Health monitoring of zebrafish

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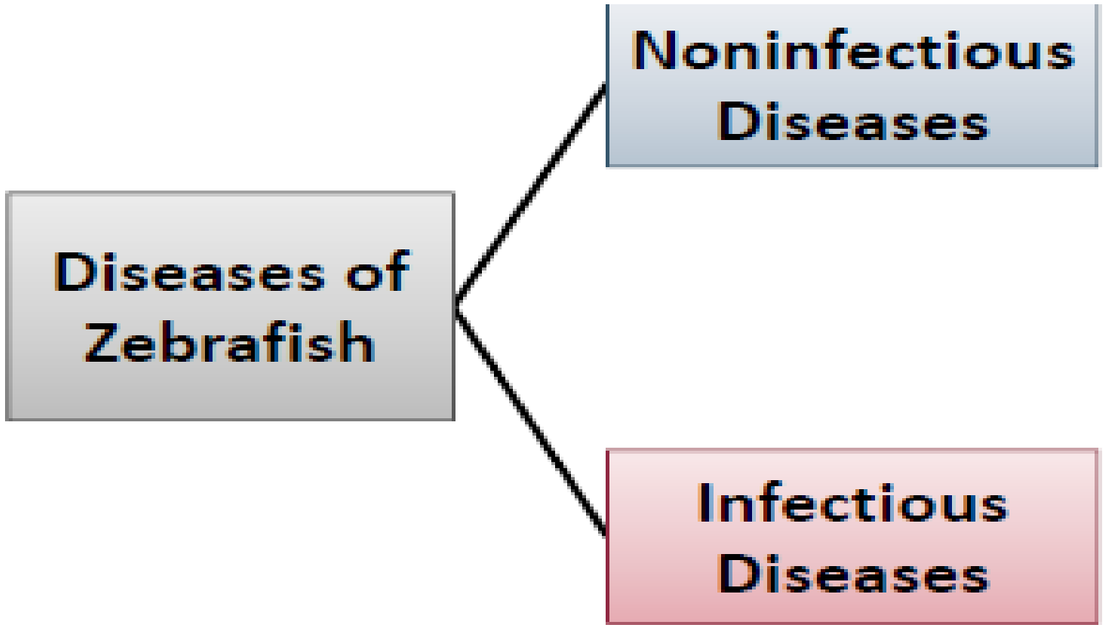
Screening vs diagnosis

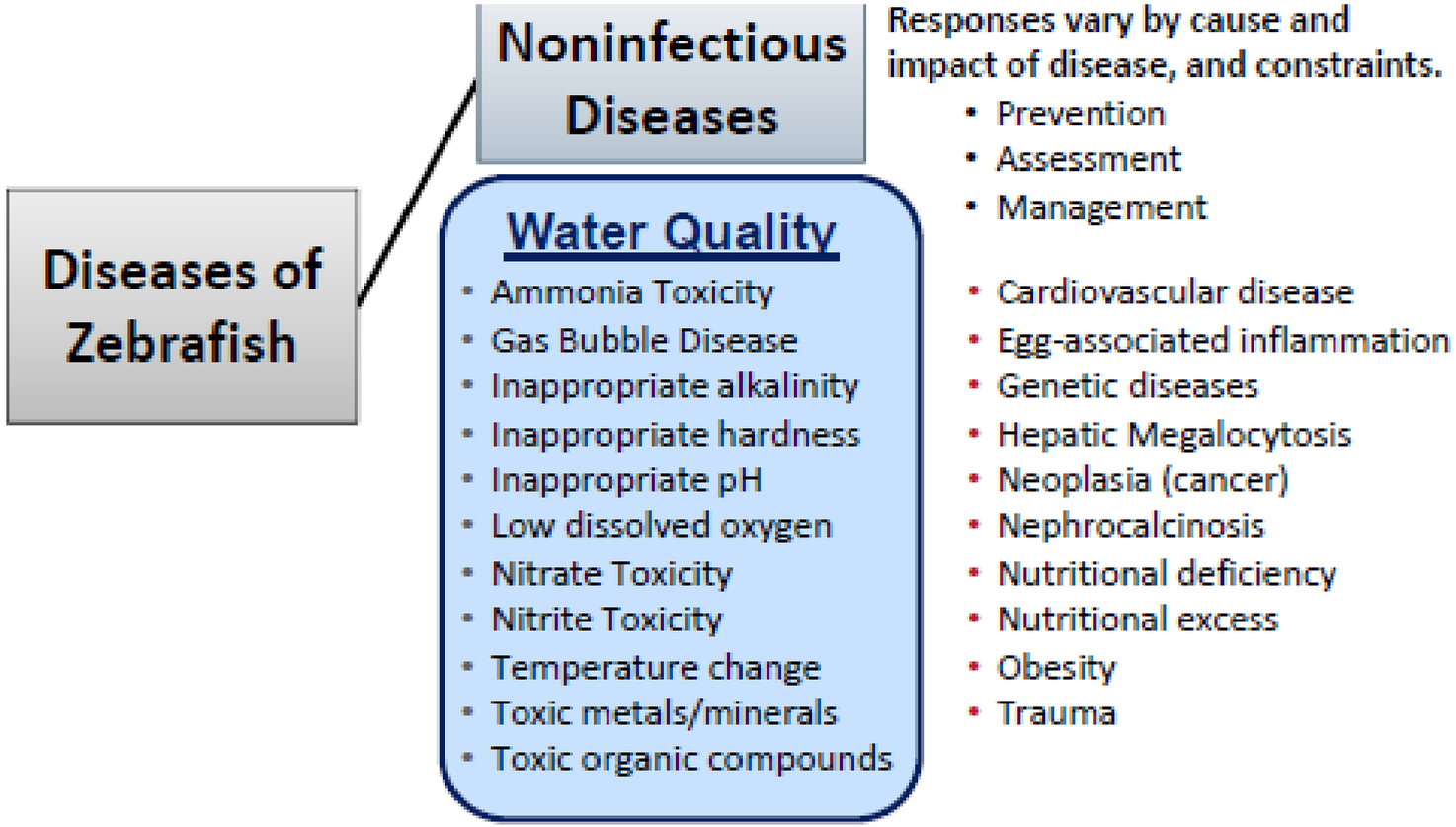
Differentiate Between Screening and Diagnostic Tests

Screening	Diagnostic
Asymptomatic	Suggestive clinical picture
Large group	Single subject
Less accurate	Accurate
Not conclusive	Conclusive
Less expensive	Expensive
Not basic for treatment	Basic to treatment

Husbandry and zebrafish colony health



Husbandry and zebrafish colony health



Zebrafish colony health monitoring

1. Why is zebrafish health monitoring important?
2. What are some important factors that influence health monitoring for zebrafish colonies?
3. What are the most important zebrafish pathogens?
4. What diagnostic platforms are available for zebrafish?
5. What are the strengths and weaknesses of each platform?
6. How can different diagnostic platforms be applied optimally in zebrafish health monitoring?

Keys health monitoring objectives

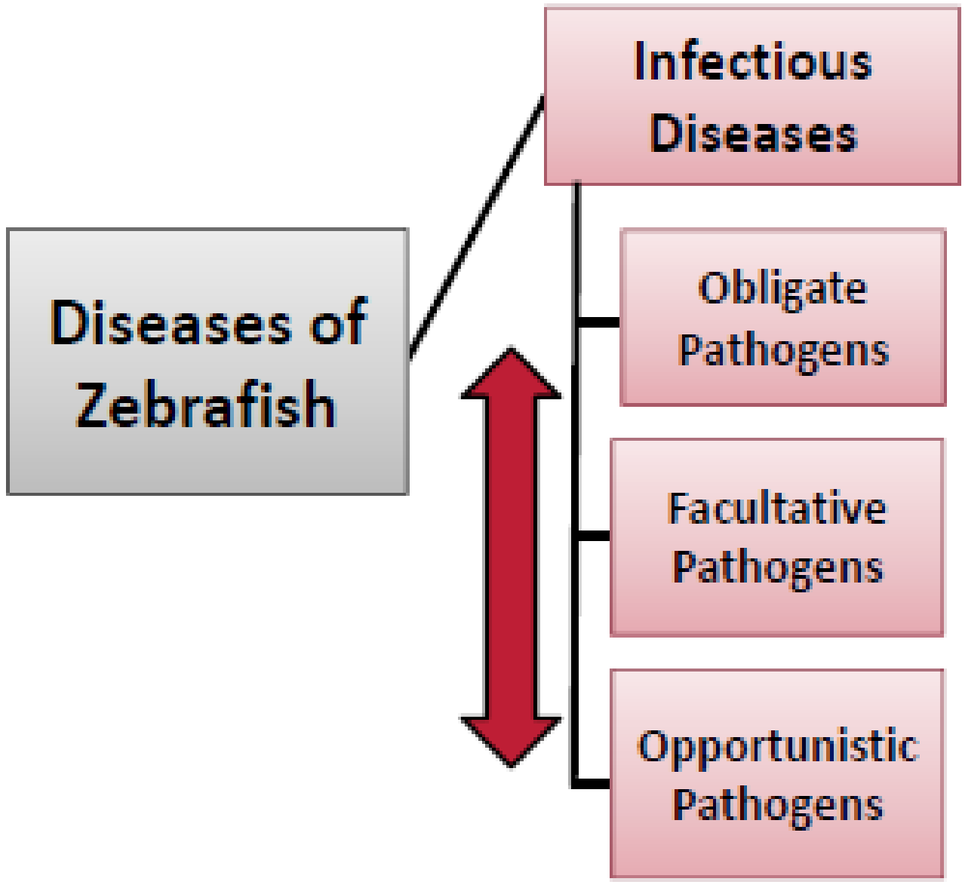
1. Improve **zebrafish health**
2. Ensure the **validity of experimental data**
3. **Biosecurity**:
   - Determine **entry measures** for the introduction of new zebrafish/embryos into a colony
   - **Prevent spread** of pathogens within or between facilities
   - Provide health status information for **import and export**
4. Protect **human health**

Images from

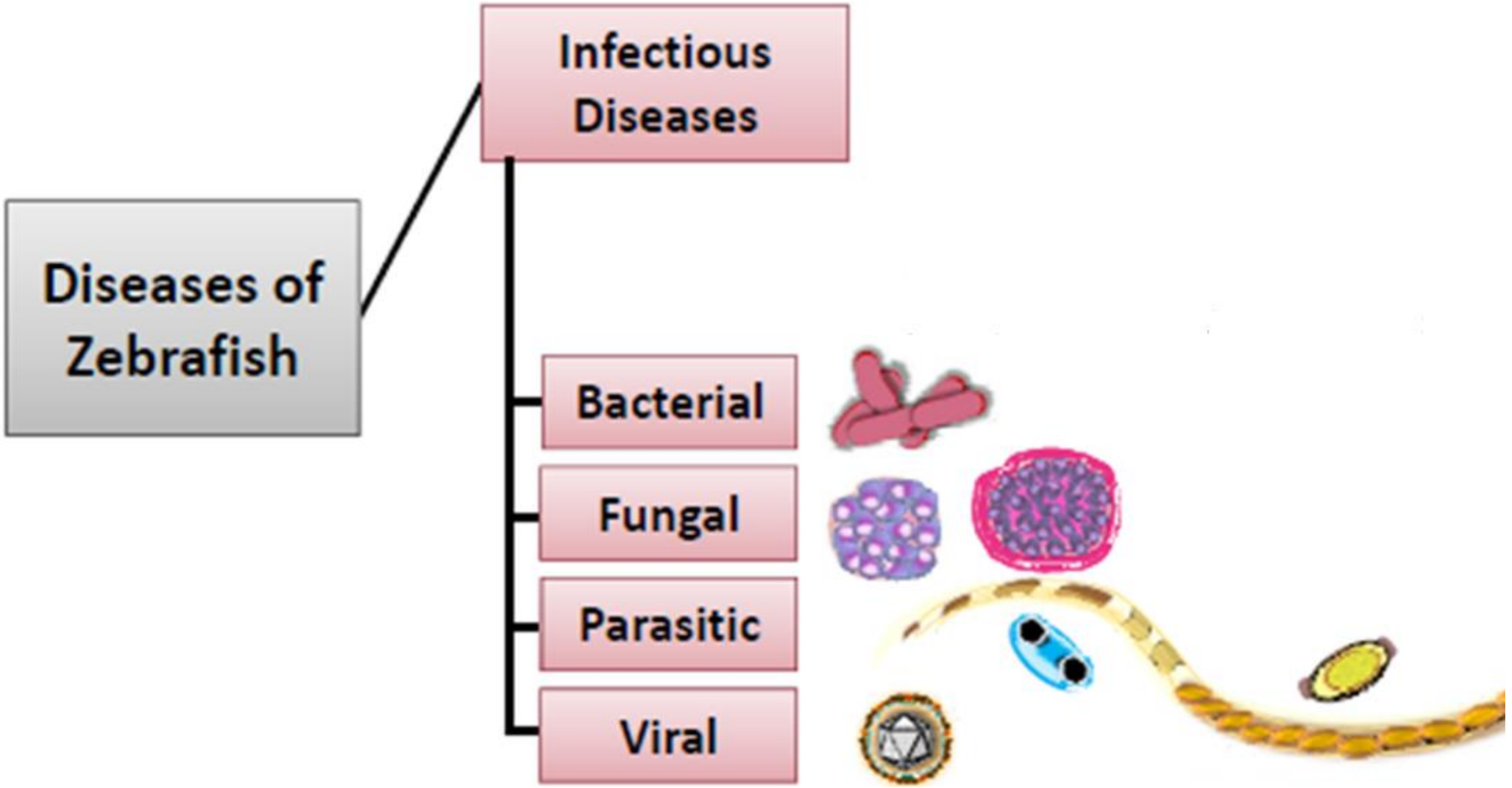
1. Wu, et al. Fish tank granuloma caused by *Mycobacterium marinum*. *PLoS ONE*. 7(7): 1-6.

