EMERGE

European Mountain lake Ecosystems: Regionalisation, diaGnostic & socio-economic Evaluation

Workpackage 5:

Fish Ecotoxicology

The EMERGE Fish Sampling Manual for Live Fish

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General

It is very important to reduce the time for catching the fish to the sampling. The least stressful catch method will be by rod, but it is necessary to use gillnets to provide enough fish for sampling. In lakes which has not been a part of AL:PE or MOLAR, gillnet sampling is necessary to get a picture of the population structure. Dead fish from gillnets should therefore be sampled for measurements of general fish data (length, weight, sex, spawning stage, stomach filling, flesh colour, scales and otholits).
NB. Number these fishes as a series different from the live fish!

To be able to interpret histology data, it is very important to note the time the gillnets have been in the lake to give a maximum time for gillnet stress, as well as the time from release from gillnets to the actual time of sampling. Fish that need to be stored before sampling must preferably be kept in keepnets in the lake. The time in the keepnets must also be noted for each fish.

The time between setting and pulling of gillnets should theoretically be as low as 2-4 hours. Since the dark nights are the best fishing period, this means use of headlights and 1-2 gillnet controls during night-time. Be careful and use floating protection!!!

Equipment for sampling

Before starting with a fish, prepare aluminium foil, paper, protocol forms, dissection tools (tweezers, scissors, razor blades, syringes etc). You need the kits for blood sampling (from J-C Massabuau), gill EM (from J-C Massabuau), glass vials for bile sampling (from J. Grimalt) and light microscopy (from R. Lackner), plastic vials for muscle, kidney, gills (from B. O. Rosseland), plastic tubes for adipose fin and Eppendorf tubes for liver and plasma (from R. Lackner).

Following equipment must also available: a centrifuge for Eppendorf type rection tubes (1.5 ml tubes) or a Bayer microspin with plasmakapillary equipment (60 µl). It is of big advantage if some ice, cold box or crushed ice (snow!) is available. Throughout all preparation keep temperature of fish and fish samples as low as possible, but do not freeze. You also need a dewar flask with liquid nitrogen or similar equipment (dry ice) to freeze some of the samples. Almost 10 litres of frozen sample storage place will be required for each sampling!

IMPORTANT MARKING:

1. **On all aluminium foil, use paper sticks for marking.**
2. **On all Eppendorf tubes for N_2 – use CRYO-pens**
3. **On all scintilation tubes, use paper sticks and write with pencil (no ink)**

Cleaning.

Correct cleaning of the material is a very important step a.o. in the analysis of organic compounds. Plasticizers are present in most recipients and material of common use (*e.g.*, gloves, bottles -distilled water is usually kept in plastic bottles). The hands and hair can also be a source of organic compounds in the sample. For this reason the cleaning procedure needs to be followed carefully during sampling and analysis.

Before starting, and inbetween the individual fish, follow the special manual of J. Grimalt, (as follows):

1. Find a flat horizontal surface to work comfortably.
2. Put the gloves on (if possible).
3. Fill a plastic tray with **EXTRAN** (Extran MA01, ref. 7555.1000, alkaline, Merck) solution 20 g per liter of distilled water) or equivalent.
4. Clean the glass bottle with this solution (to prepare a bottle with cleaning water).
5. Rinse the glass bottle with lake water (3 times)
6. Fill the glass bottle with lake water.
7. Spread an aluminium sheet over the grass or table.
8. Put in the tray the material to be used and clean it.

9. Once cleaned, rinse it with the lake water stored in (6).
   IMPORTANT: Do not throw the first rinsing water nor the cleaning mixture in the catchment. Store it in a recipient and throw it when being back in the lab. EXTRAN products can be discarded in the inorganic salt recipient for residues of the lab.
10. Rinse again the material with distilled water and later with **acetone**, to ensure that no contamination of the sample would be obtained.
11. Put the material on the aluminium sheet spread in (7)

Cover the clean material with aluminium if not to be used immediately.

Blood sampling .- Heparinization of syringes

2 or 5 ml syringes are used for blood sampling. They must be coated with heparin to avoid coagulation of blood. The best way to do this is to fill the syringe with the needle attached with a heparin solution (1000 Units/ml; dilute with water if necessary), empty syringe and remove as much liquid as possible.

EMERGE FISH SAMPLING ORDER – LIVE FISH ONLY

Before sampling label all vials, aluminium foil, plastic bags and so on with an unique code for the fish and name the organ which will be placed inside. This protocol applies to live fish only! Dead fish may be used for population data and stomach content, POP and metal samples only. The time to process one fish will be between 40-60 min dependent on assistance.

