HIPRA

ICTHIOPATHOLOGY

FISH NECROPSY MANUAL

START >

CONTENT

1 INTRODUCTION

The aim of this manual is to provide a step-by-step sampling procedure for when a disease investigation is undertaken on a fish farm.

Irrespective of the aetiological agent, many of the observable symptoms in a sick fish will be common to most diseases (darkening, exophthalmia, ascites, haemorrhage, splenomegaly, change in colour/shape of the liver, kidney, heart, gills, etc.), hence diagnosing on the appearance of clinical signs alone can lead to incorrect treatment.

Observation of the fish in the cage or tank, their reflexes/behaviour, description of the lesions, necropsy, bacteriology sampling and parasitological examination are key clues for the diagnosis of a disease, but collating information about transportation/past treatments/ handling/possible stressful events (heavy rain, predators, power off …) and water parameters is fundamental to paint a full picture of the clinical history.

2 SAMPLING

2.1. Sample selection

Which fish do I sample?

A. Select fish which present any disease symptom or moribund fish, never dead fish as the condition and chemistry of body tissues rapidly deteriorate and ectoparasites detach, giving a false picture of the condition of the fish.

B. Sick fish are easy to spot at the sides or on the surface of the tanks or close to the water inlet or outlet pipes. They are usually separated from the group (check picture 1: rainbow trout showing pop eyes, lethargy and skin darkening).

C. When possible, avoid batches of already medicated fish.

D. Other clues to select sick fish and the problems they might have:

- Gasping, rapid opercular movement and aggregation at the water inlet: gill problems, low dissolved oxygen

- Darkening: very common for most fish diseases, indicates severe stress.

- Loss of buoyancy or balance: swelling of the swim bladder (infection)

- Flashing movement or rubbing against walls: ectoparasites.

- Whirling (circling): infection affecting the brain (septicaemia / IPN/VNN)

E. How many? Not too many for the farm, not too many for the lab:

- Minimum of 5 fish with signs of disease from each affected tank/cage.

Picture 1: Sick fish.

2.2. Working area

If there is no laboratory on site, set aside an indoor area (check pictures 2.A and 2.B) which can be used for sample collection. Avoid sampling outdoors: heat, wind and rain can affect samples.

· Prepare the working area: disinfect the working surface, cover the surface where you will work with bed pads or filter paper. Also, have some buckets in which to dispose of the dead fish, and consider how you will disinfect the dissection equipment and also how you will dispose of the waste.

Ensure that all the dissection equipment (check table 1)

Dissection equipment	
Dissection kit: forceps, scalpel and blades, and scissors	Disposable sterile loops (for bacteriology)
Gloves	Dissection board (easy to clean and disinfect) and/or disposable material such as bed pads or filter paper.
Waterproof notebook/ sampling form-template	
FTA cards or PCR tubes (with RNAlater or alcohol 70%)	Coverslips
Ruler	Microscope slides
Pen	Paper towels
Agar plates (check **page 14** to choose the right agar medium) and sealant, or swabs with transport media	A spray bottle or disinfectant holder with 70% alcohol, isopropyl alcohol , bleach or other industrial disinfectant.

Table 1: Disection equipment.

Picture 2.A: Improvised working area over a workbench.

Picture 2.B: Improvised working area over a desk.

Picture: Dissection kit

3 EXTERNAL EXAMINATION

This is a visual inspection of the fish (Fig 1). If necessary, samples will be taken for further analysis: bacteriology and PCR from skin lesions or gills using aseptic procedures **(pages 14, 15 and 16).** Recording biometric parameters such as weight and length is advisable. Also, taking notes on the batch or lot of fish, origin (hatchery) etc. will be important.

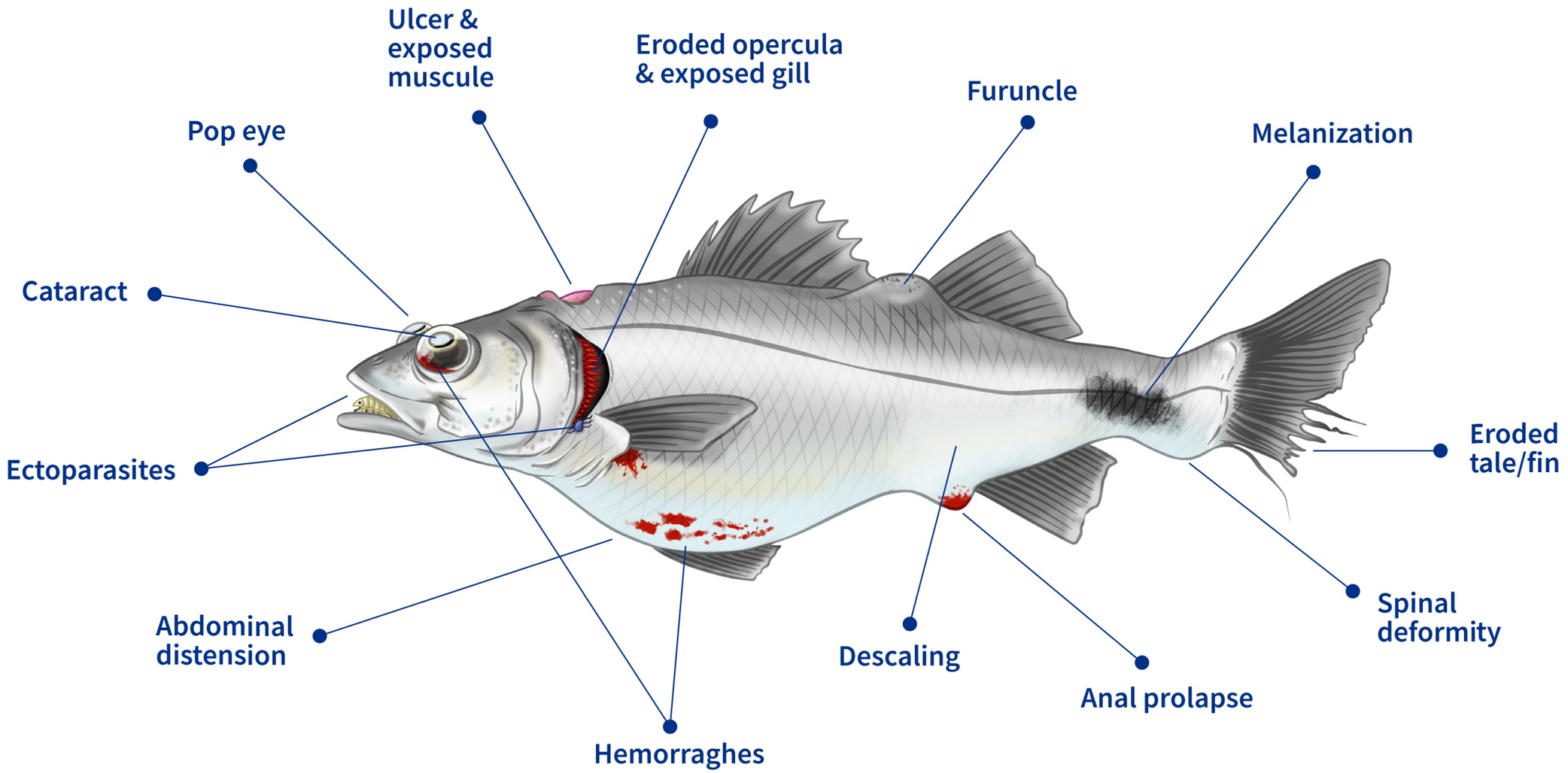


Figure 1: Possible visible external changes.

Check and record any changes in:

SKIN:

- Ulcers/ lesions/ furuncles (abscess), and their location on the body **(Picture 3)**.
- Skin haemorrhages: blood leaks over the surface of the skin, and their location **(Picture 4)**.
- Spots, and their colour and position.
- Descaling: scales are noted on the dissection board or the hands. Stress-related.
- Excess production of mucus. Stress-related.
- Macroscopic parasites and their location.

MOUTH:

- Check for haemorrhages, macroscopic parasites or stomatitis= biofilms of yellow bacteria (*Flavobacteria* spp. in fresh water and *Tenacibaculum* in sea water)

EYES:

- Haemorraghes: these can be bilateral (viral or bacterial diseases) or unilateral (traumatic injury).
- Corneal opacity/cataracts: nutritional deficiencies (tryptophan and pantothenic acid), trematodes, osmoregulatory problems **(Picture 5)**.
- Exopthalmia (pop eye) caused by systemic infection (viral or bacterial), heavy metal toxicity and gas bubble disease **(Picture 6).**

GILLS:

Remove the operculum and check for

- Macroscopic parasites
- Changes in colour/ shape
- Pale red: anaemia, asphyxia
- Brown chocolate colour: nitrite toxicity
- Congested dark red colour: septicaemia
- Presence of white patches (amoebas) or biofilm (*Flavobacterium* spp. or *Tenacibacullum* spp., **picture 7**).
- Excess mucus: poor gill health (different factors).