2. Nguyen C. 2004. *Mycobacterium marinum*. *N Engl J Med*. 350(9):e8.

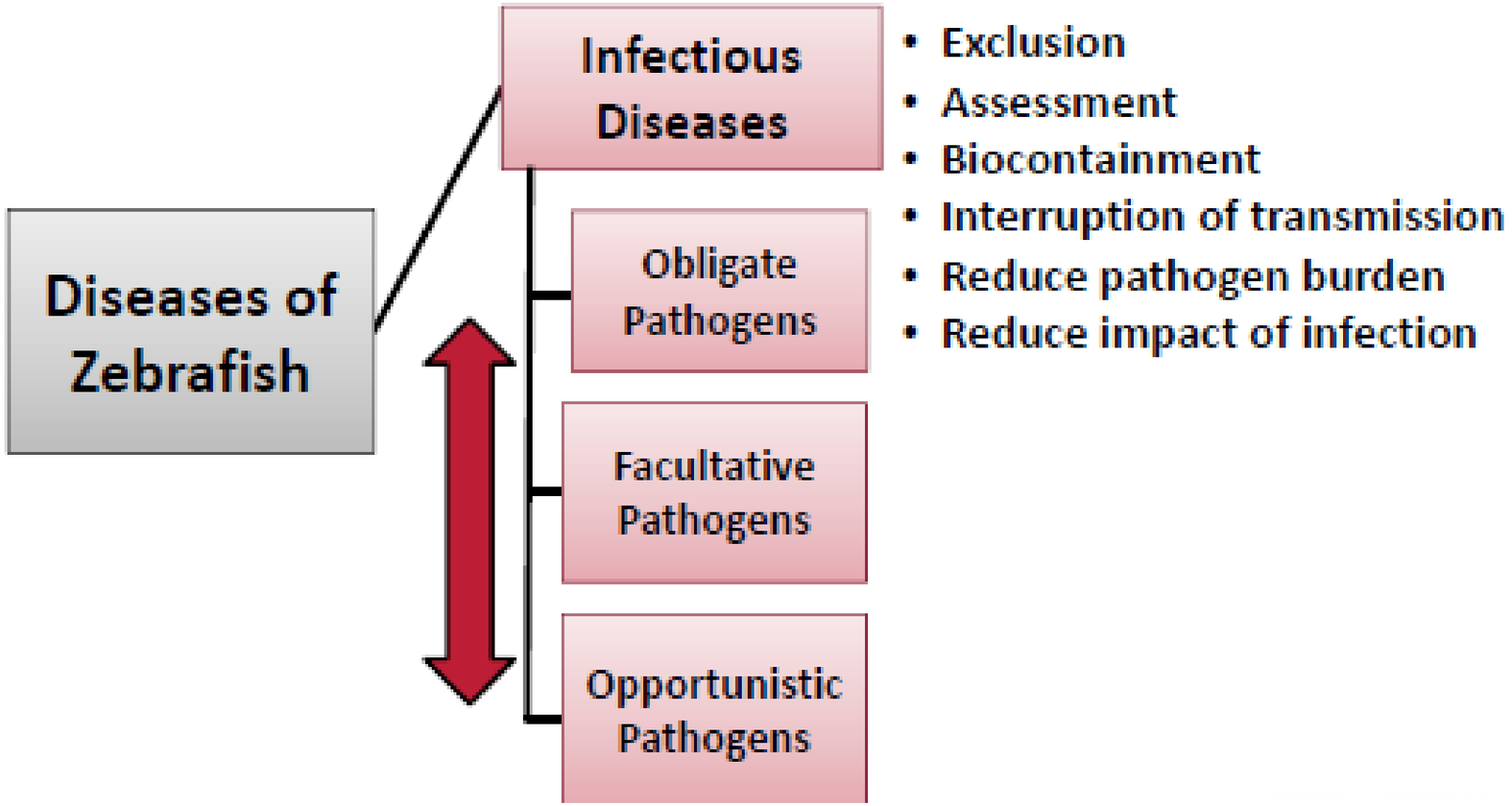
Husbandry and zebrafish colony health



Husbandry and zebrafish colony health



Husbandry and zebrafish colony health goals



Husbandry and zebrafish colony health: exclusion

- Entry Measures: Prevent/reduce introduction of new pathogens
  - Exclusion list (Define acceptable health status by biological unit)
  - Quarantine
  - "Eggs only" policy:
    - Adapted from salmonid aquaculture
    - Adults euthanized in quarantine
    - Allow only **surface-disinfected embryos** into main systems

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- Biosecure Husbandry Practices
  - Work flow (people)
  - Materials flow
  - Live feeds
  - Source Water
  - Disinfection practices

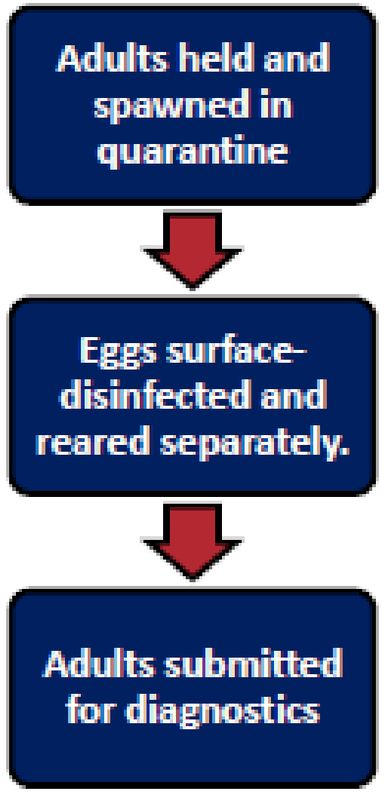
Husbandry and zebrafish colony health: exclusion – receiving adult fishes

- Usually as breeding pair(s)
- Colony health status may be unknown
- May be preferred by a lab that is relatively new to zebrafish
- Adult may be individually very valuable to PI

  e.g., mutant or transgenic lines:
  - May not spawn easily
  - Rarely survive past the larval stage
  - Adults are weak or short-lived

Husbandry and zebrafish colony health: exclusion – receiving adult fishes

- Adults held and spawned in quarantine
- Test adults to allow entry of embryos:
  - No Pathogens Detected ➡
    - Release offspring from Quarantine
  - Pathogen(s) detected ➡
    - Raise to maturity in quarantine
    - Spawn in Quarantine
    - Surface-disinfect embryos
    - Euthanize adults and submit for diagnostic testing



Husbandry and zebrafish colony health: exclusion – receiving embryos

Receive embryos, but they are too old for surface disinfection

(Embryos are often disinfected prior to shipping)

Decision: Should you trust other institutions? Risks:

- Introduction of fish pathogens
- Introduction of *M. marinum* (zoonotic)
- Entrenchment of new pathogens*

- Embryos raised to maturity and spawned in quarantine
- Test adults to allow entry of embryos:
  - No Pathogens Detected →
    - Release offspring from quarantine
  - Pathogen(s) detected →
    - Raise to maturity in quarantine
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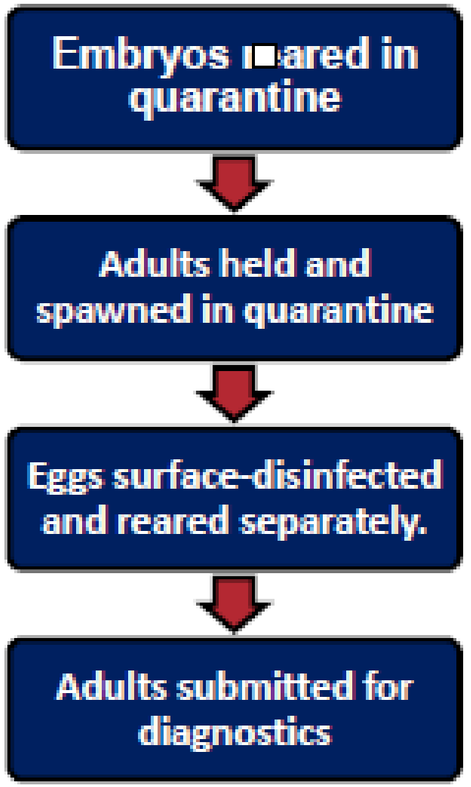
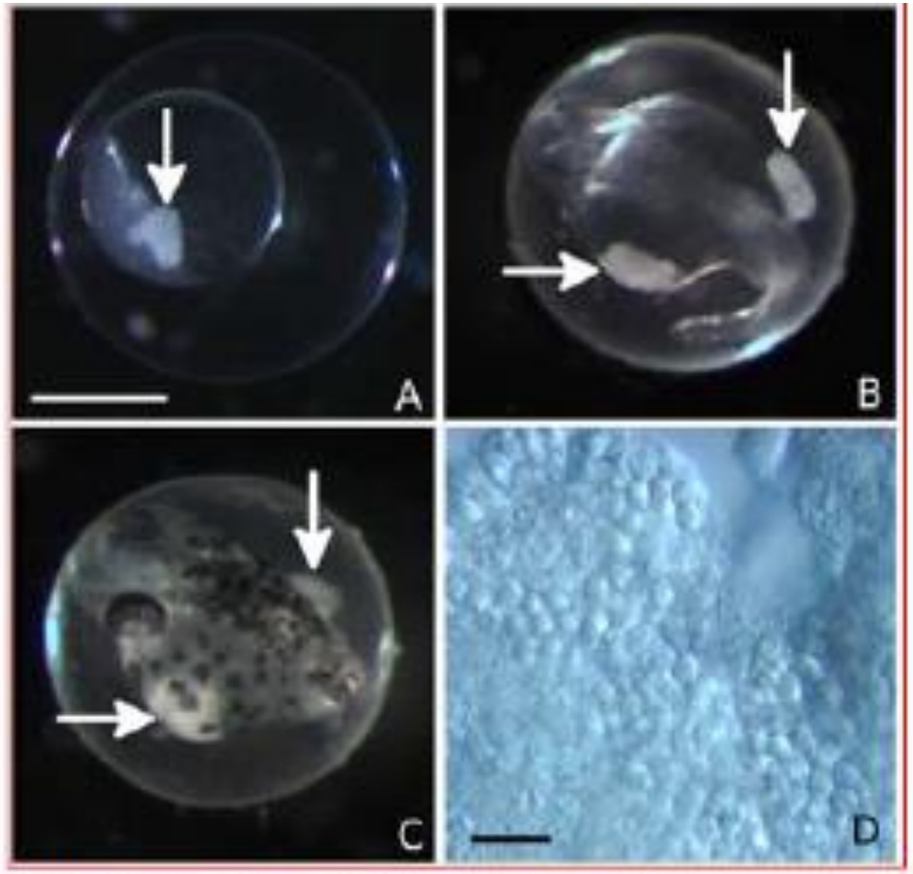


Entry measures. Surface disinfection of embryos

Entry measures. Surface disinfection of embryos

Pseuloloma neurophilia: vertical (intraovum) transmission

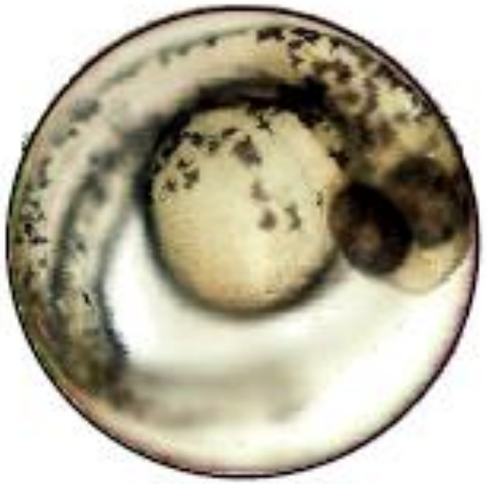


Sanders JL, *et al.* 2013. Verification of Intraovum Transmission of a Microsporidium of Vertebrates: *Pseudoloma neurophilia* Infecting the Zebrafish, *Danio rerio*. *PLoS ONE* 8(9): e76064.

