INDUSTRIAL MONITORING

STANDARD OPERATING PROCEDURE

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http://www.finefish.info/

1. Sample reception and handling

2. Sample preparation

2.1 Radiographies

2.2 Staining protocol

3. Sample analysis

1. Sample Reception

Procedure

- Check labels on samples and confirm they correspond to listed samples on sampling spread sheet.

- Verify the sample integrity/quality and label each sample tube, with a paper label written with pencil and sealed with impermeable tape. Check and register the solution in which samples are preserved (eg. 70% ethanol, formol or paraformaldehyde).

- Register date of sample arrival, list of samples and integrity in a designated note book – **Industrial Monitoring**. All data should also be registered in a computer database (word document in a folder labeled **Companies)**.

- Write an email to the person in charge of sampling and mailing of the samples, confirming sample arrival and listing the samples received and sample integrity/quality. The names, addresses and e-mail contacts of each participating company should be included in the note book – **Industrial Monitoring**.

****Important note:** If samples are still in fixative (formol or paraformaldehyde), rinse 2 to 3 times in distilled water and then transfer into ethanol 70% for long term preservation.

2. Sample preparation

- In each sample tube count all the individuals and quickly check the external morphology. For example, gill-cover deformities (operculum shortening) or *pughead*. This first quick evaluation will give a general idea of the overall quality of the sample and incidence and severity of malformations. For specimens in samples which will be radiographed the number of individuals with gill cover deformities should be registered.

- For radiography select at least 60 individuals. Selection should be random.

- For whole mount bone and cartilage staining, using Alcian Blue & Alizirin Red S, isolate a sub-sample of at least 100 individuals. The selection of the sub-sample for whole mount staining should be random.

2.1 Radiographies

- Make a random selection of specimens (approximately 60 of a similar size and age).

- Cut a piece of cardboard with appropriate dimension for the X-ray area. If the machine utilised is a Senographe DMR Mammography System (General Electric Medical Systems, Slough, UK), dimensions are 21 cm by 14 cm. Identify each piece of cardboard with the code indicating the sample batch etc.

- Place fish in several rows on the cardboard and place a ruler at the base of the cardboard and photograph each group of fish ensuring the batch code and ruler are clearly visible.

- At the bottom right hand side of each piece of cardboard with the specimens to be analysed place a coin (to serve as a reference between each sample) and then seal the fish under a layer of thin plastic to ensure samples are held in place and to aid preservation.

- Transport the specimens on ice to the X-ray facility.

- Radiograph the samples (manual exposure mode to give 40 mAs and 40 kV), it is unnecessary to remove the plastic cover.

Radiography conditions:

- Senographe DMR Mammography System (General Electric Medical Systems) and manual exposure mode to give 40 mAs and 40Kv. In order to read and process the digital X-rays a FUJIFILM Computed Radiography CR Console should be connected to the Mammography Workstation MV-SR657 FCR PROFECT CS. The digital X-rays can then be archived as JPEG 8bit images and DICOM format until subsequent analysis.

2.2 Whole Mount Staining Protocol

Acid-free Cartilage and Bone stain (adapted for sea bream and sea bass from; *MB Walker, CB Kimmel 2006)*

- ➔ If samples are in fixative, discard the solution and wash twice with distilled water and place into ethanol (70%) for 1-2 hours prior to staining.
- ➔ Samples which are stored in ethanol (95% or 70%), there is no need to wash.

1. Remove the specimens from the preserving solution and place in acid-free double stain solution. Samples should be stained overnight, rocking gently on an agitator at room temperature (25^{o}C).
2. Rinse the specimens in 2 changes of water to remove excess dye.
3. Bleaching – Bleach is used to remove pigmentation and facilitates observation of stained specimens. Add bleach solution to the tubes containing the stained and washed specimens and leave the tubes with the lid open at room temperature for ≈ 20 min (this time varies according to size and the time in bleach should be carefully monitored for small larvae).
4. Rinse the specimens in 2 changes of water to remove excess bleach.
5. Clearing – tissues are cleared through a gradient of glycerol and KOH. The time is variable in this step and depends on the size of the specimens; solution 1 can take 30 minutes to overnight. Solution 2 takes 2 h up until overnight.
6. Larvae are then stored in a storage solution at 4°C in the dark.

Solutions

1. Preparation Acid-free double stain solution

This solution is made up in two parts that are mixed together immediately before staining.

Part A

- 0.02% Alcian blue 8 GX (Sigma-Aldrich)

- 150 mM MgCl2
- Ethanol 70%

First make a stock of 0.4 % Alcian blue in 70 % ethanol. As the Alcan blue powder is poorly soluble in 70% ethanol, start by preparing a solution of Alcian blue in 50% ethanol (eg. 0.4g alcian blue powder in 50 ml of 50% ethanol (z)(25ml of 100% ethanol + 25ml distilled water) and incubate for 1h at 37°C and mix occasionally to dissolve the dye). To make up the final solution add to the alcian blue solution (z), 20ml of 100% ethanol and 30 ml of distilled water and mix to obtain the final concentration of 0.4% Alcian blue in 70% ethanol.