ABDOMINAL DISTENSION

- Anterior abdominal distension: swollen swim bladder induced by an inflammatory condition (viral, bacterial or parasitic infection)
- Middle abdominal distension: accumulation of ascetic fluid in the abdominal cavity (ascites) (viral, bacterial, parasitic and toxic aetiology)
- Posterior abdominal distension: enlargement of gonads during spawning stages.

ANUS & UROGENITAL PAPILLA REGION

- Check for prolapses or inflammation, reddening or pseudofaeces.

3.1. Rapid diagnostic methods used during external examination

1. Unstained wet mount smears from skin and gills.

A) PREPARATION OF A SKIN SCRAPING

Perform skin scraping from head to tail direction (picture 8), thus reducing the number of scales in the preparation, as the aim is to gather just the mucus and ectoparasites. The material collected is then smeared (Fig. 2) on to a microscope slide and covered with a coverslip. The smear should not be too thick, or identification of parasites may be difficult.

1. Introduce a drop from the culture water or 1 to 2% saline under the coverslip with a pipette until the area beneath the coverslip is completely saturated.

2. The preparation must be free of air bubbles and should have no excess of fluid at the edges of the coverslip.

3. Examine under a light microscope.

Picture 8: Skin scraping following the direction of the scales. Note mucus accumulating g on the blade.

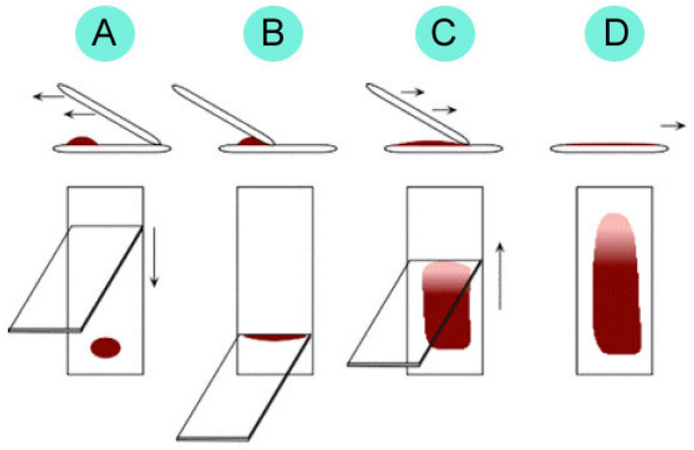


Figure 2: Smear preparation procedure is the same for any fluid: blood or digestive, skin or gill mucus. Taken from http://mt-lectures.blogspot.com/2017/08/lectu-re-13-morphological-examination-of.ht

3.1. Rapid diagnostic methods used during external examination

1. Unstained wet mount smears from skin and gills.

B) PREPARATION OF A GILL MOUNT

1. Remove the second gill arch from the fish using forceps and scissors. The second gill arch will be less damaged than the first one and will contain less organic material from the water. **(Fig.3)**

2. Using a scalpel cut the cartilage away from the filaments. **(Fig.4)**

3. Place a section of the gill on a microscope slide and coverslip it. Press down.

4. Introduce a drop from the culture water or 1 to 2% saline under the coverslip until the area beneath the coverslip is saturated.

5. Examine under a light microscope for parasites, swollen gill lamellae, white spots on the filaments, excess mucus, filamentous bacteria (*Tenacibacullum*, *Flavobacteria* etc). **Picture 9, Picture 10 and Picture 11.**

FIGURES

PICTURES

3.1. Rapid diagnostic methods used during external examination

1. Unstained wet mount smears from skin and gills.

C) PREPARATION OF A GILL SMEAR
Using this technique, it will be possible to detect both parasites and bacteria.

1. Perform gill scraping vigorously: it is important to do this in order to gather mucus, blood cells, epithelial cells and any bacteria present on the gills (Picture 12)

Picture 12: Gill scraping, recover mucus on the blade.

2. Once on the slide, the tissue should be spread properly, leaving a thin layer (check figure 2)

3. Allow the smear to air dry.

4. Stain using Gram stain, Diff-Quick, Giemsa, Fuchsine, etc. and examine under a compound microscope.

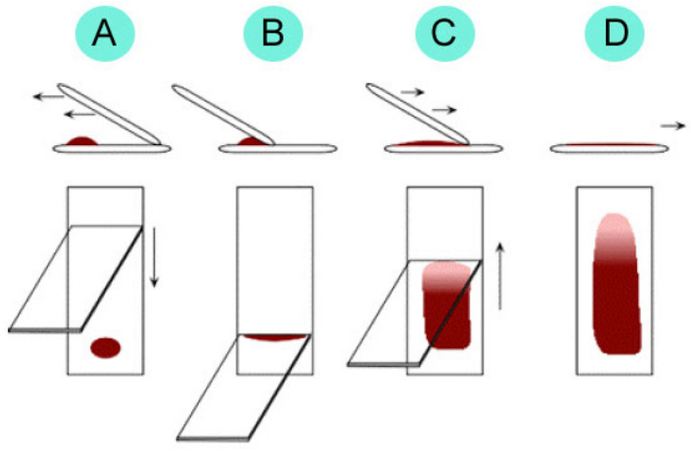


Figure 2: Smear preparation procedure is the same for any fluid: blood or digestive, skin or gill mucus. Taken from http://mt-lectures.blogspot.com/2017/08/lectu-re-13-morphological-examination-of.ht

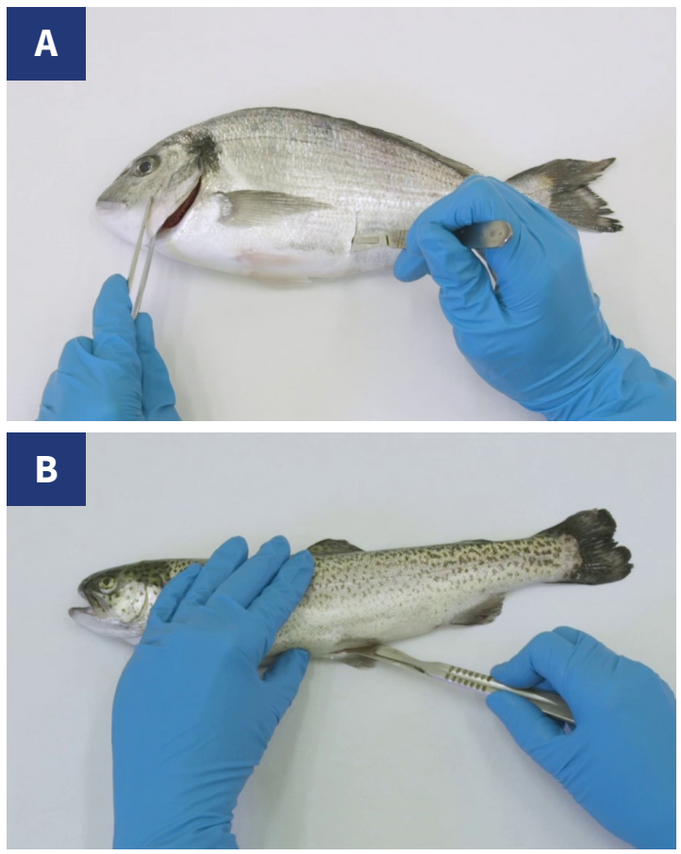
4 INTERNAL EXAMINATION

STEP 1

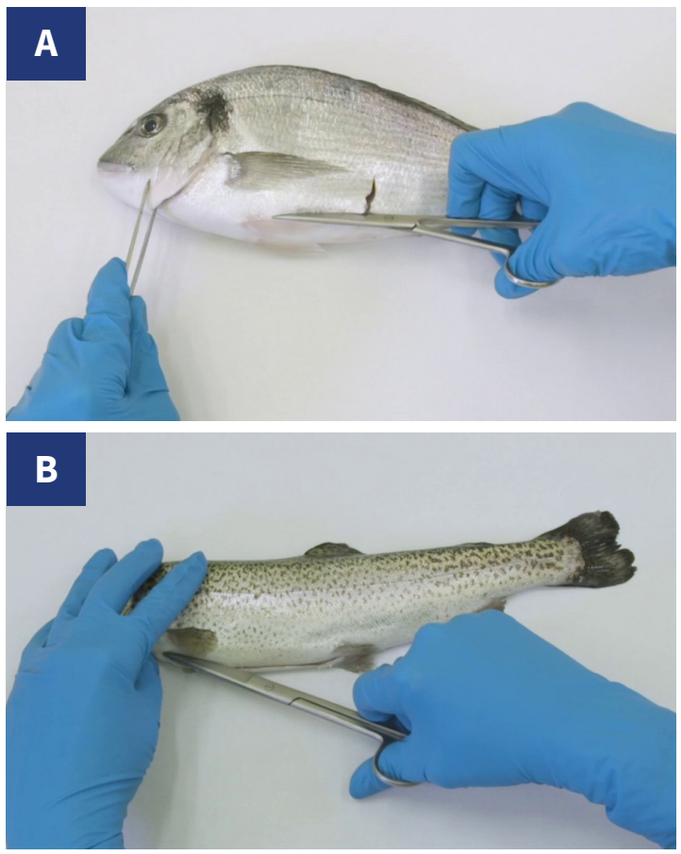
1. Place the fish on its right flank and using a sterile scalpel (new or dipped in alcohol or bleach), make an incision in the abdominal area (Picture 13), avoiding perforation of the digestive system.