1- Blood sampling
Kill a fish by a blow on the head. Take the blood from the ventral aspect of the tail (caudal vein) with a heparinized syringe. You should get 1.5 – 2 ml of blood. Note the actual blood volume taken.
The remaining blood is transferred to **either**:
a 1.5-2 ml centrifuge tube and centrifuged (10 min, 10000 rpm).
or 2 X 6 plasmatubes for Bayer Microspin.

Collect 150 µl plasma. This equals to 6 filled microspin plasmatubes at a blodcell % (hematocrit) of 50, where the glasstubes are cut and pure plasma "blown into the a vial (1.5 ml eppendorf, stick a small hole in the locker!). Freeze it in $\mathbf{N_2}$ for vitellogenin analysis (NIVA). The remaining plasma (same amount) is collected and also frozen in $\mathbf{N_2}$ for blood electrolytes (J-C).
Be careful not to get any part of the pellet (packed red blood cells) into the plasma. This caution is more important than the actual volume of plasma obtained. The total amount of plasma for ions should still exceed 100 µl.

2- Weigh the fish and measure its **length** from the nose to the end of the tail. Add the blood volume to the fish weight and enter both values to the protocol. Turn the animal onto the side, and take **scales** from the area above the midline, between the dorsal and adipose fin. Eliminate the mucus and then collect with a scalpel or your knife some scales, and put in special envelope. Note fish code and all physical/morphological data also on the envelope.

3- Gill electron microscopy (6 fish only) Excise the 1st gill arch , cut to 6 to 8 segment with the razor blade provided in J-C C Massabuau's kit: **follow the special manual provided with the Gill-EMkit! This is a time-dependant job, and you have to note and control time!!!**

4- Metal analyses of gills. Excise the 2rd gill arch and put it in a small plastic tube (red cork and pre-numbered for weight calibration) for metal analysis. Be sure the bag is labelled correctly with the specific fish number from your lake) and freeze it. This will go to NIVA, Norway.

5- Open the abdomen and note the flesh colour (red, pink, white), sex, maturation stage (I-VII, see Figure 1) and stomach filling (0-5, 0= empty, 1 = only food remains in the end of the intestine, 5 = full)

6. Bile bladder. Dissect the bile bladder from the liver. Be careful not to damage the bile bladder and remove it carefully with sharp scissors, and place in glass container from J.Grimalt, and freeze it. (If bile is spilt on liver, do not use the contaminated liver tissue!)

7- Liver. From the liver:

- Cut a piece of about 100 mg and put it into the vial containing formalin (special kit from R. Lackner). Do not squeeze the tissue and check immediately that the tissue is **in** the liquid.
- **Two** more liver samples (each about 100 mg) are placed into vials (1.5 ml Eppendorf with a tiny hole in cap) and freez in liquid N_2. This two samples will be sent to Innsbruck for oxidative stress parameters and enzyme measurements.
- The remaining liver (need to be more than 0.5 g) are wrapped in aluminium foil and frozen. This sample will go to J Grimalt, Spain, for POP and PAH analysis.

8- Stomach. Remove the stomach of the fish and keep it at cool place for further dissection (continues as stage 14).

9- Kidney. The whole kidney is removed from the fish an collected in a white scintilation tube with screw cap for metal analysis. This sample is frozen and sent to NIVA, Norway.

10- Muscle sample. Tear off some dorsal skin to expose axial muscle from the area above the lateral line and between the dorsal and adipose fin. Remove the “red muscle” (a bit darker superficial muscle layer) along the lateral line by scraping with a scalpel.

- Take 5 g of muscle is put into a white scintilation tube wit screw cap and freeze for metal and stable isotope analyses at NIVA
- Collect about 15-20g of muscle, divide into two pieces, and wrap both in aluminium foil and freeze it. This sample will be sent to Barcelona and will be shared for POP-analyses and endocrine disruptor studies.
- From the remaining muscle put about 1-2 g in aluminium foil and freeze it. This sample will be sent to Innsbruck for pigment analysis. Obs, the eye (next stage) can be put together within this package). Freeze in N_2

11- Eye. For analyses of UV-effects, take out the lens of the eyes on both side. Wrap them in Al-foil and put them within the muscle packadge for Innsbruck. Freeze in N_2.

12- Adipose fin. For genetic studies, cut the adipose fin and put it into a 3 ml plastic tube with screw cap and fill with alcohol ($\geq$70%)

13- Otoliths: Open the roof of the head in order to see the brain. Collect the two otoliths (see appendix) and put them into the paper envelop together with the scales.