Part B

- 0.5% Alizarin Red S
- Distilled water

Parts A and B can be stored at room temperature for up to a year.

➔ Prior to staining specimens mix 10 µl of Part B and 1 ml Part A.

2. Bleach solution

Fish >35 dph

Use equal volumes of 3% H_2O_2 and 2 % KOH, to give a

- Final concentration of 1.5% H_2O_2 and 1% KOH.

Fish < 35 dph

- Final concentration of 1% H_2O_2 and 0.25% KOH.

3. Clearing solutions

Solution 1 (Fish > 35 dph)	Solution 1 (Fish < 35 dph)
20% glycerol	20% glycerol
0.25% KOH	0.125% KOH

Solution 2 (Fish > 35 dph)	Solution 2 (Fish < 35 dph)
50% glycerol	50% glycerol
0.25% KOH	0.125% KOH

4. Storage solution

Fish > 35 dph	Fish < 35 dph
50% glycerol	50% glycerol
0.1% KOH	0.05% KOH

5. Sample Analysis

Cranium and associated structures

To facilitate analysis and classification of abnormalities in sea bream an illustrated "score card" was produced and is used to evaluate frequency and severity (example, table 1 and figure 1). Each alcian blue/alizarin red stained specimen was observed carefully under a dissecting microscope and characterized according to each template one for the head and another for the vertebral column (table 2).

Sample	Head					
Age	Pughead	Crossbite	Lower jaw	Branchiostegal	Hyoind	Gill cover
1	√	×	√	√	√	×
2	×	√	√	√	×	√

Table 1 – "Score card" for registering the principal abnormalities of the cranium and associated structures. (Schemes prepared by *Isabel Penisga*)

The evaluation of abnormalities using the "score card" system was supplemented by consultation of the on line FINEFISH manual (http://www.finefish.info/finefish/diagnostic_manuals/bassandbream_en.asp) and also sets of images of the most common abnormalities collected during the screening of various thousands of individual sea bream.