2. Carry the incision forward to the throat (Picture 14) and later backward to the anus using a pair of sterile scissors (take care not to cut the gut on the inside).

3. Carefully cut away the musculature overlaying the internal organs to expose the intestine (picture 15).



Picture 13: Pelvic incision in seabream (A) and trout (B)



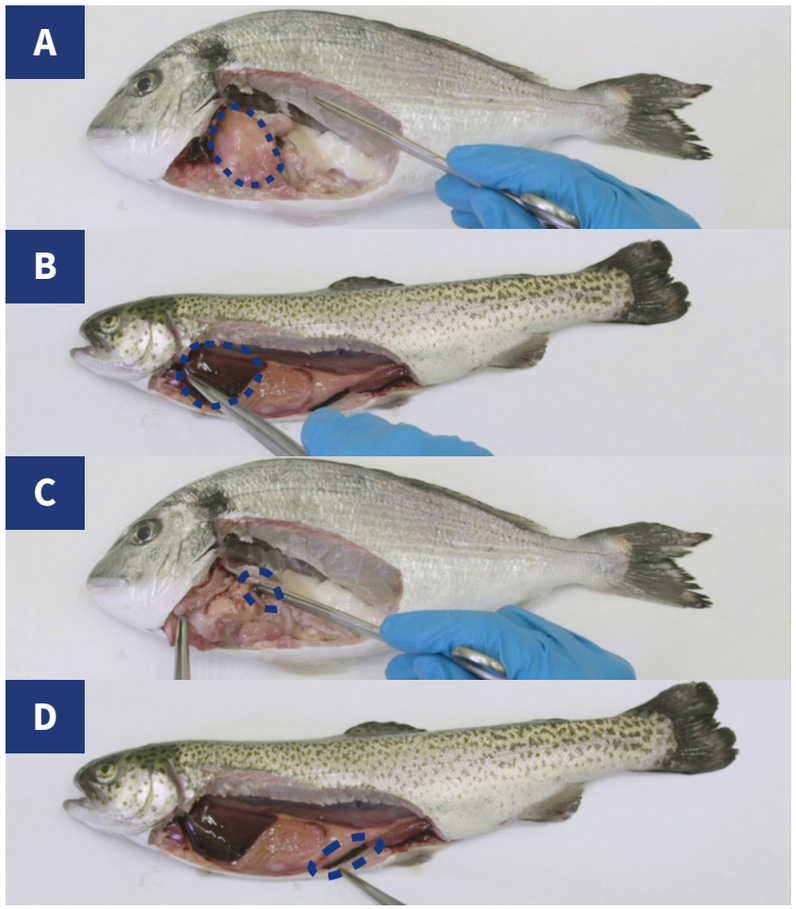
Picture 14: Incision forward to the throat in seabream (A) and trout (B)

Picture 15: Expose the abdominal cavity

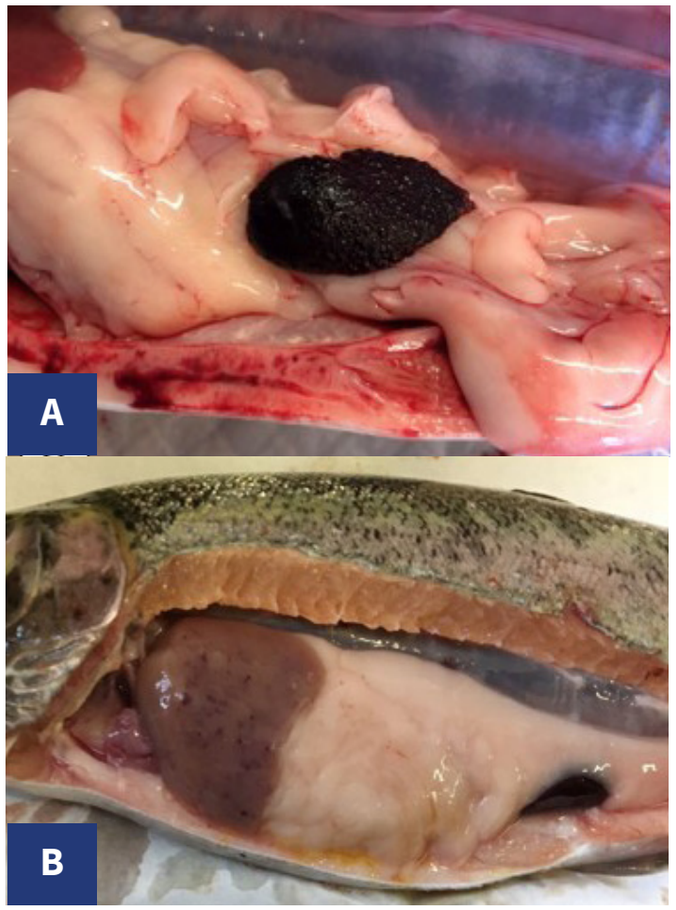
STEP 2

1. Look over the surface of the gut and the muscle flanks, checking for any abnormalities (haemorrhages, ulcers, changes in colour etc.) and note the position of any. You will be able to see vaccine residues and adhesions (if present) if the fish has already been vaccinated

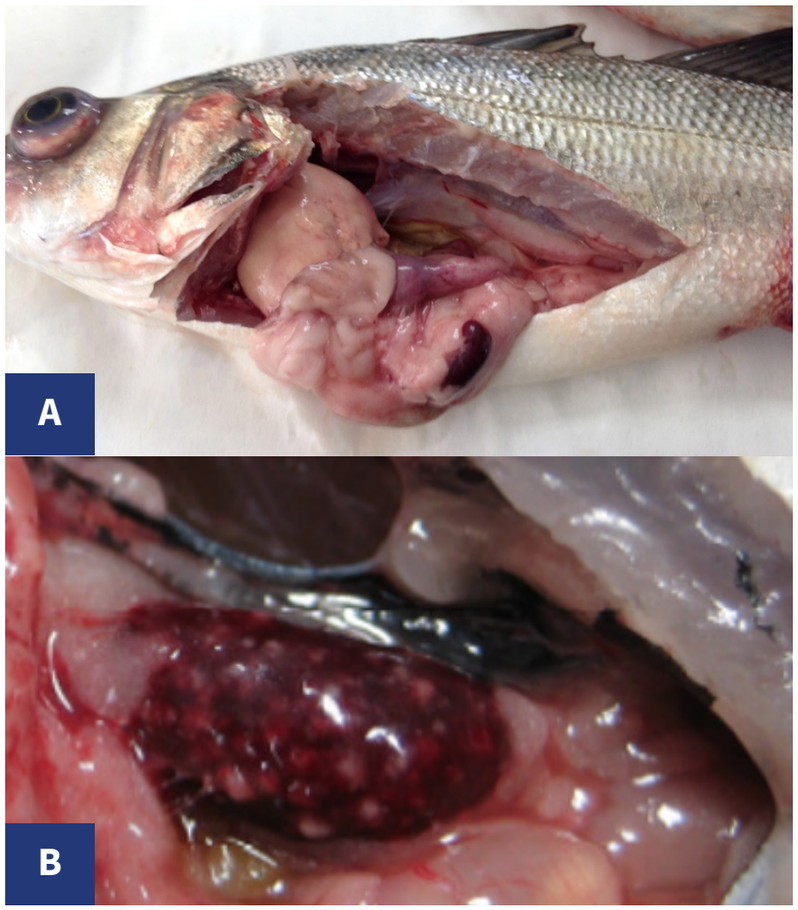
2. Locate the main organs (picture 16): Check and record any changes in colour and/or size/shape of the liver, spleen and gut (pictures 17 and 18).