Possible (partial) solution

Zebrafish embryos:

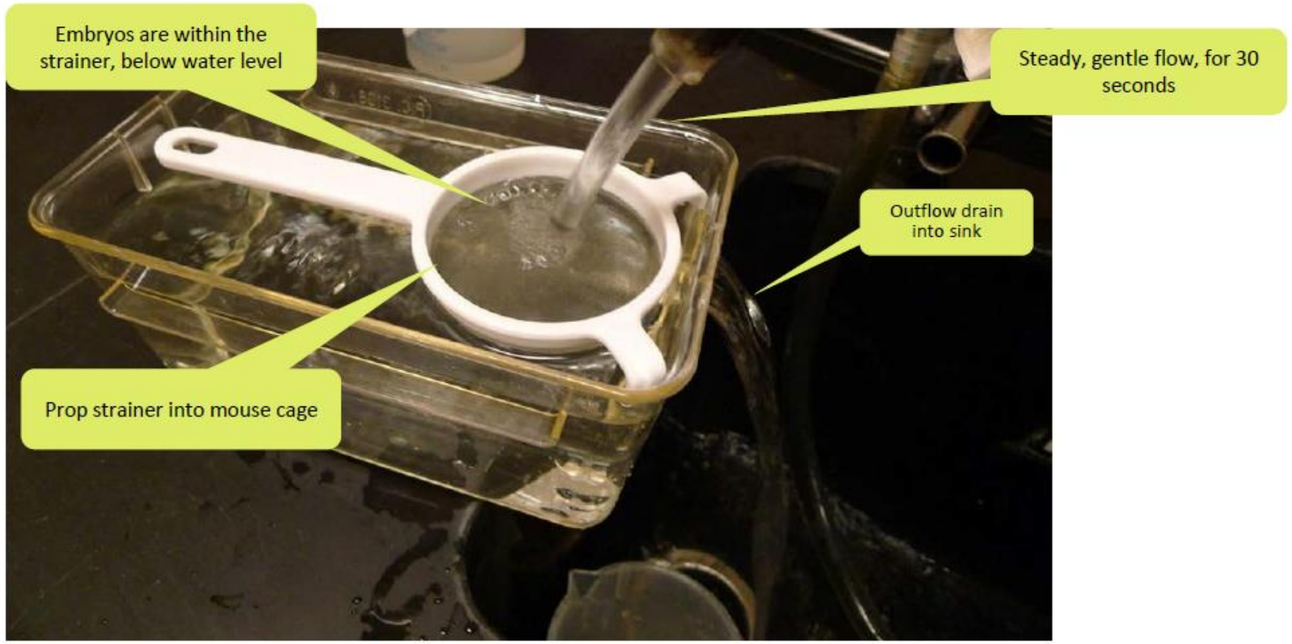
- Usually easy to obtain
- Sample of embryos in a **clutch**
- Antemortem sample type for **adults**



Fluidized rinsing of embryos:

- Can be utilized after 6-24 hour window for surface-disinfection
- Remove pathogens from the surface of embryos?
- How does fluidized rinsing compare to surface disinfection?

Gently rinse the bleached embryos with fish water. The best approach is to use a large tea strainer that “fits” into a sanitized mouse cage. Turn on fish water hose and run it directly over the embryos in the strainer. Flow should be gentle, but enough to swirl the embryos within the strainer basket. The embryos should always be submerged during the rinsing process. Place the cage on the edge of the sink so that water flows from the tank overflow hole into the sink. Rinse in this way for 30 seconds.



Pathogen detection in embryos of subclinical zebrafishes

Tankmates of clinically diseased zebrafish from two colonies with multiple pathogens.

20 Unrinsed embryos

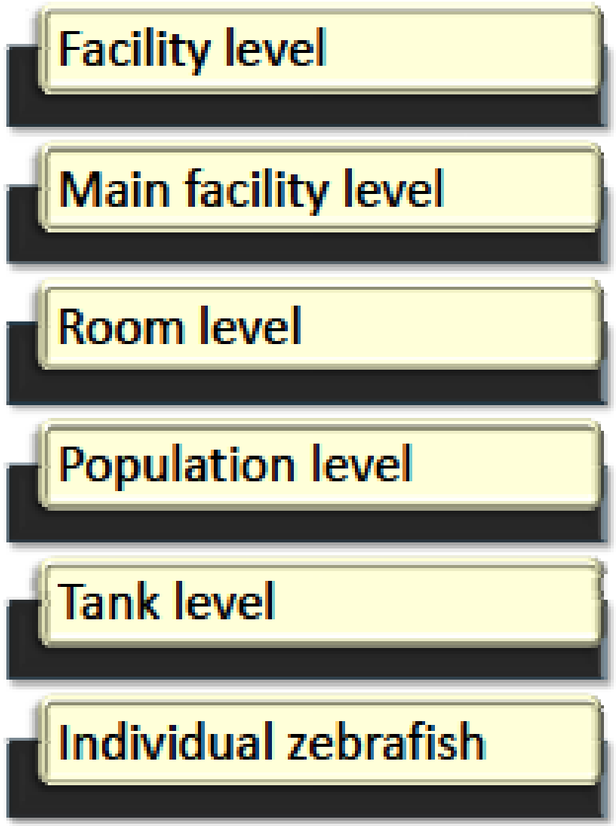
20 Rinsed embryos

20 Bleached embryos

Detection in embryo when parent(s) infected

	Mycobacterium chelonae	*Mycobacterium marinum*	*Pseudocapillaria tomentosa*	*Pseudoloma neurophilia*	*Pleistophora hyphessobryconis*
Unrinsed	0/1	0/1	1/12 8.3%	1/10 10.0%	1/7 14.3%
Rinsed	0/1	0/1	0/12	0/10	1/7 14.3%
Bleached	0/1	0/1	0/12	0/10	1/7 14.3%

Husbandry and zebrafish colony health: health status assessment



Colony Health Monitoring:

- **Sentinel Health Monitoring**
  - Intentionally placed sentinels
  - "Sump" fish
- **Postmortem evaluation**
  - Euthanized
  - "Found dead"
- **Retired (old) zebrafish**
- **Daily health checks (clinical signs)**

Husbandry and zebrafish colony health: health status assessment

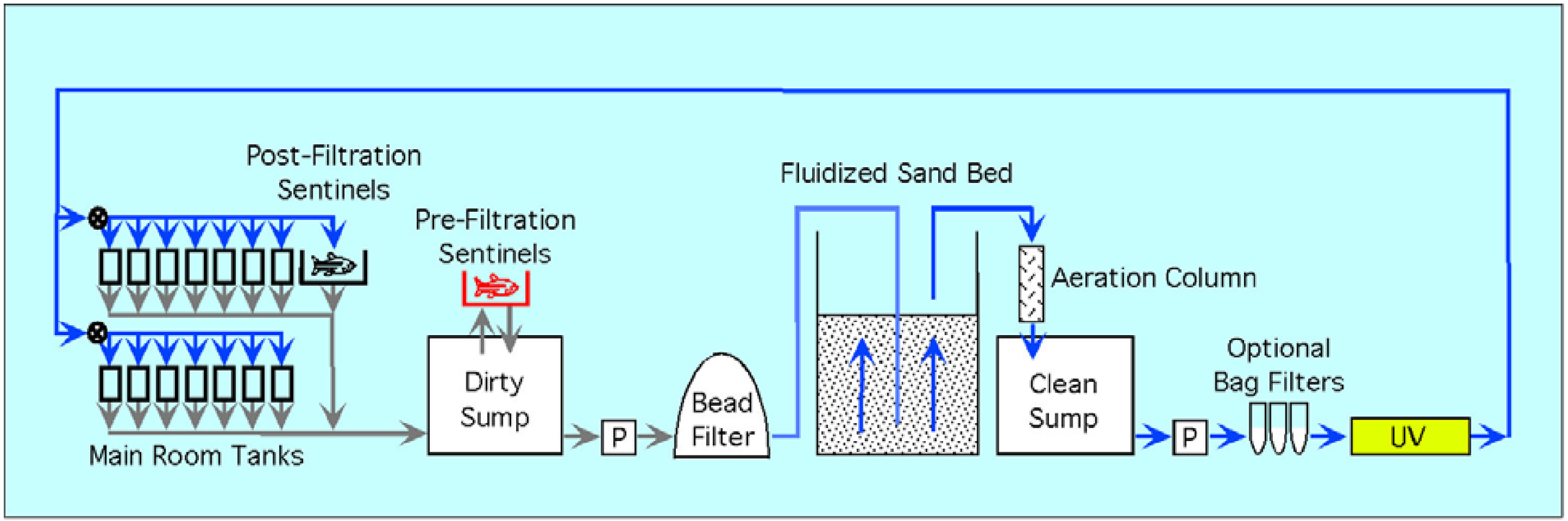
Sentinel health monitoring

- Best sentinels?
  - Source
  - Best sentinel line?
  - Immunocompromised?
- Best placement
- Effluent exposure time
- Testing interval

Postmortem evaluations

- Euthanized clinical fish
  - Real-Time PCR
  - Microbiology
  - Histopathology
- Retired (old) fish
- “Found dead” fish
  - PCR only

DOUBLE SENTINEL SYSTEM



Sentinel system

BIOFILTERS!

Husbandry and zebrafish colony health: assessment

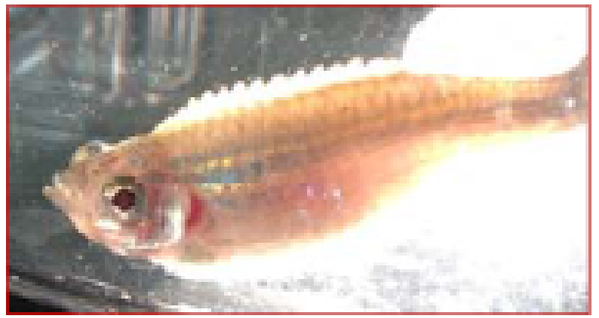
Infections may be:

- **Acute (rapid)**
- **Chronic (continue for weeks-years)**

- Most zebrafish infections are chronic
- Adults have had longer to acquire chronic infections

Clinical signs and disease

- Some infections cause mortality (death)
- Some infections cause morbidity (disease)
- Many are subclinical (no clinical signs)
- Many infections cause inflammation



Husbandry and zebrafish colony health: assessment

Behavioral clinical signs:

- Loss of appetite/anorexia (going “off feed”)
- Lethargy
- Flashing (rubbing)
- Equilibrium disturbances
- Swimming in isolation
- Hiding
- Clamped fins
- Respiratory rate changes
- Piping (gasping at surface)
- Opercular flaring
- Thrashing/ erratic swimming

Physical clinical signs:

- Skin lesions
  - Ulcers
  - Erythema (redness)
  - Hemorrhage
  - Loss of scales
  - Scale protrusion
- Changes in color
- Dropsy (ascites + scale protrusion)
- Improper buoyancy
- Death

Marked haemorrhage (Edwardsiella ictaluri)

emaciation

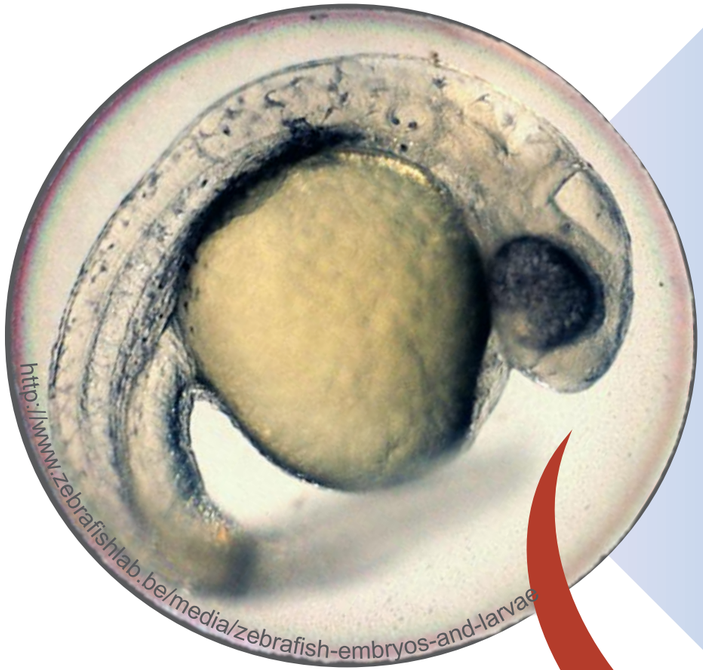
“tail rot” (Flavobacterium columnare)

“Dropsy” (Mycobacterium haemophilum)

Ulcer

Tumor

Rapidly Increasing Need for Zebrafish Health Monitoring



Zebrafish expanding from developmental genetics to a wide array of new research areas, including:

- Aging
- Behavior
- Immunity
- Infection
- Neoplasia
- Toxicology

Zebrafish pathogens: impact on research

Zebrafish are susceptible to naturally-occurring infectious diseases that can invalidate studies.