14- Continue with the fish stomach: Open the stomach and collect the food items which are not yet digested. If possible, divide the stomach content into 3 parts:

- Put the first 1/3 of the stomach content into a white scintilation tube wit screw cap and freeze for metals and isotope analysis NIVA
- Put the 2nd part for species analyses, for G. Raddum, Norway, into a glass container filled with 70% alcohol and put a piece of paper with the fish number written with pencil.
- The 3rd part goes into a glass container for organic analyses for J. Grimalt, Spain

15- One fish is finished: start over again using the next fish.

Sample overview and recipients after one sampling

NIVA, Norway

20-25 gill arches, **frozen**, for metal analyses
20-25 **N_2-frozen** plasma samples for vitellogenin analyses,
20-25 **frozen** muscles (about 5 g each) for metal and stable isotope analyses
20-25 **frozen** kidney samples for metal analyses
20-25 envelopes containing scales and otoliths (store dry and at room temperature)
20-25 vials containing about 1 g or the first 1/3 of the food in the stomach for metals and stable isotope analysis (**frozen**).
1-10 vials (scintillation glasses): additionally representative samples of invertebrates, macropytes and mosses are taken in the lake for metals and stable isotope analysis (**frozen**).

Univ. Innsbruck

20-25 vials, containing liver floating in formalin. **DO NOT FREEZE.**
40-50 **N_2-frozen** liver samples for oxidative stress and enzymes (2 samples from each fish).
20-25 Al-foil packages with muscle and eyes**, N_2-frozen**

CID-CSIC, Barcelona

10-25 gall bladders, **frozen**, for OH-PAH
20-25 livers, **frozen**, for PAHs and POPs
20-25 muscles, frozen, about 15 g each, shared sample for POPs and endocrine disruptors
20-25 glass containers with stomach contents, frozen, for PAHs
and POPs

CNRS, Bordeaux

20-25 **N_2-frozen** plasma samples
6 fixed gill arches for electron microscopy, **DO NOT FREEZE**

Univ. Bergen

20-25 glass vials containing about 1 g or the first 2/3 of the food in the stomach for species determination. Fill up with 70% alcohol and put a piece of paper with the fish number written with pencil.

SECURITY!
To ensure that badly marked samples are mixed, gather all samples for freezing from one fish into one single plastic bag. Split when returning to institute.

MATURATION INDEX OF FEMALE SALMONIDS

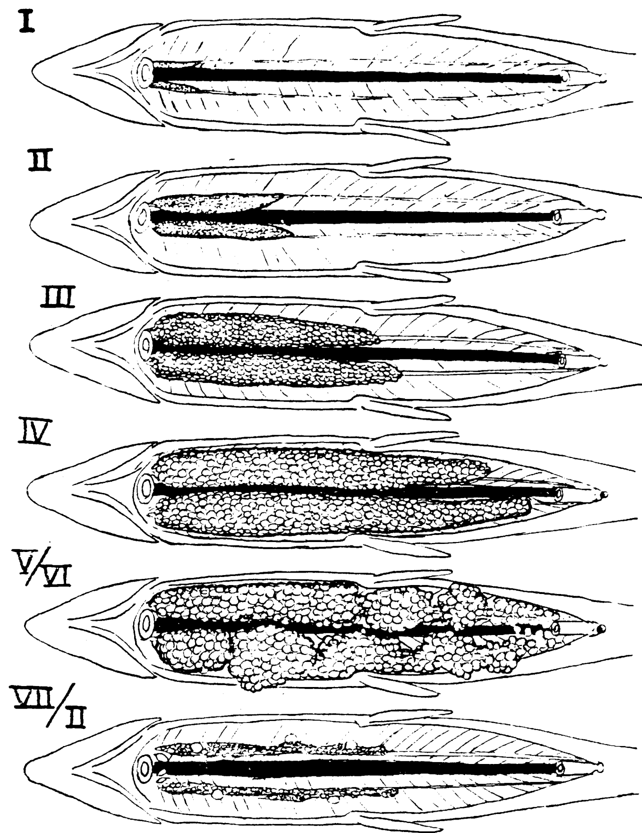

Figure 1. Determination of maturation stage of female fish.

Stage I-II means it will not spawn this year.
Stage III-IV means it will spawn this year, but it is still early in season (June-August)
Stage V-VI is very close to spawning, and eggs are becoming loose in the gut.
Stage VII/II means the fish has spawned before, and often have residual eggs or "egg ghosts" (perivitelin membranes) in the gut.

The male maturation stage is similar, but at stage V sperm can be pressed out of the distal part . Stage VII/ (spawned earlier), is indicated with a very rich blood supply (thick arteries) along the gonads. Usually the colour of the fish is darker, and scales are sitting very strong.