Picture 16: . A) Liver position in seabream B) Liver position in trout. C) Spleen position in seabream. D) Spleen position in trout.



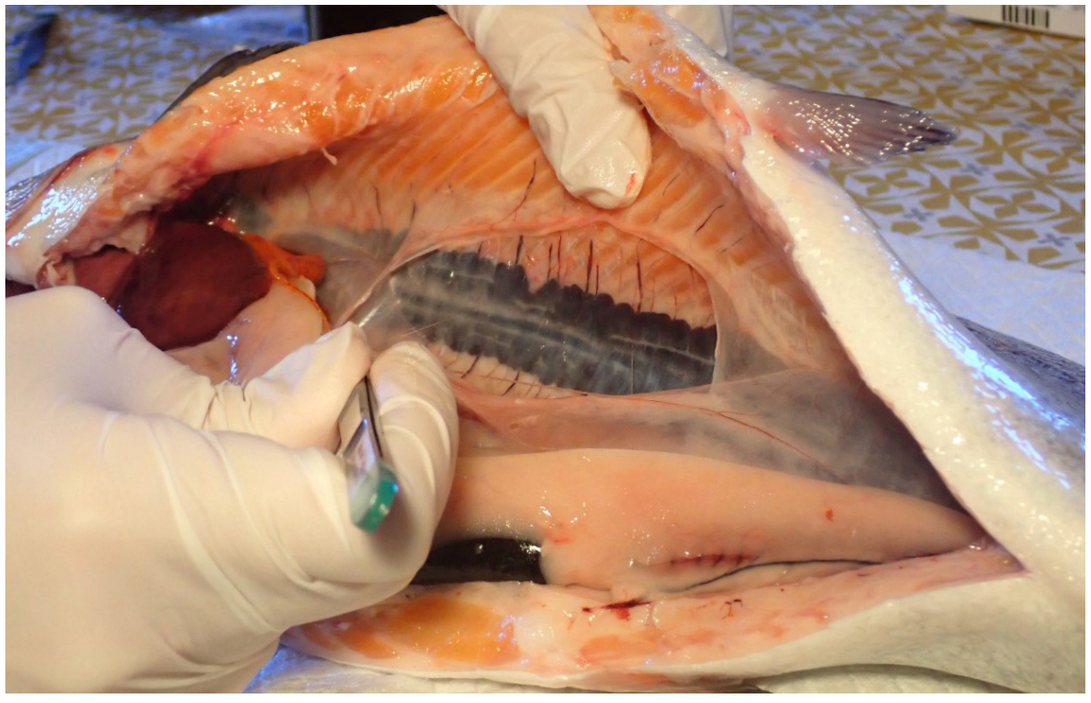
Picture 17: A) Granulated and enlarged spleen in Arctic char due to atypical Aeromoniasis. B) Changes in liver colour and presence of petechiae (red spots caused by minor bleed), pale heart, ascites, enlarged spleen and bile coming from an enlarged gallbladder (typical of anorexia) (Lactococcus gariveae outbreak)



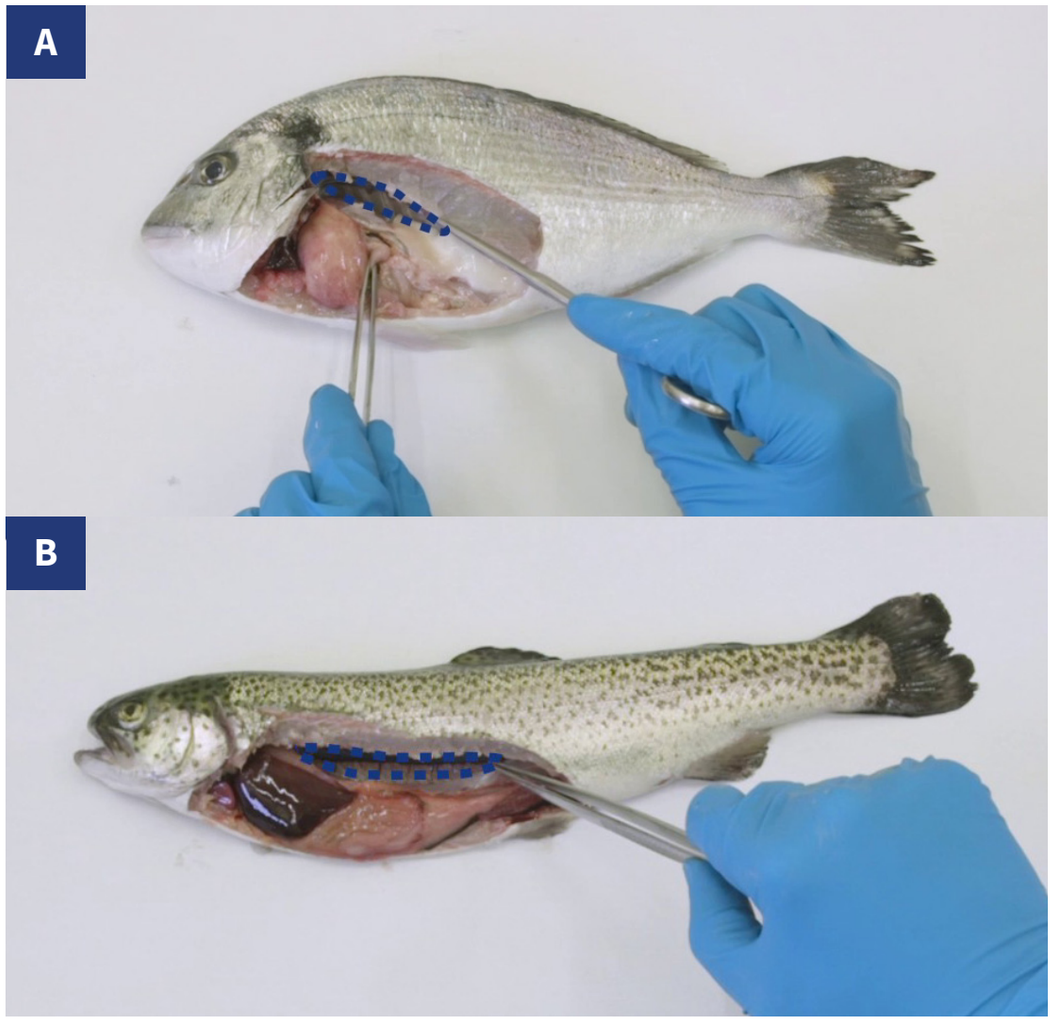
Picture 18: A) Pale liver and enteritis in seabass. B) Nodules on enlarged spleen in seabass (Photobacteria damselae)

STEP 3

1. Then move the alimentary tract to one side until the kidney is exposed, covered by the swim bladder (pictures 19 and 20). Also check and record any abnormality in colour, shape and consistency of the kidney. This is the best time to take bacteriology samples.



Picture 19: Peeling back the swim bladder in large trout and exposing the kidney.



Picture 20: A) Kidney position in seabream B) Kidney position in trout.

! Change gloves, or at least rub them with alcohol, each time a new fish is internally inspected.

4.1. Bacteriology sampling

NOTE: Use aseptic procedures for collecting bacteriology samples.

Samples for bacteriology will be taken before any other samples are taken (PCR) to avoid cross contamination from one organ to another.

WHICH AGAR MEDIUM SHOULD I USE?

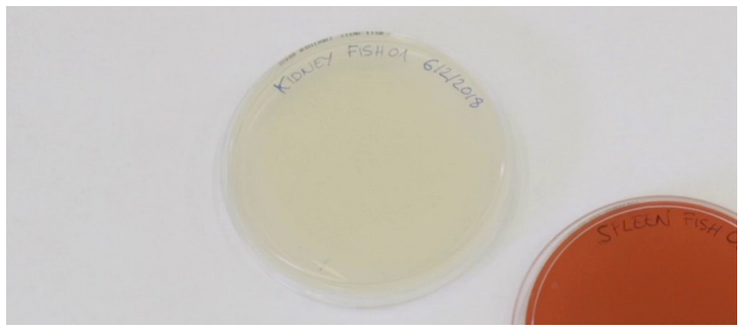
- If there is no evidence of any visible internal lesions, a kidney sample should be taken and inoculated into a general growth medium (blood agar)

- Samples of other organs will be taken if evidence of abnormality is observed, e.g. granulated/ enlarged spleen, congested liver etc.

- Selective agars such as TYES or marine agar will be used if sampling for specific bacteria such as Flavobacterium spp. and Tenacibacullum spp. respectively, TCBS for vibriosis or blood agar plus 2% salt for some marine pathogens such as Moritella, Pasteurella and Pseudomonas genera.