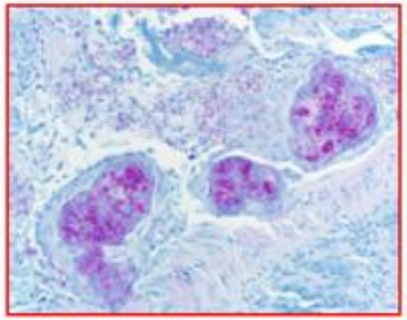
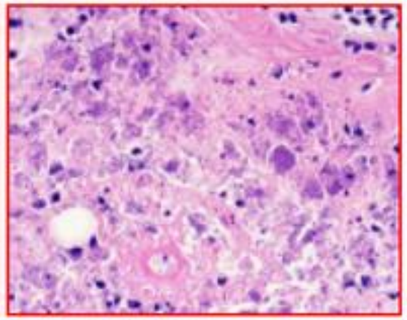
- **Mortality**
- **Clinical disease (morbidity)**
- **Subclinical infections**

Possible Impacts:

- **Loss of an animal model**
- **Misinterpreted data** (Type I & Type II Errors)
- **Repetition** of failed experiments
- **Inability to replicate** experimental results
- **Loss of balanced experimental design**
- Unexplained **variability** in experimental data
- **More animals required** to demonstrate statistical significance

Zebrafish pathogens: impact on research

1. **Damage due to pathogen virulence factors**
2. **Damage due to the host immune response**
   - Energy expenditure
   - Possible induction or exacerbation of autoimmunity
   - Collateral damage (incidental damage to tissues)
   - Tissue repair and remodeling
   - Alteration of future immune responses
   - Possible epigenetic effects

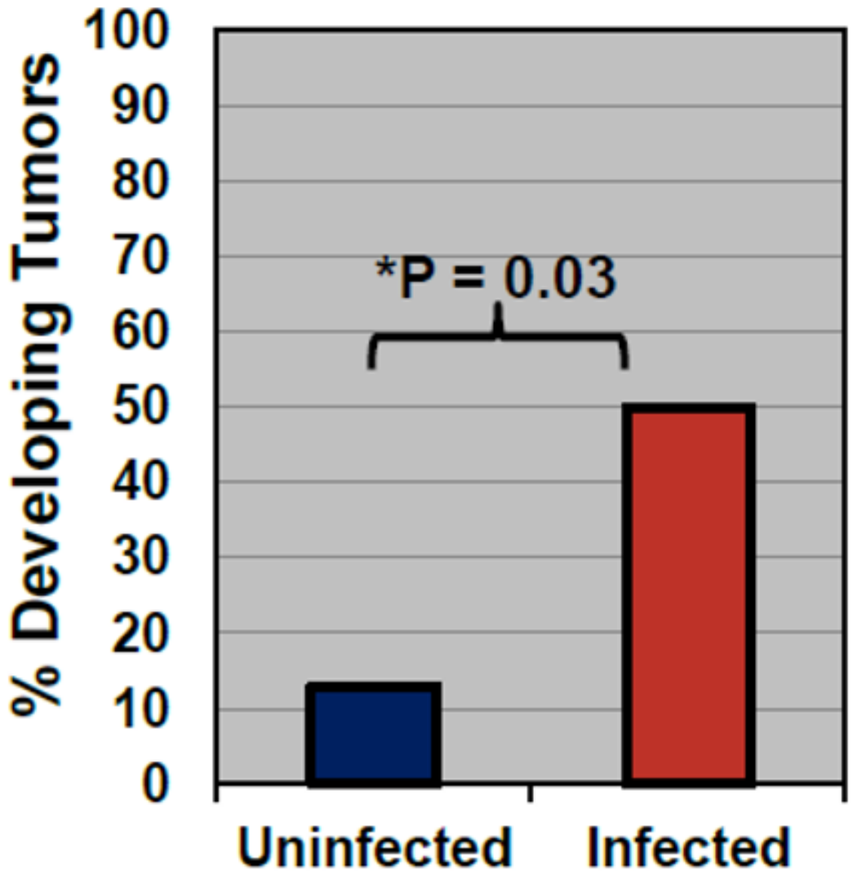




Impact on research using embryo and larvae

- At least some subclinical infections reduce fecundity of zebrafish.
- Some zebrafish pathogens are transmitted vertically.
- In general, embryos are much less likely to be infected than adult fish.
- Embryos and larvae are susceptible to infection and mount an immune response to infectious agents.
- Infections alter gene transcription.
- Infections alter cytokine levels.
- Many cytokines have important roles in both the immune response and development.

Example: subclinical infection confound neoplasia experiment

Intestinal Neoplasia in *P. tomentosa*-infected and uninfected DMBA-exposed zebrafish



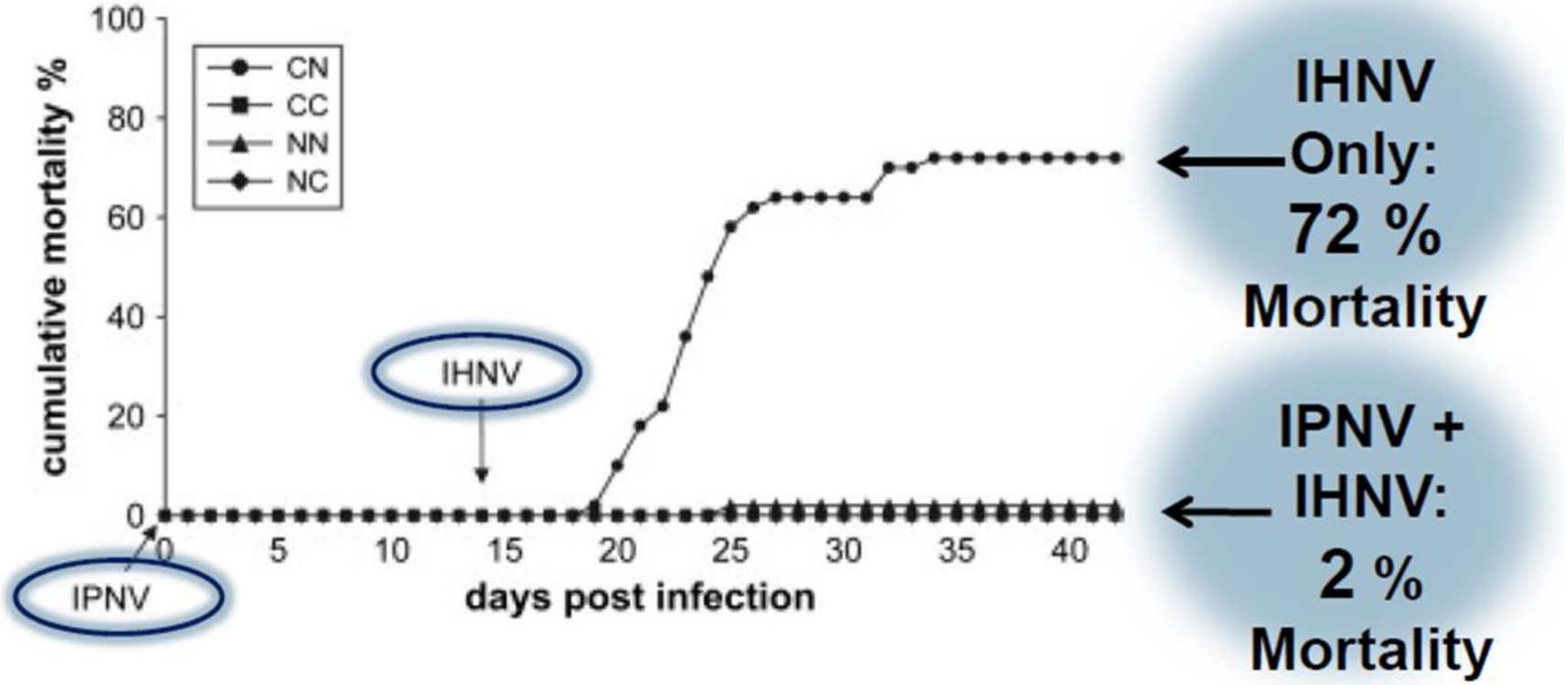
Confounded Carcinogenesis study:

- Undetected *Pseudocapillaria tomentosa* infection
- Only one treatment group was affected: 5ppm 7,12-dimethylbenze[a]anthracene (DMBA)
- Worms located within or adjacent to tumors.

Significantly different outcome between infected and uninfected fish within the same treatment group.

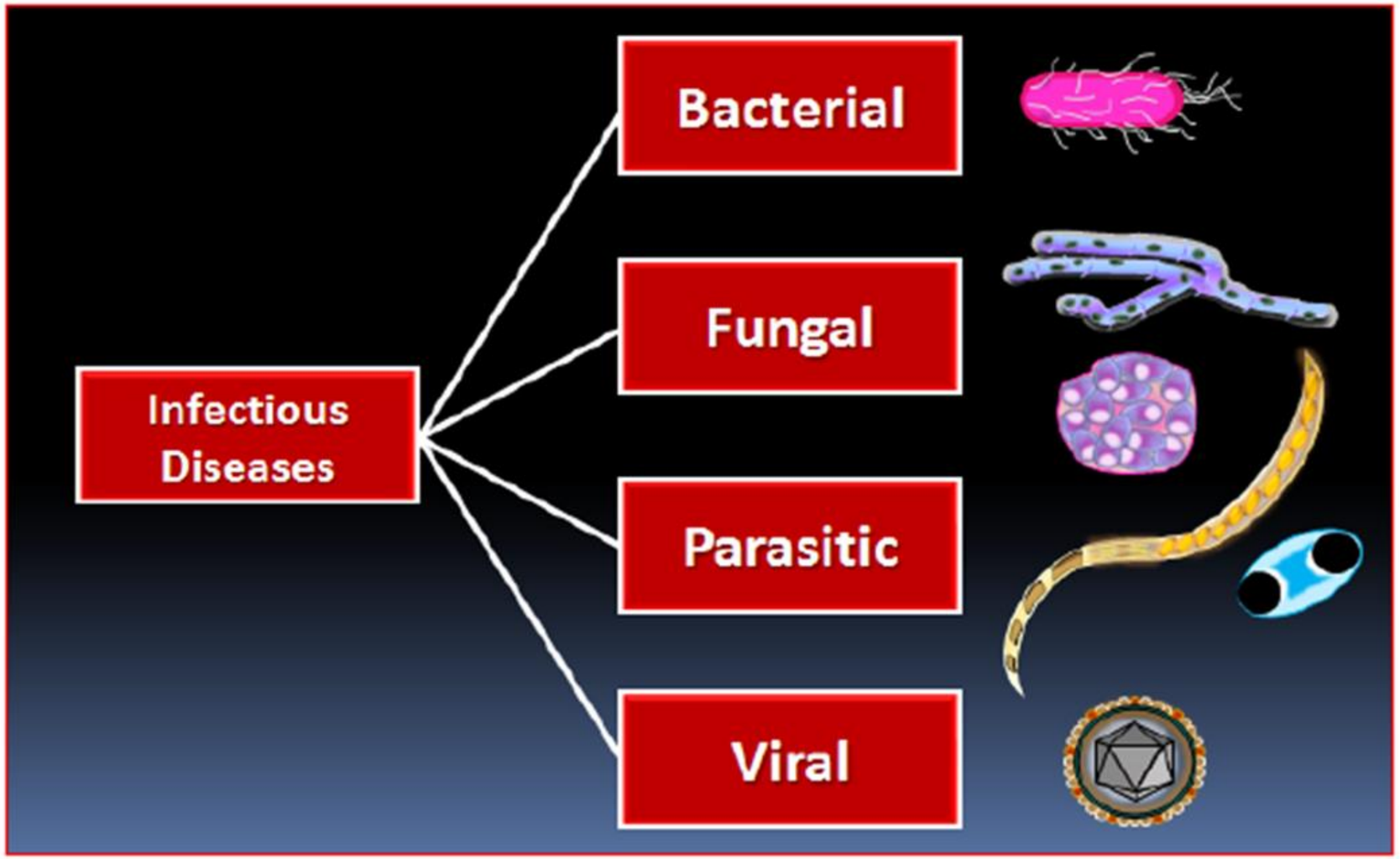
Data from: Kent, ML, Bishop-Stewart JK, Matthews JL, Spitsbergen JM. 2002. *Comparative Medicine* **52**(4): 354-358.

subclinical infection alters the outcome of subsequent infection



Adapted from:
Byrne N, Castric J, Lamour F, Cabon J, Quentel C. 2008.. *Fish and Shellfish Immunology* **24**(5):489-497.

