyter

Faculty of Veterinary Medicine – Department of Morphology
Faculty of Bioscience Engineering – Laboratory of Aquaculture & Artemia Reference Center



UNIVERSITEIT GENT
Faculty of Veterinary Medicine – Department of Morphology
Faculty of Bioscience Engineering – Laboratory of Aquaculture & Artemia Reference Center