INOCULATION ON AGAR PLATE

- Label around the edge of the base of the agar plate (not the lid) with a waterproof marker: note sampling date, cage/pen number, fish number and organ.



- As mentioned on previously: after exposing the kidney, carefully peel back the swim bladder (picture 19).

- Push in and insert a new/sterile loop into the kidney.

- Remove the loop from the kidney, taking care not to touch any other part of the fish (proceed in the same way for any other target organ) to avoid cross contamination.

- Inoculate the sample by placing the loop on the agar surface and spreading the sample with zigzag movements across a small agar section. This is known as streak 1 **(Figure 5)**.

- Discard the loop and using a new loop, spread streak 1 (blue) over the rest of the plate by making 4-5 parallel streaks (streak 2, orange).

4.1. Bacteriology sampling

It is possible to use one single agar plate to inoculate two samples (2 organs from the same fish, for example) . Each semicircle must be clearly labelled to properly identify each sample (check figure 6)

Leave the plate to incubate on the lid side. By doing this, condensation from the humidity trapped in the lid (picture 21, A) will not drop on to the agar and the bacteria, thereby avoiding contamination later (picture 21, B)

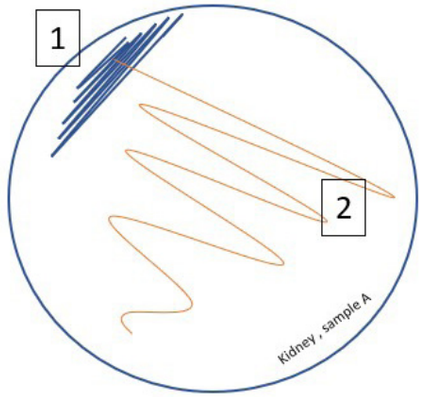


Figure 5: Streaking in agar plate

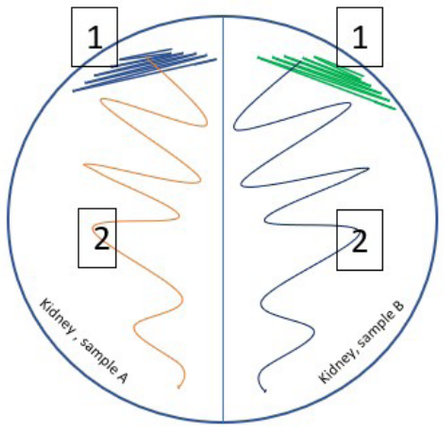
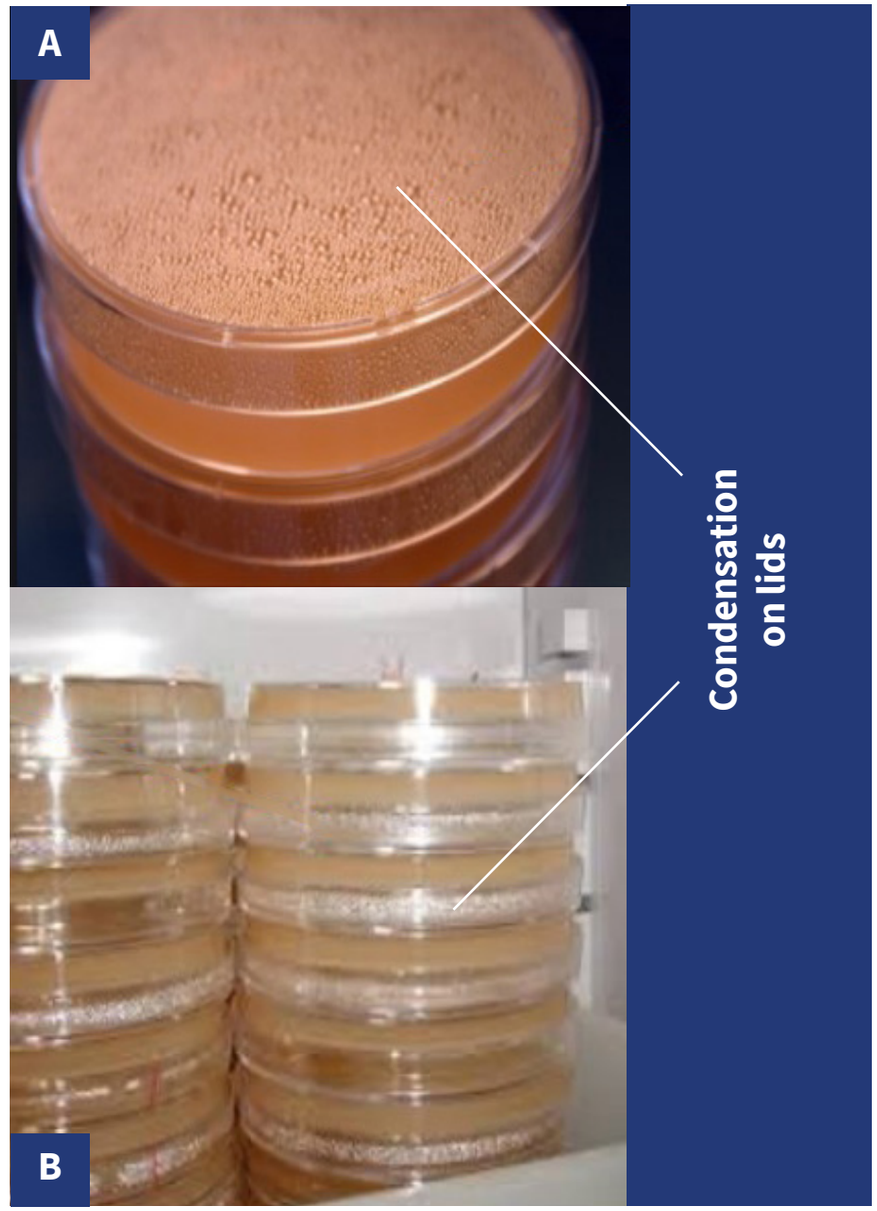


Figure 6: Streaking 2 samples

! Do not leave plates uncovered or exposed to the sun.

- **Check media expiry date & condition of the media. Have the media been refrigerated during storage?**
- **Do not use media if expired, contaminated or if they have been frozen.**