Pathogens of zebrafish



Pathogens of zebrafish

Bacterial Pathogens:

- *Edwardsiella ictaluri*
- *Flavobacterium columnare*
- *Mycobacterium abscessus*
- ***Mycobacterium chelonae***
- *Mycobacterium fortuitum*
- *Mycobacterium haemophilum*
- ***Mycobacterium marinum***
- *Mycobacterium peregrinum*

Parasites:

- *Ichthyophthirius multifiliis*
- *Myxidium* sp.
- *Piscinoodinium pillulare*
- *Pleistophora hyphessobryconis*
- ***Pseudocapillaria tomentosa***
- ***Pseudoloma neurophilia***

IHNV Virus

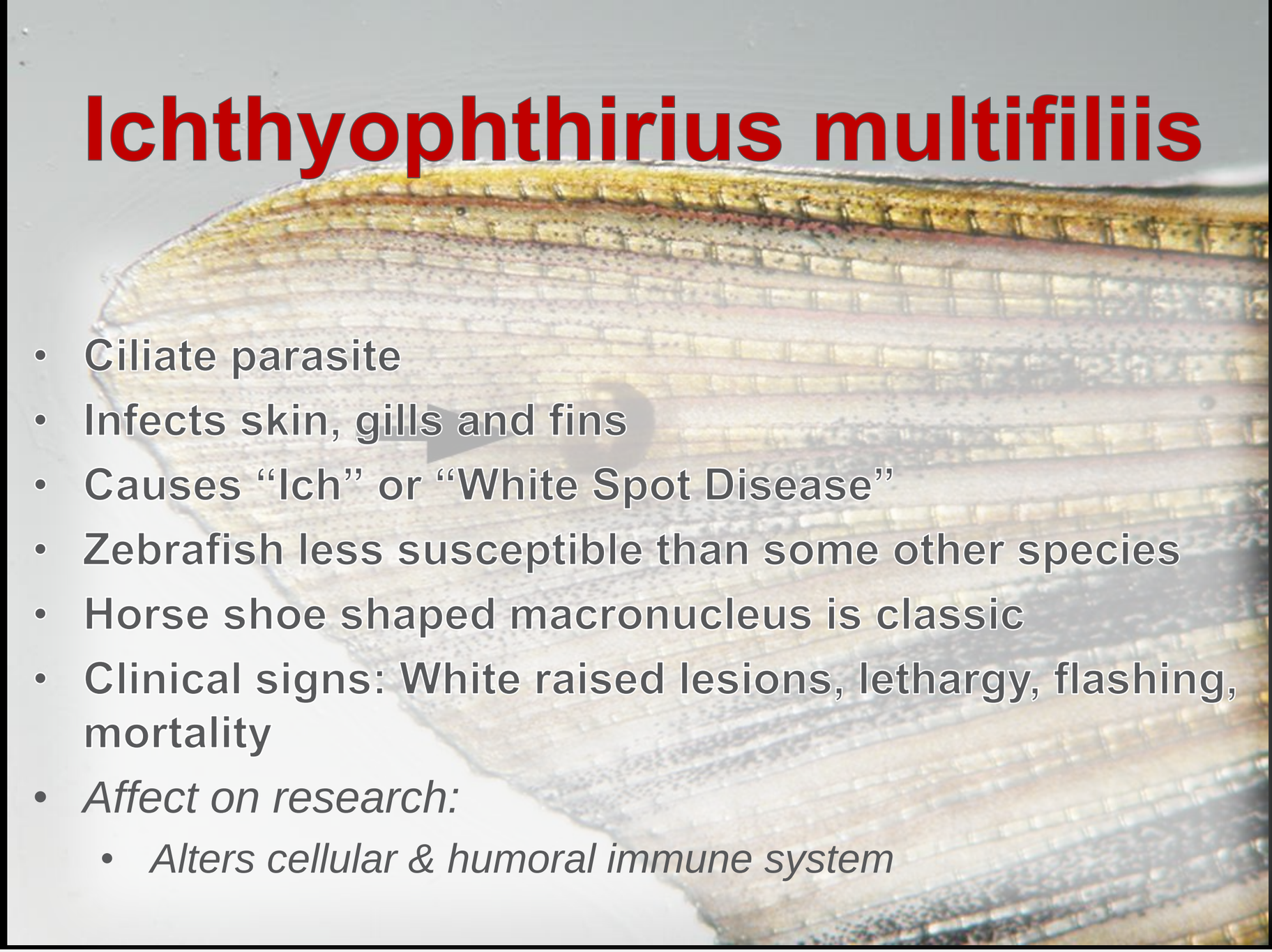
Edwardsiella ictaluri

- Emerging Pathogen
- Intracellular Gram(-) Bacillus
- Severe Acute Necrosis in Multiple Organs
- Sepsis, Hemorrhage, Ascites
- Clinical signs: Lethargy, Skin lesions, Edema
- High Mortality
- Can be zoonotic
- *Affect on research: Alters the immune system*.

Piscinoodinium pillulare

- Dinoflagellate Ectoparasite
- Infects skin and gills
- Causes “Velvet or Gold Dust Disease”
- Variable pathogenicity—can be very high, especially if crowding or water quality is poor
- Clinical signs: Lethargy, flashing, labored breathing
- Mortality

Ichthyophthirius multifiliis

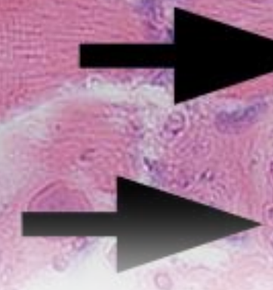


- Ciliate parasite
- Infects skin, gills and fins
- Causes “Ich” or “White Spot Disease”
- Zebrafish less susceptible than some other species
- Horse shoe shaped macronucleus is classic
- Clinical signs: White raised lesions, lethargy, flashing, mortality
- *Affect on research:*
  - *Alters cellular & humoral immune system*

Pseudoloma neurophilia

- Microsporidian (Fungal) Parasite
- Extremely common—74% of research facilities
- Historically associated with emaciation +/- scoliosis
- Infects central nervous system, kidney, muscle, ovary,
- Spores Are Environmentally Resistant
- Spores Are Resistant to Disinfectants, Including Bleach
- *Affects on Research*
  - *Modifies growth*
  - *Reduces fecundity*
  - *Profound effects on behavior*

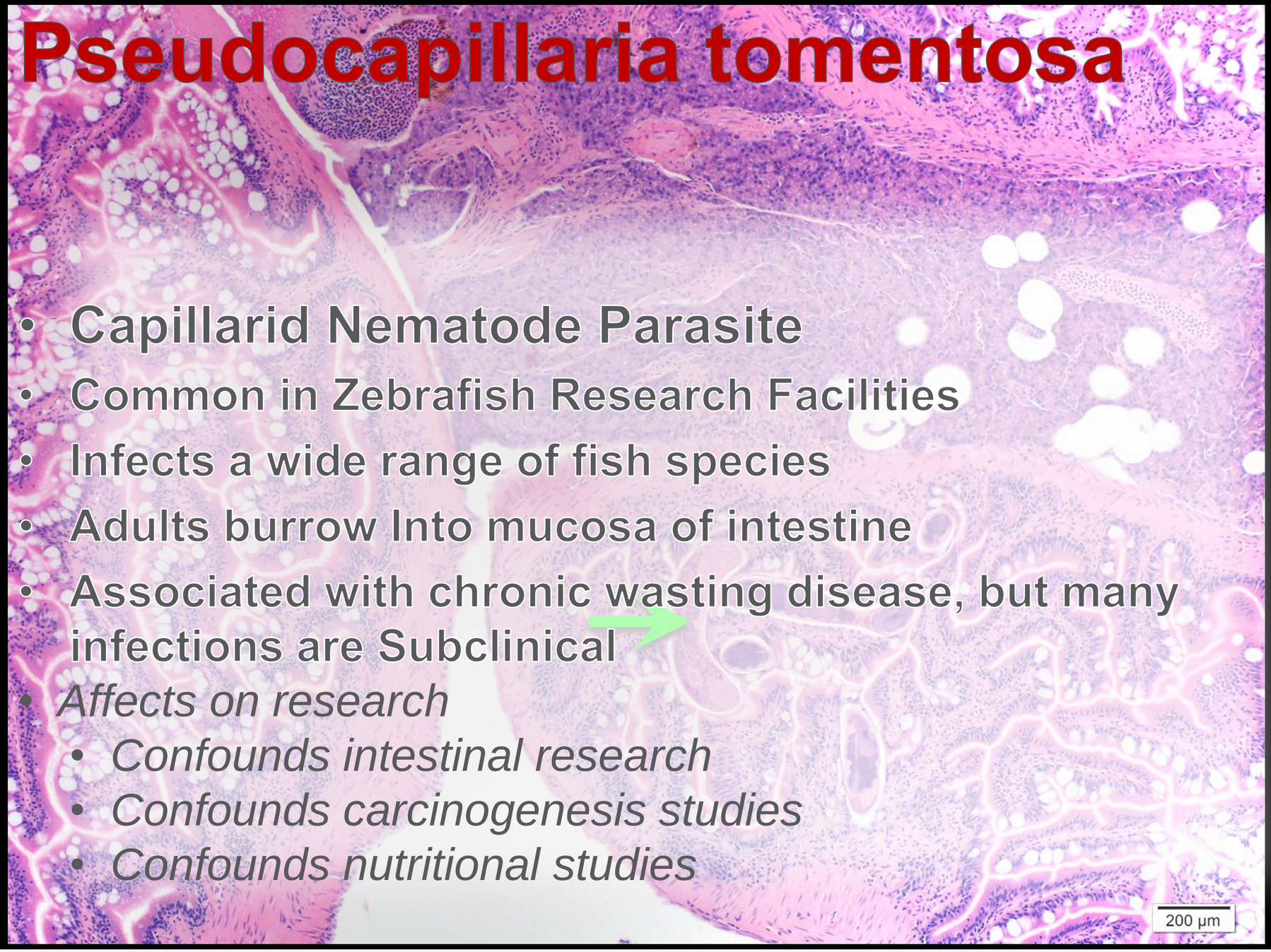
Pleistophora hyphessobryconis



- Microsporidian (Fungal) Parasite
- Causes Neon Tetra Disease
- Clinical signs: Whitish, swollen muscle tissue
- Causes muscle necrosis, difficulty swimming, death
- VERY severe disease in certain lines (*e.g.*, CG1)
- Affects on research: Profound effects on behavior

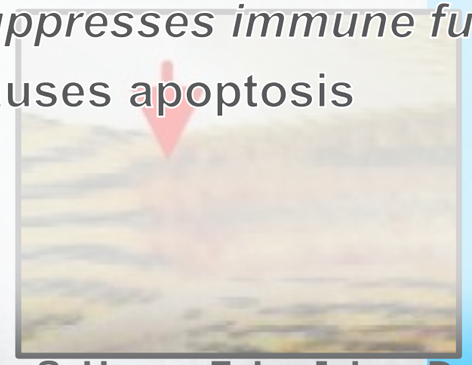
Pseudocapillaria tomentosa

- Capillarid Nematode Parasite
- Common in Zebrafish Research Facilities
- Infects a wide range of fish species
- Adults burrow Into mucosa of intestine
- Associated with chronic wasting disease, but many infections are Subclinical
- *Affects on research*
  - *Confounds intestinal research*
  - *Confounds carcinogenesis studies*
  - *Confounds nutritional studies*