Picture 21 A&B: Agar plates showing condensation in the lids

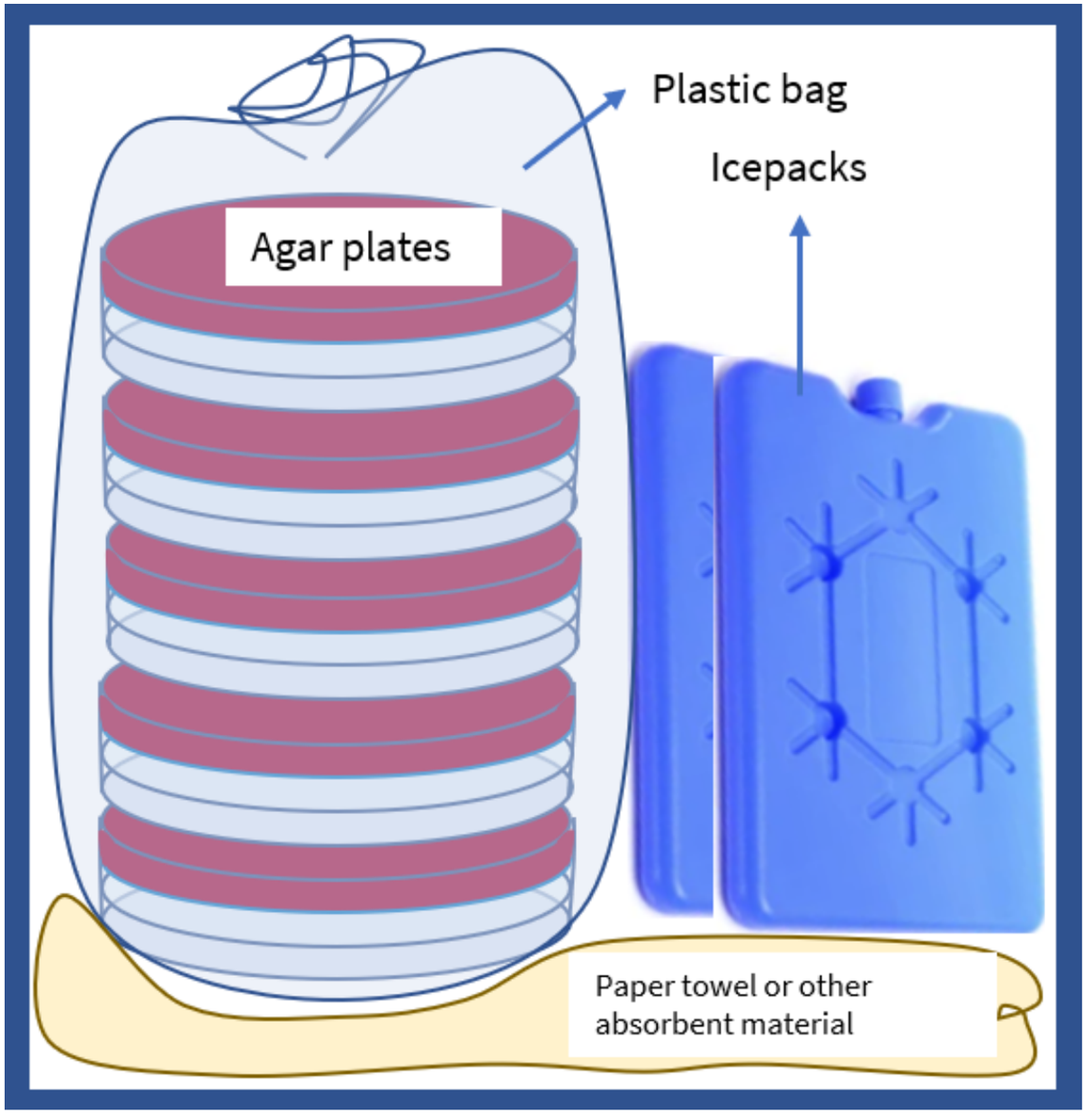
4.1. Bacteriology sampling

SHIPMENT OF BACTERIOLOGY SAMPLES

- Seal the agar plates with parafilm (or tape) and place them inside a plastic bag, always with the lid facing down as explained above.

- Place icepacks inside the parcel (avoiding direct contact with the plates, wrap them in towel paper or newspaper). Line the bottom of the box with crumpled paper towel, newspaper or bubble wrap. Then nestle the bag or bags into the box and fill the remaining empty spaces with more crumpled newspaper / paper towel.

- Complete appropriate sample submission form and notify the recipient lab of the sample submission. Follow their advice.



4.2. Samples for PCR

NOTE: Use aseptic procedures for collecting the PCR samples.

Samples for bacteriology will be taken before any other samples are taken (PCR) to avoid cross contamination from one organ to another.

WHICH ORGAN SHOULD I TARGET?

- If there is no evidence of any visible internal lesions, a kidney sample will be taken for PCR analysis.

- However, other tissues should be collected to target specific diseases, for example the brain for Nodavirus, ulcers or biofilms over gills or mouth for *Flavobacterium* or *Tenacibaculum* species, spleen for *Photobacterium damselae*, etc.

PCR SAMPLING PROCEDURE

- Extract/cut a small piece of the tissue/organ and place it into a PCR tube (with alcohol or RNAlater) or press it on to an FTA Card.

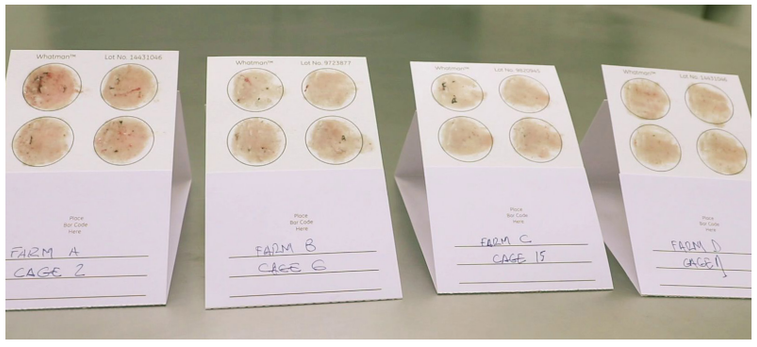
- Remember to disinfect, with bleach or alcohol, the scissors, forceps and scalpel, each time you target a different organ. Otherwise PCR samples will be contaminated.

- Label the lid of the tube or the FTA card, noting down the sampling date, cage/pen number, fish number and organ.

4.2. Samples for PCR

FTAs cards are cards made of special paper which in contact with a tissue or organ will absorb and fix its genetic material to the cellulose. The nucleic acids will remain immobilised and are preserved for transport at room temperature.

Each FTA card has 4 circles or wells. Three samples can be pooled in each well, therefore 12 samples can be taken on the same card.



When working with small fish:

- Salmonid alevins: remove the yolk sac and immerse 1 or 2 fish in the RNAlater/alcohol.

- Fingerlings: open the body cavity and scoop out the kidney and also the liver (target easy organs for collection)

Pooling samples

- 5 to 10 fry or fingerlings may be pooled in alcohol or RNAlater and treated as one sample.

- For larger fish, tissues from max. 5 fish may be pooled.

SHIPMENT OF PCR SAMPLES

Complete appropriate sample submission form and notify the recipient lab of the sample submission. Follow their advice (labelling of the shipment, dates etc).

Samples in RNAlater/ 70º alcohol:

- Both liquids can stabilise the samples at room temperature for a period of a week, meaning that transport and shipment of these samples do not need icepacks. Nevertheless, if possible, it is recommended that the samples should be refrigerated if shipment is delayed.

- Do not place the tubes directly in a box, pack them in a plastic bag with a piece of absorbent paper. Seal the bag and the box.

FTA cards ensure easy transport and shipment:

- Inactivate the pathogen so it is not categorised as Biological samples type B, therefore there are no paperwork requirements (import licences).

- Ambient transport temp.

- Up to 4 weeks for shipment.

- Use normal mail for shipment, pack them in an envelope)

4.3. Rapid diagnostic methods used during internal examination

NOTE: Use aseptic procedures for collecting bacteriology samples.

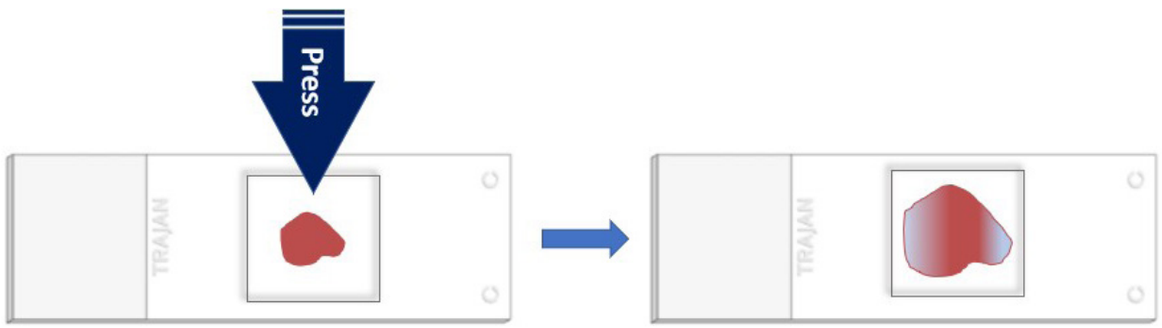
A) PREPARATION OF A TISSUE SQUASH

The practical application is the same as for gill imprints.

1. Cut a small section of tissue and place on a microscope slide (when working with the spleen and kidney, remove excess blood by placing them on absorbent paper).

2. Cover the tissue with a coverslip and gently press down allowing the tissue to spread out on the slide. If the tissue is hard or will not spread fairly easily, tease it apart carefully using a scalpel.

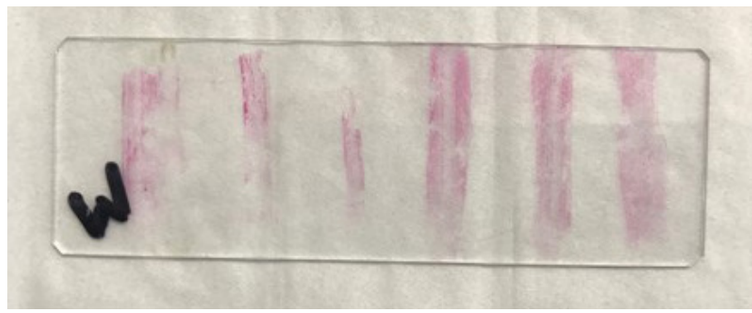
3. Introduce saline (or fresh water) under the coverslip until the area under the coverslip is saturated. Examine under a microscope. Bacterial cysts, sporozoa, fungal cysts and encapsulated larvae of various worms may be encountered.



B) PREPARATION OF A TISSUE IMPRINT AND TISSUE SMEAR

1. Tissue Imprint:
Cut a small block (1 cm3) of the tissue and press it on to a microscope slide, leaving an imprint. Repeat 2–3 times with each piece of tissue. Allow the imprint to air dry and then fix it to the slide by passing it through a flame 2–3 times. Stain with fuchsine.