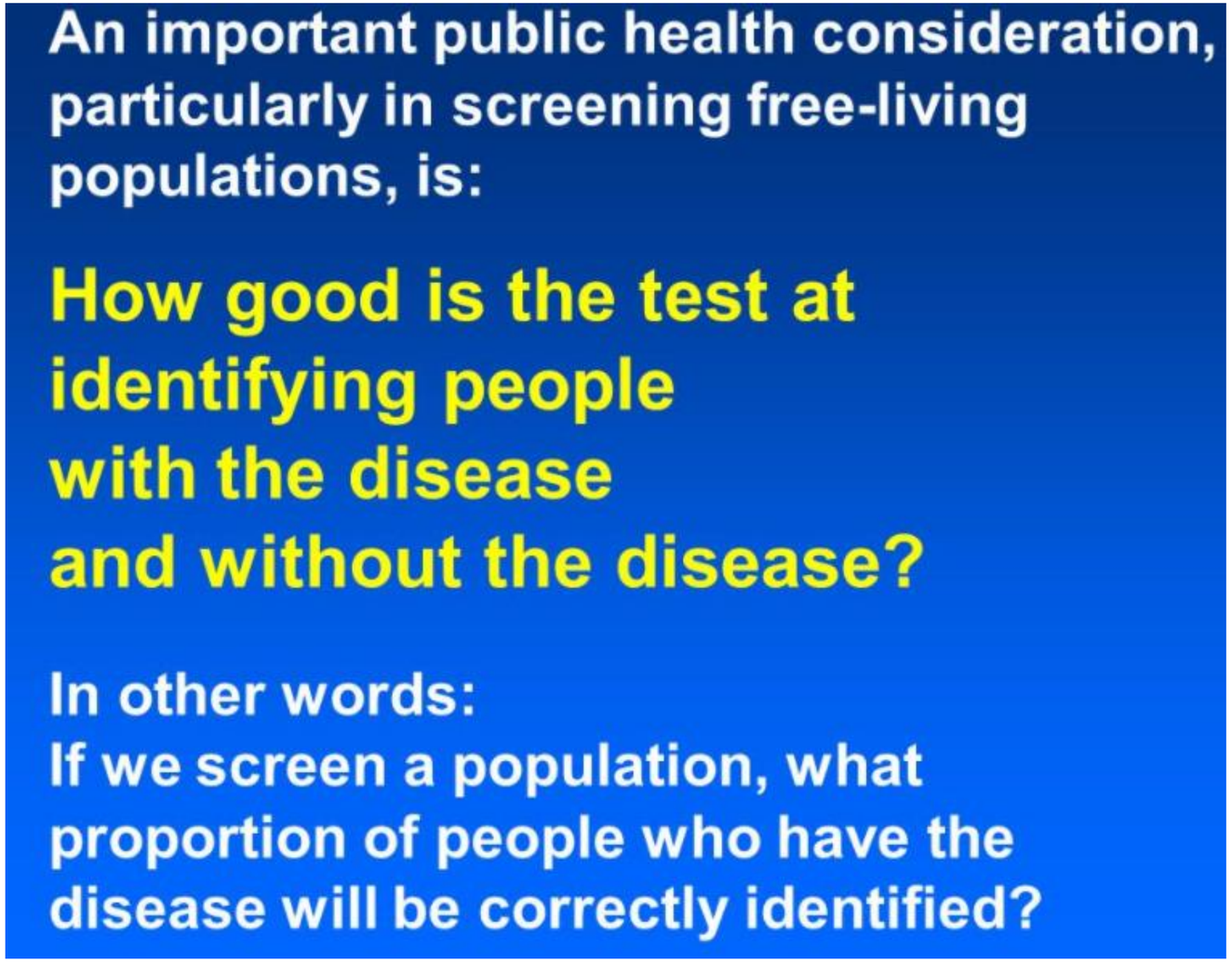
Infectious spleen and kidney necrosis virus

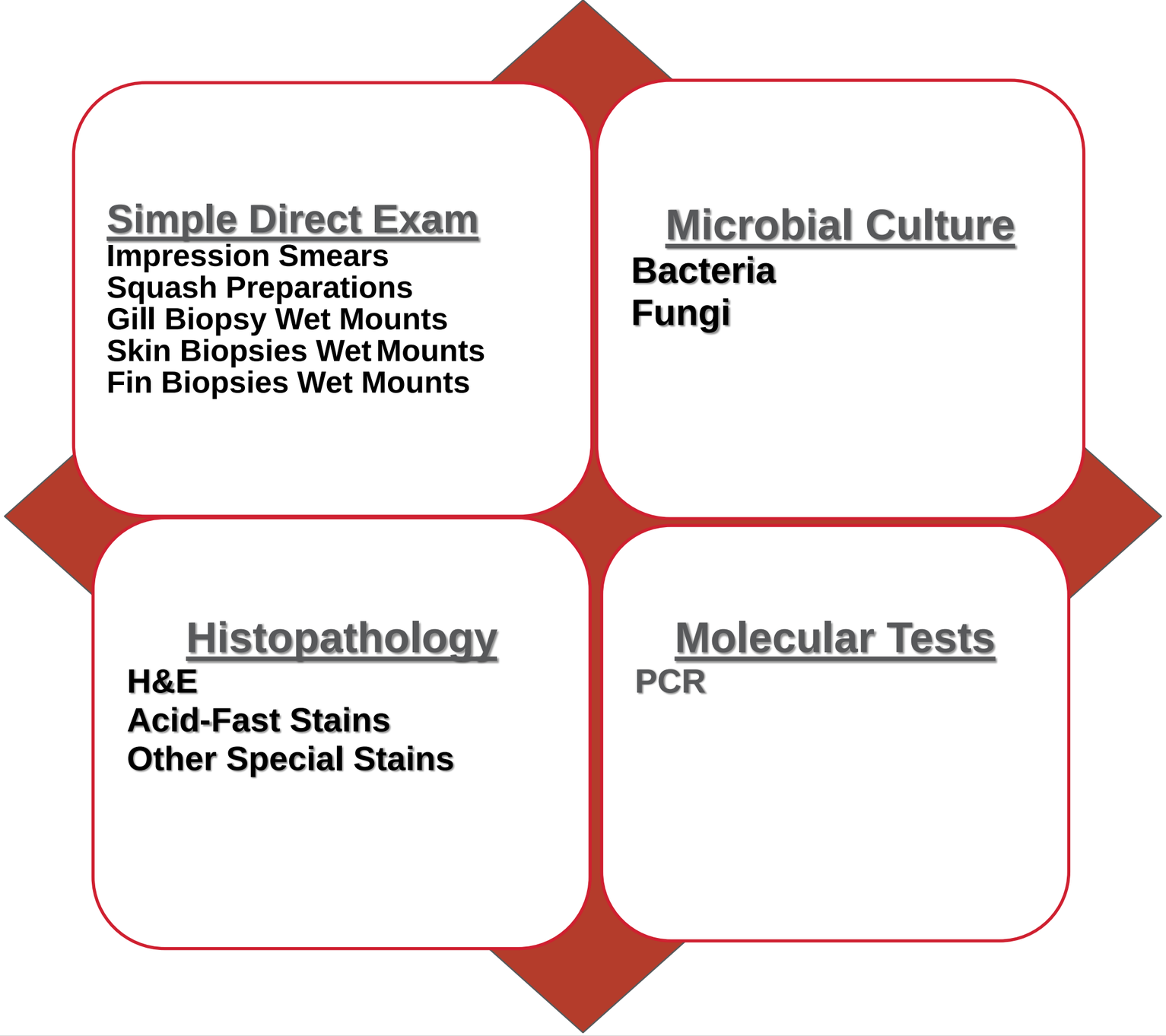
- Iridovirus
- Infects a broad host range of fish species
- Zebrafish highly susceptible
- Spleen and kidney necrosis
- Hemorrhage at base of fins, abdomen, eyes
- Scale Protrusion
- *Affects on research:*
  - *Modifies host gene expression*
  - *Suppresses immune function*
  - Causes apoptosis

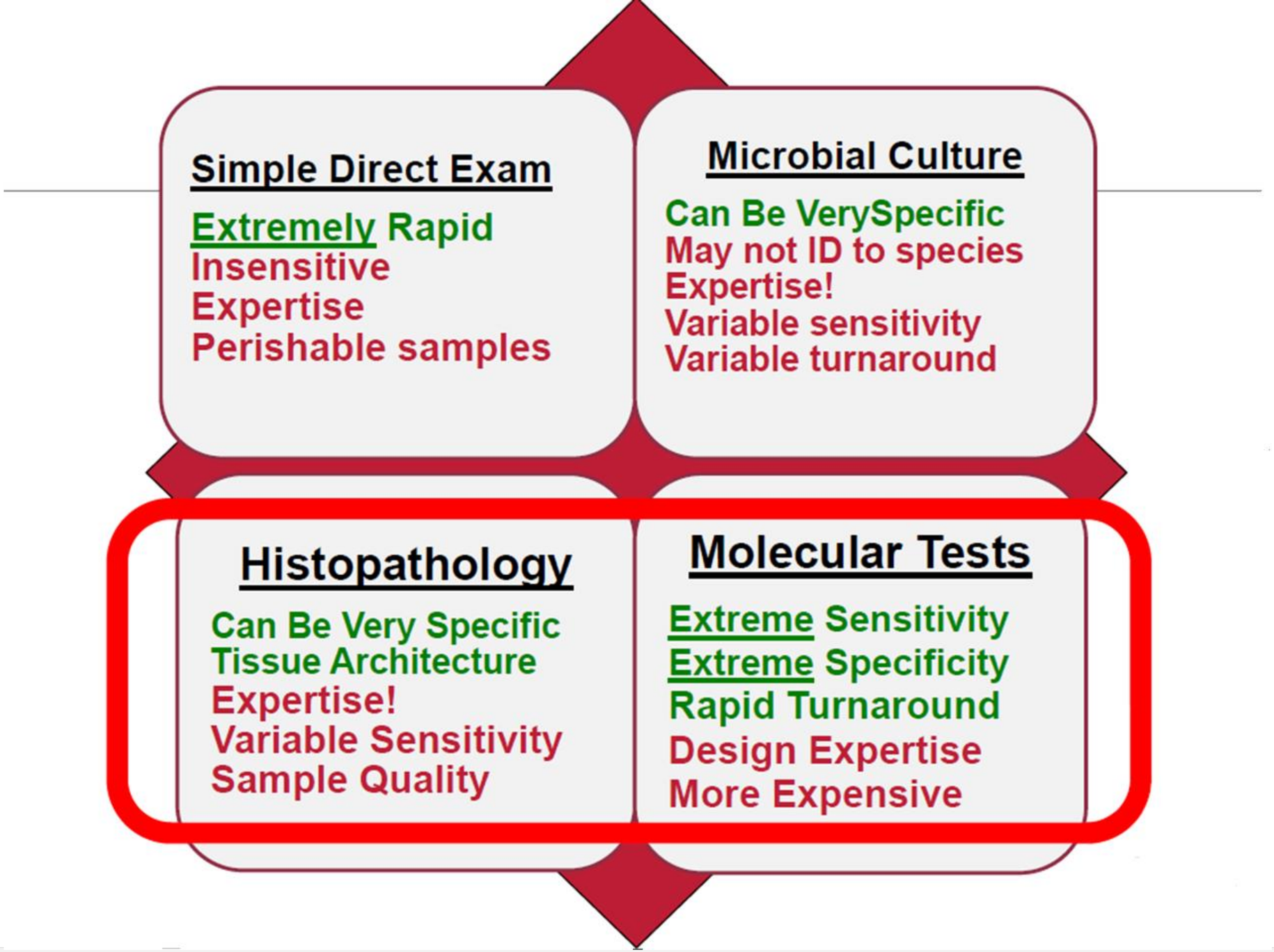


Modified from:
Xu X, Zhang L, Weng S, Huang Z, Lu J, Lan D, Zhong X, Yu X, Xu A. 2008. A zebrafish (*Danio rerio*) model of infectious spleen and kidney necrosis virus (ISKNV) infection. Virology 376(1):1-12.