2. Tissue Smear:
Scrape the tissue with a clean scalpel blade. Smear the material on to a microscope slide and allow it to air dry. Do not make the smear too thick or it will be difficult to see anything. When the smear is dry, pass it through a flame 2–3 times. Stain with fuchsine.



5 SHIPMENT OF LIVE OR REFRIGERATED FISH

If necessary, fish can be shipped live or refrigerated to a recipient lab.

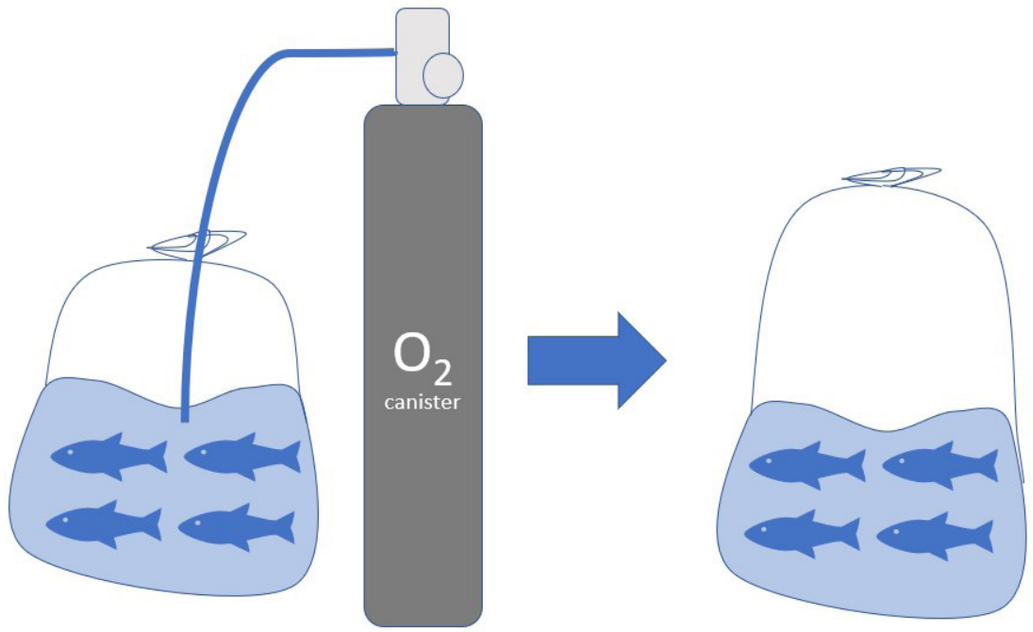
5.1. Shipment of live fish

Fill a bag less than half full of tank water, then add the fish.

Add pure oxygen or air to the bag, if possible. If you have access to a canister of liquid oxygen, use it to inflate the bag after you've added the water and the fish. Otherwise, use a hand pump to fill the bag with ambient air. This should still be sufficient to sustain the fish for 2-3 days in most cases.

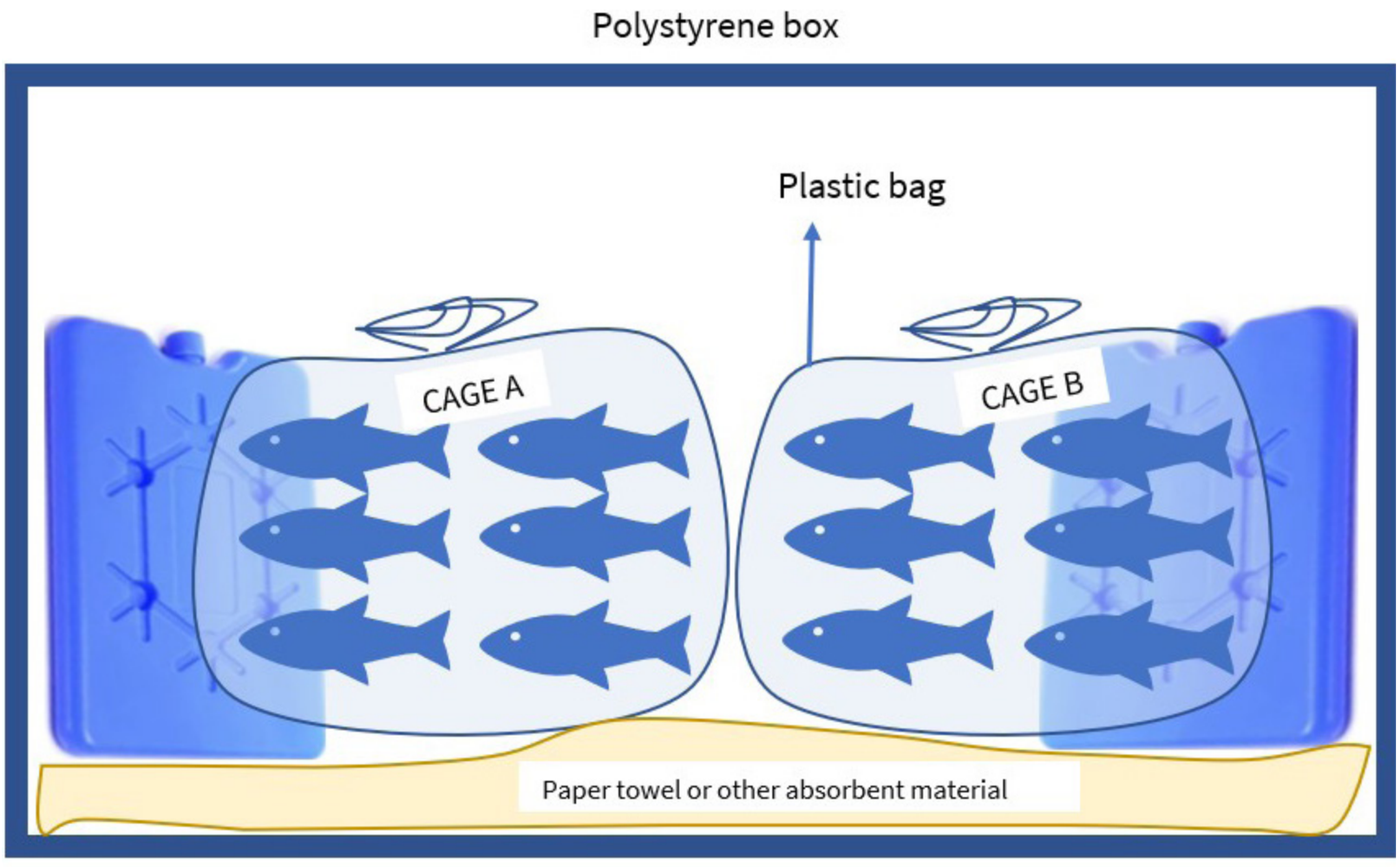
Place the bagged fish in the Styrofoam box. Line the bottom of the box with crumpled newspaper or bubble wrap. Then nestle the bag or bags into the box and fill the remaining empty spaces with more crumpled newspaper.

Add heat/cold packs wrapped in newspaper if necessary. Do not freeze the cold packs or overheat the heat packs—instead, cool or heat them so that they are just slightly below or above the current water temperature. Then, wrap them in newspaper and nestle them in the box.



5.2. Shipment of refrigerated fish

Place freshly killed fish in plastic bags, label each bag with the appropriate information on the group and nestle them in a polystyrene box, together with icepacks (wrapped in newspaper or paper towel). Line the bottom of the box with crumpled paper towel, newspaper or bubble wrap. Then nestle the bag or bags into the box and fill the remaining empty spaces with more crumpled newspaper / paper towel.

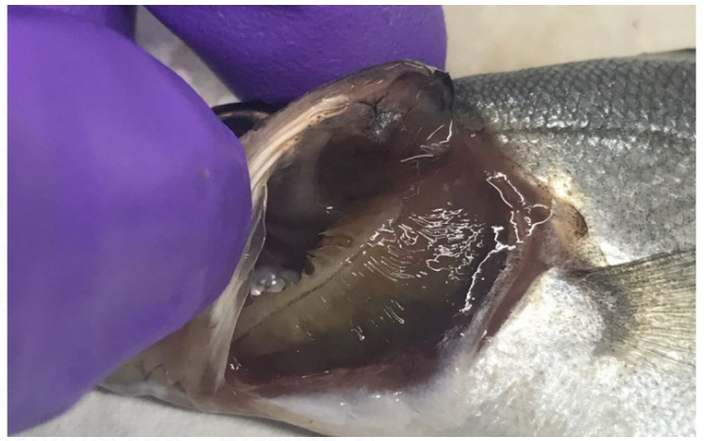


HIPRA

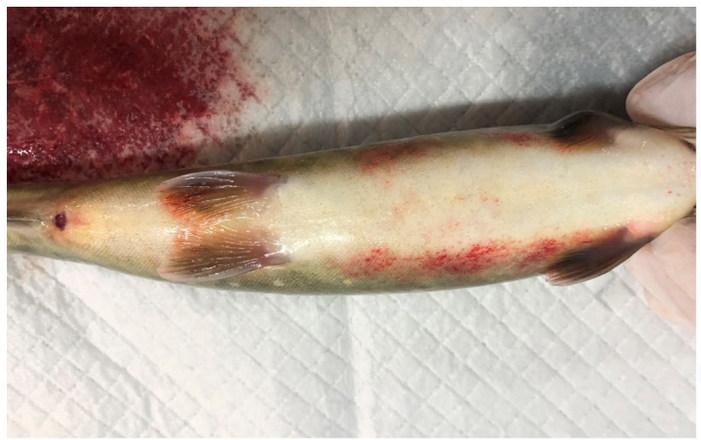
HIPRA
Avda. La Selva 135 · 17170 Amer (Girona), Spain
Tel. (34) 972 43 06 60 · Fax (34) 972 43 06 61 · hipra@hipra.com · www.hipra.com

t flank in seabass.