screening





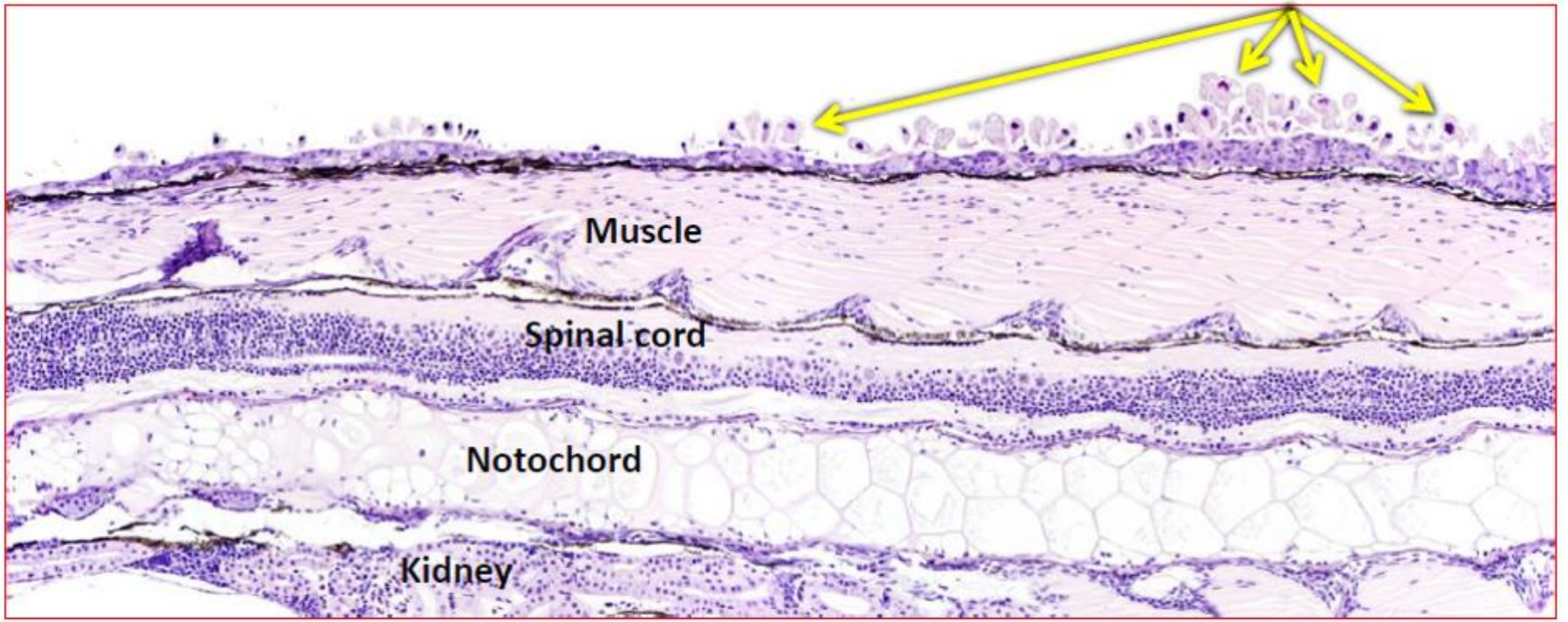


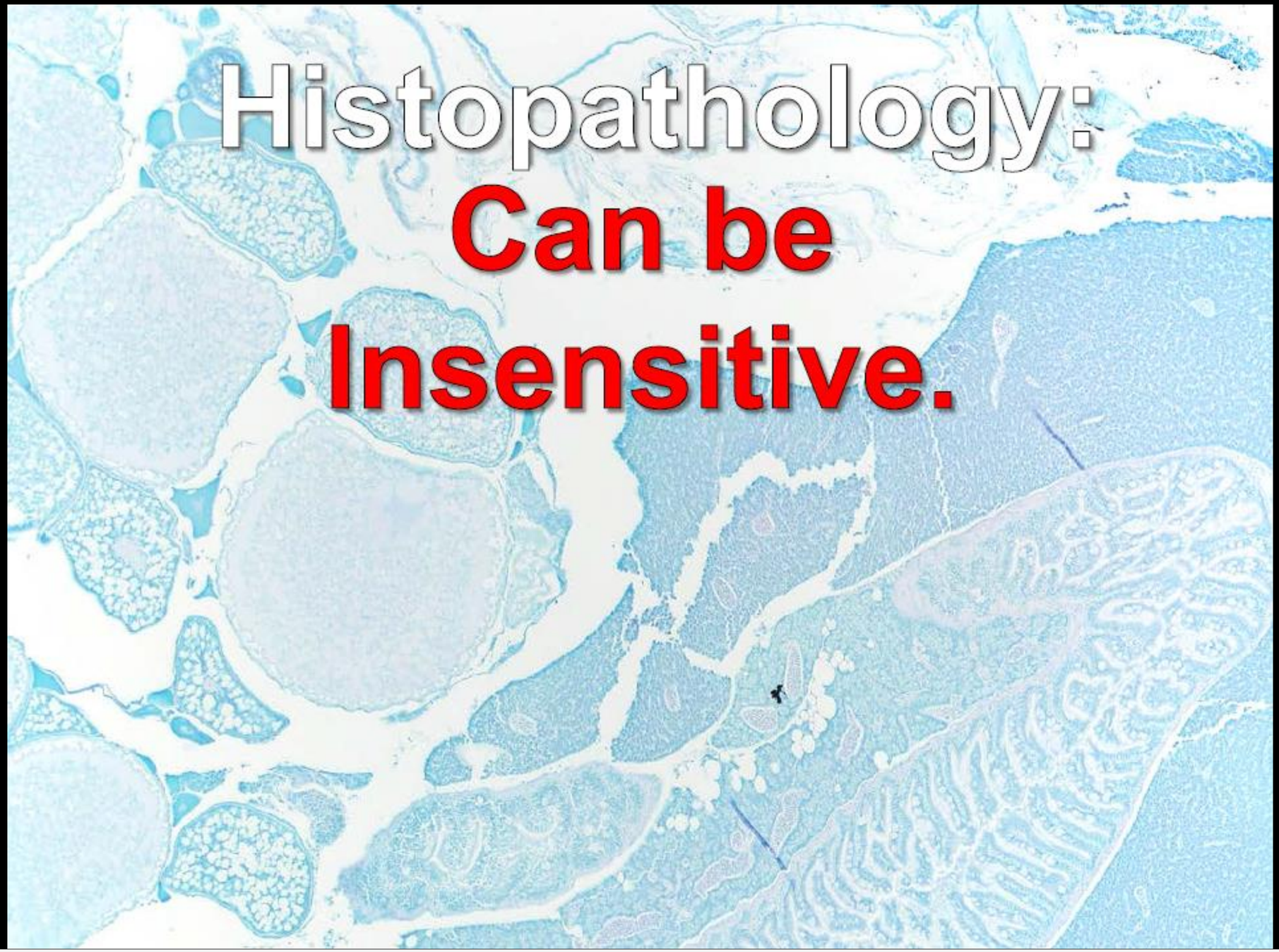
Histopathology

Can be Very Specific!

Histopathology. A lot of specific information

Zebrafish larva heavily infected with ectoparasites (***Piscinoodinium pillulare***):





PCR

Right sample, better results

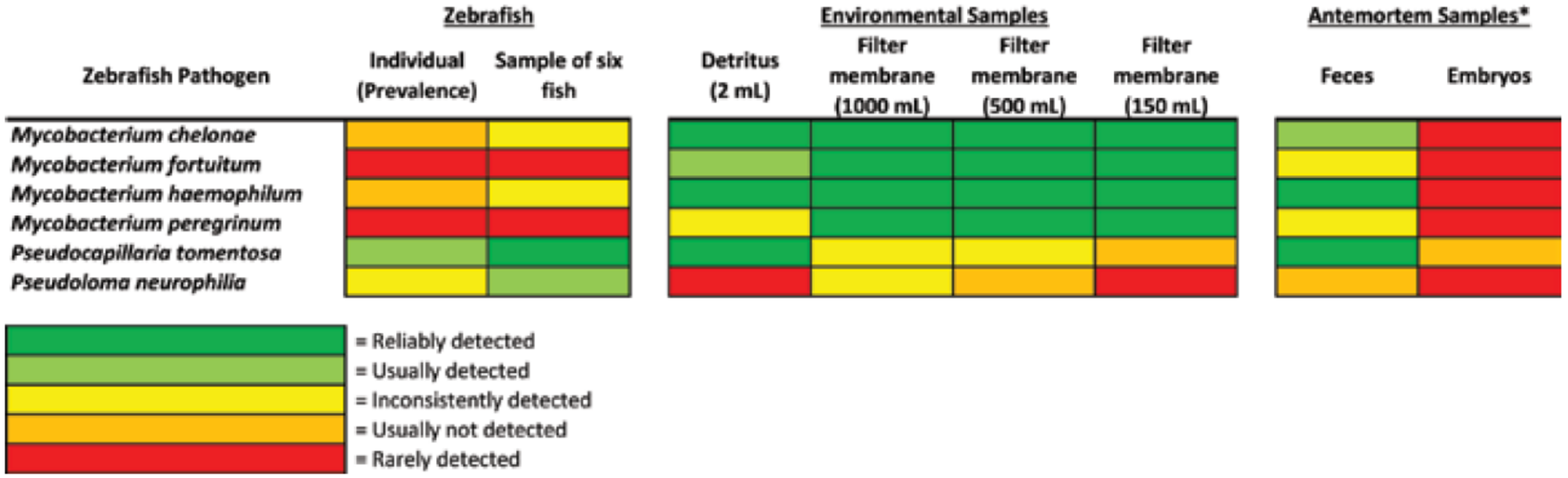


Figure 2. Heat map summarizing the relative diagnostic sensitivity of the different environmental and antemortem real-time PCR sample types for the detection of each pathogen studied: dark green, reliably detected; light green, usually detected; yellow, inconsistently detected; orange, usually not detected; red, rarely detected; *embryos collected from a small subset of the population and used to represent the entire population.

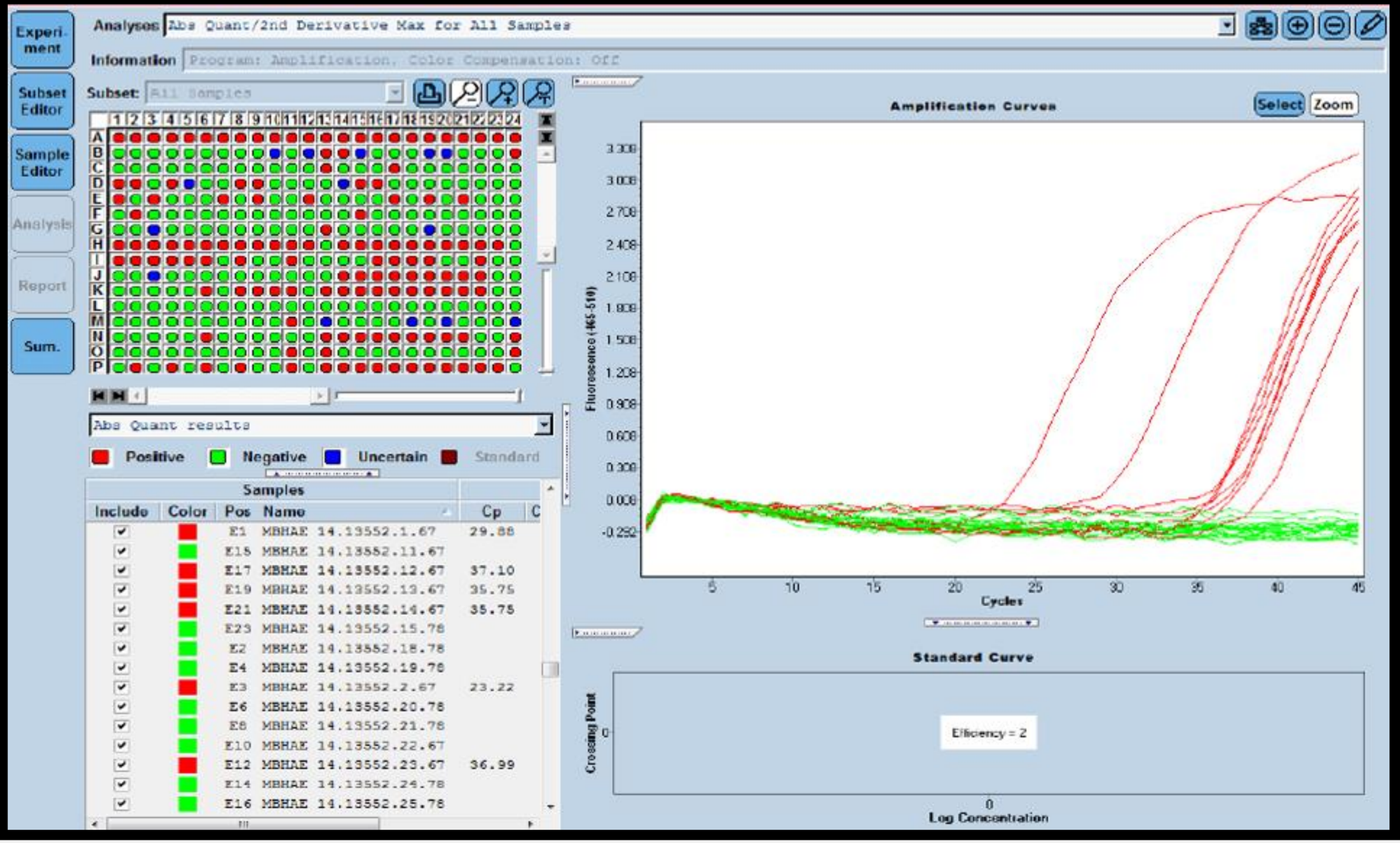
Strenghts of PCR

- **Extraordinary sensitivity**
  - A few target molecules ⟶ billions of copies
  - Samples can often be pooled
- **Excellent specificity**
  - Primers designed to amplify unique genome segments
  - Probes add an additional level of specificity
- Detects **active infections in fish**
- Useful for **many sample types**:
  - Environmental samples (e.g., biofilm swabs)
  - Microbial cultures
  - Live and manufactured feeds
  - Embryos, sperm, etc.

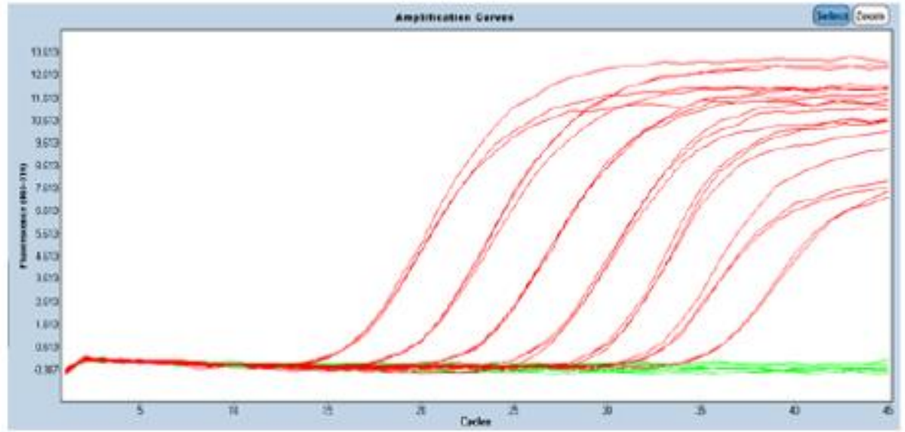
Challenge:

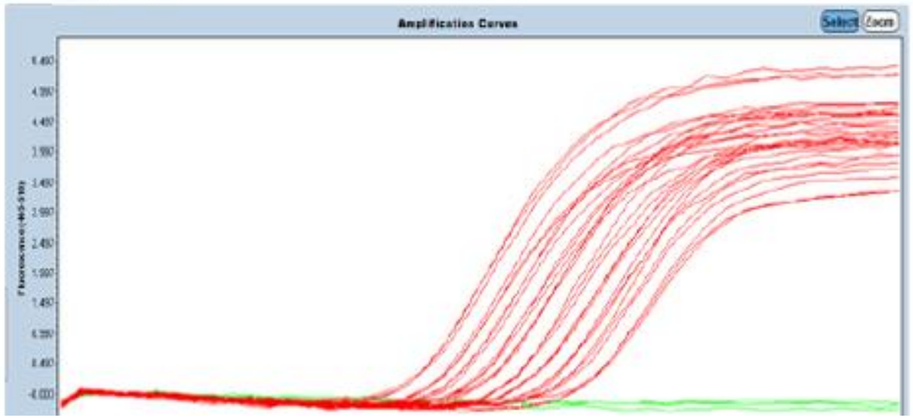
Obtaining a representative sample

Real time PCR



Assay validation and controls

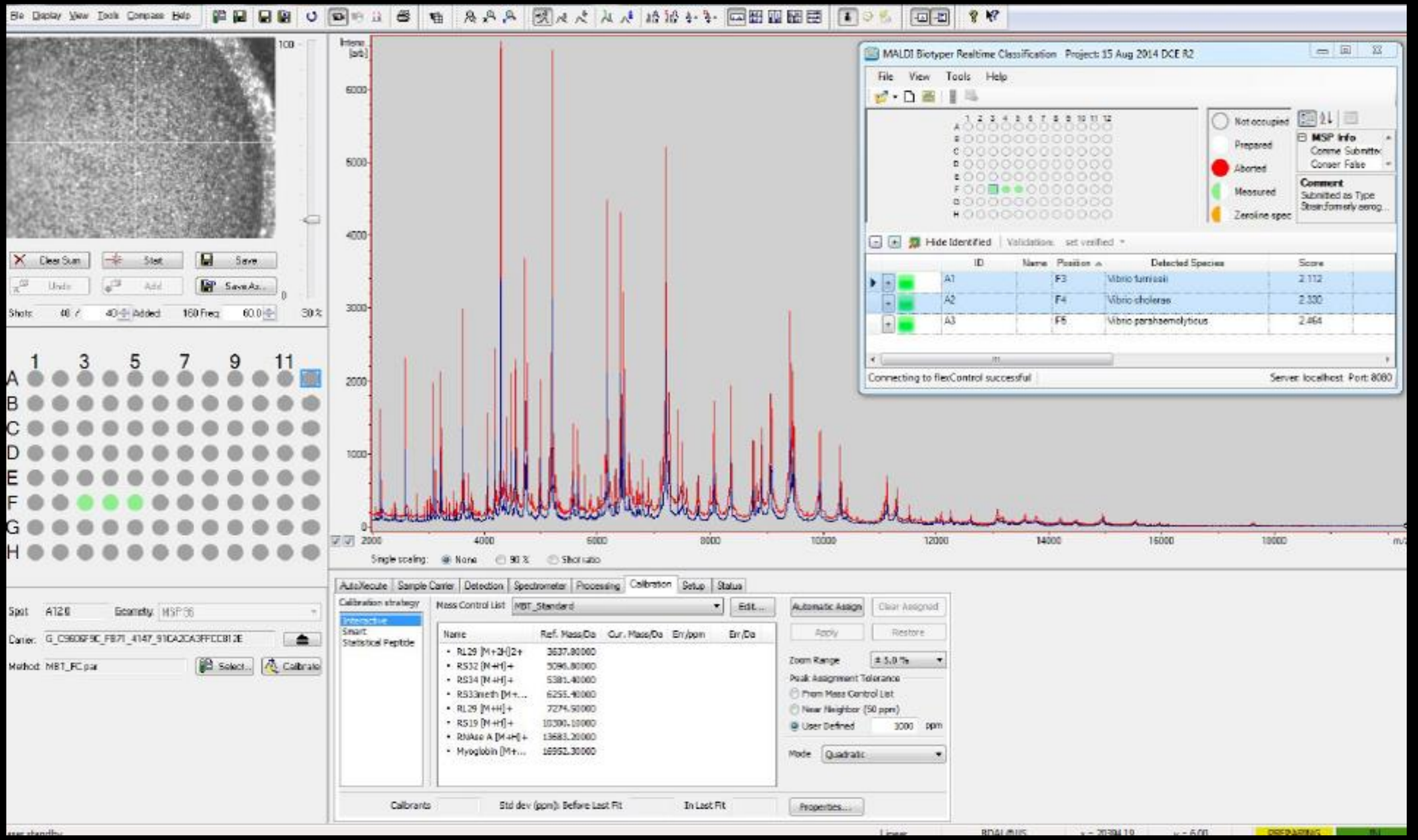




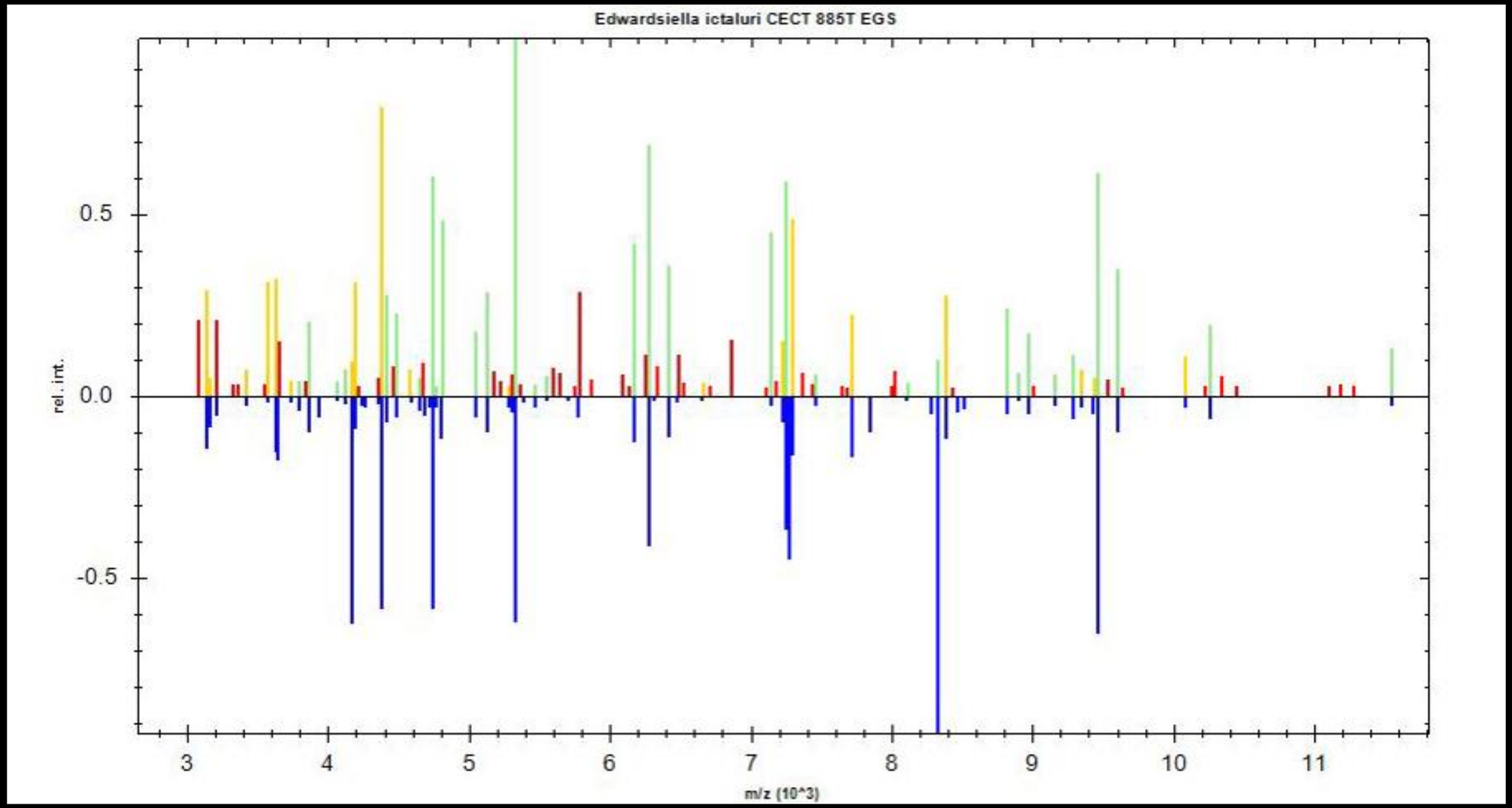
- Optimized extraction chemistry
- Analytical validation
- Clinical Validation
- Adequate Controls:
  - Positive control
  - Negative control
  - Reference gene
    - Extraction
    - PCR inhibition

microbiology

Matrix **A**ssisted **L**aser **D**esorption **I**onization-
Time **O**f **F**light **M**ass **S**pectrometry
(MALDI-TOF MS) for microbial identification



Edwardsiella ictalurid spectrum



Edwardsiella ictaluri best matches

	Detected Species	Log(Score)
🟢	Edwardsiella ictaluri CECT 885T EGS	2.334
🟡	Edwardsiella ictaluri DSM 13697T HAM	1.904
🟡	Edwardsiella tarda ATCC 35 1 EGS	1.777
🟡	Edwardsiella hoshinae DSM 13771T HAM	1.763
🟡	Edwardsiella tarda HL23 1 EGS	1.726
🔴	Edwardsiella tarda ATCC 36 1 EGS	1.673
🔴	Edwardsiella tarda DSM 30052T HAM	1.648
🔴	Edwardsiella tarda CIP 68 6 CIP	1.620
🔴	Edwardsiella tarda CIP 106473 CIP	1.604
🔴	Edwardsiella tarda CIP 68 5 CIP	1.497

Summary points

- Health monitoring is important for zebrafish health, valid data, biosecurity, and human health.
- Undetected infections can result in misinterpreted data, disruption of experiments, or the loss of a model.
- Effective health monitoring depends on knowledge of the biology and transmission of diverse zebrafish pathogens, as well as sensitive and specific diagnostic information.
- Other considerations influence success, including system design, biosecure practices, source water filtration and treatment of recirculated water.
- Histopathology, microbiology, and PCR have different applications, and usually require different samples.
- MALDI-TOF mass spectrometry is a powerful tool for microbial identification.
- Choose the best assay(s), and test the optimal sample type for that assay.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