Picture 5: Cataracts in salmon smolt.

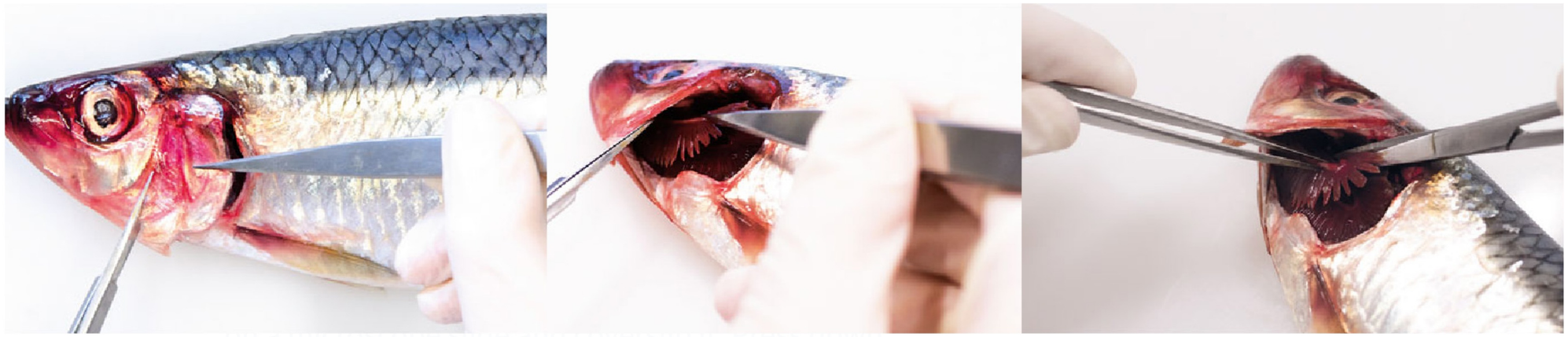


Picture 7: Yellow biofilm on seabass gills (T. m



es and haemorrhagic anus in

Picture 6: Pop eye in seabass.



f the gill cover, B) Examination of the gill and gill cavity, C) Removal of the gill (taken from Klippel. S, et all 2019. Parasites of Marine fish a cal guide. Springer)

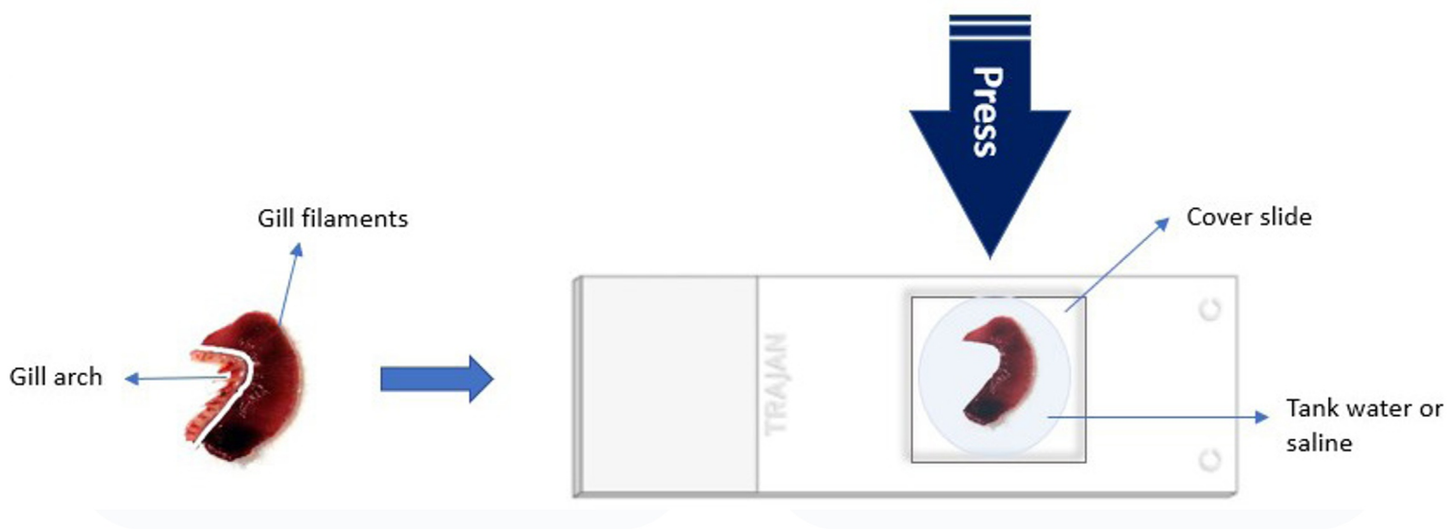
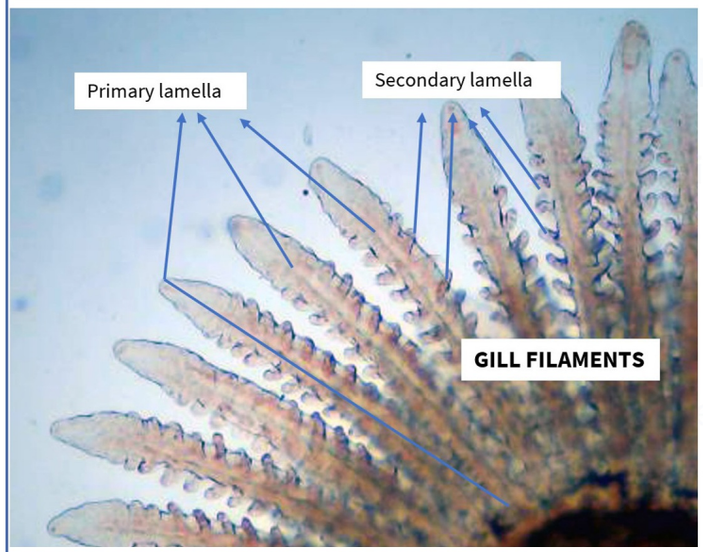
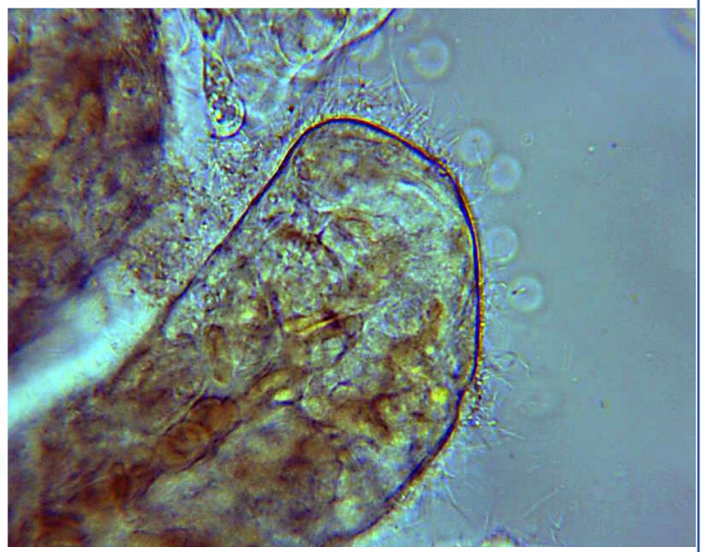


Figure 4: Remove cartilage (gill arch), place gill filaments on the slide.



fry). Water moves easilly ary and secodary lamellae.

Picture 10: Clamped filaments with excess mucus (salmon fry): O2 transport through primary and secondary lamellae is impeded.



Picture 11: Filamentous bacteria growing secondary filament