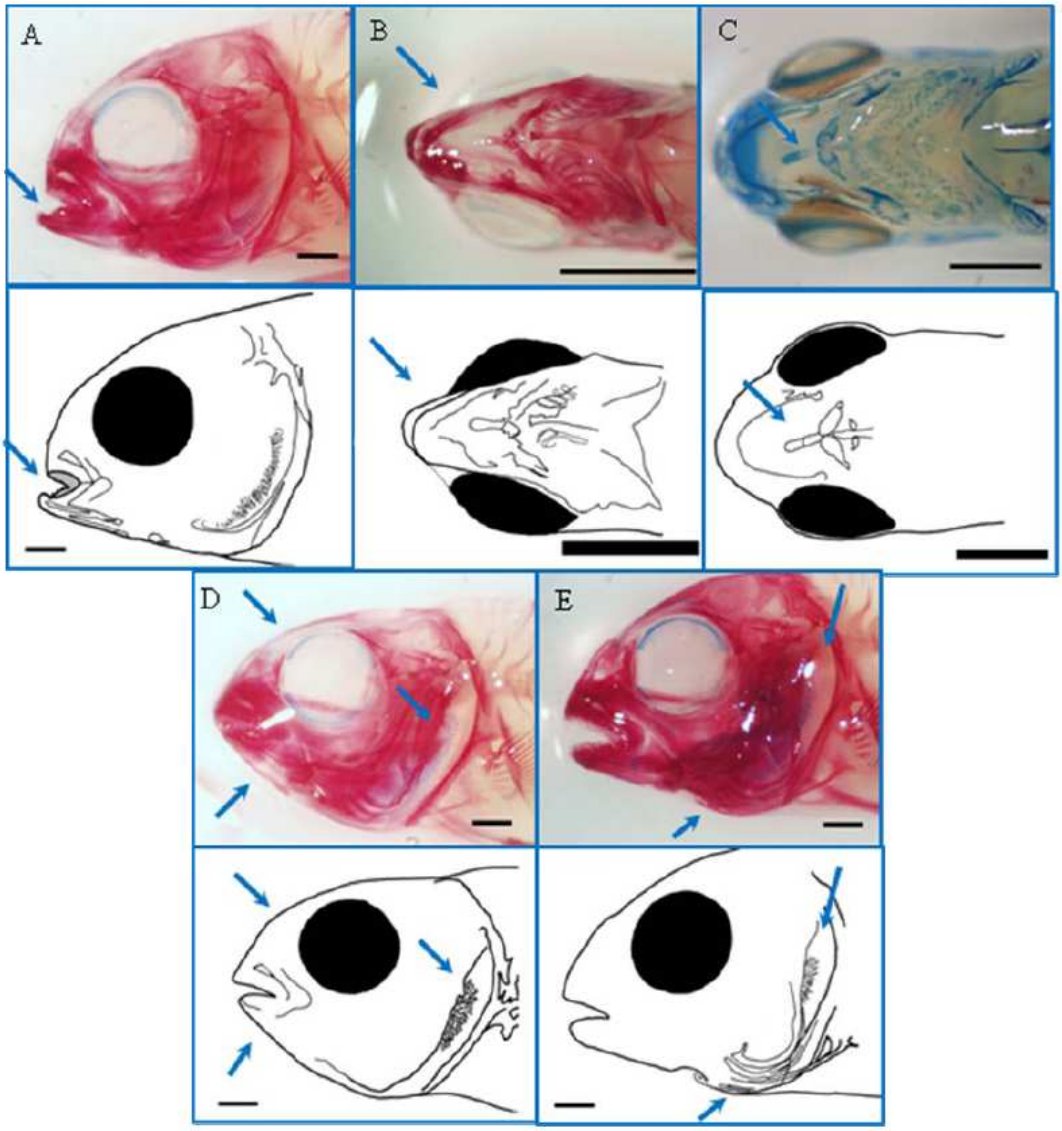

Figure 1. Photographs of individual larvae subject to "whole-mount" staining of cartilage (alcian blue) and bone (alizarin red). A simple line-scheme was also prepared to simplify the presentation and highlight the main modifications. A) example of a larvae with moderate pughead viewed from the side, note the prominent inferior dentary (→), in some cases the superior dentary and head are also shortened. This modification is visible in unstained specimens; B) an individual with cross bite viewed from a ventral angle, note the upper and lower dentary fail to meet and project in opposite directions (→). This modification is visible in unstained specimens; C) Hyoid (hypohyal) deviates from the mid-line and leads to a slight sideways deviation in the lower dentary. This malformation is only visible after staining and may be an artifact associated with poor sampling; D) Superior dentary is elongated and the frontal bone of the cranium is shortened (→).The same specimen also has a gill cover deformity (folding) which reveals the gills (stained blue). Both of the alterations are visible externally in unstained specimens. The scale bar corresponds to 1 mm. (Schemes prepared by *Isabel Penisga*)

B) Gill cover deformities

Gill cover deformities are characterized by a shortening or folding of the gill cover (operculum) (figure 2) and this may vary from very severe in which the gills are almost completely exposed or light in which the operculum is only slightly retracted. Opercular shortening may occur on one or both sides simultaneously.

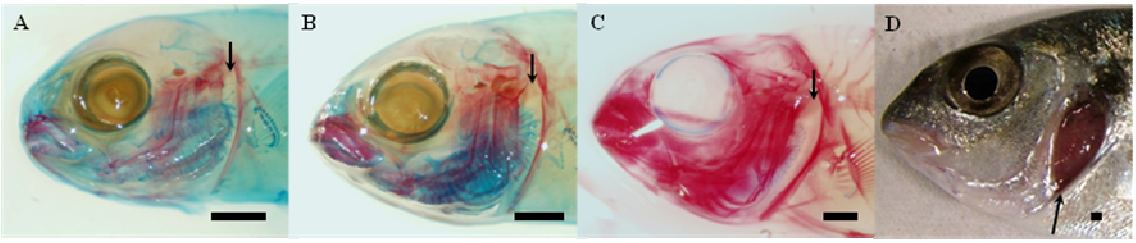

Figure 2 – Photographic representation of different gill cover malformations in the sea bream. The difference in the area of the gill filament exposed is used as a measure of the severity of the condition. A) The operculum is only slightly retracted this is a a light deformation (↓). B) Gill cover with a greater retraction exposing the gills (stained blue in the photograph); classified as Light + (↓). C) Retracted operculum (severe) (↓). D) Severe gap, exposing gills (↑). The scale bar corresponds to 1 mm.

Vertebral axis

The abnormalities in the vertebral region range from severe to light with the severe malformations of the vertebral column being evident externally and the light malformations not being visible unless fish are analyzed in depth by whole mount staining or radiography. A “score sheet” was produced and is used to evaluate frequency and severity of malformations in each batch of samples and includes abnormalities of the vertebral column which can be localized in several different regions (table 2 and figure 3 and figure 4) and fins (caudal, paired and dorsal, anal). Generally abnormalities of the fins are less common.

Sample	Vertebral column												Caudal		Fins	Obs.
	Region affected															
	A	MR	P	A	MR	P	A	MR	P	A	MR	P				
Age	Scoliosis			Lordosis			Kyphosis			Fused vertebrae			Hypural	Epural		
	L	S		L	S		L	S								

Table 2 – Template for the evaluation for vertebral column and fin deformities in sea bream and sea bass. The vertebral column deformities were classified according to body region as Anterior (A), mid-region (MR) or posterior (P). The intensity of the deformity was also classified as "light" (L) or "severe" (S).

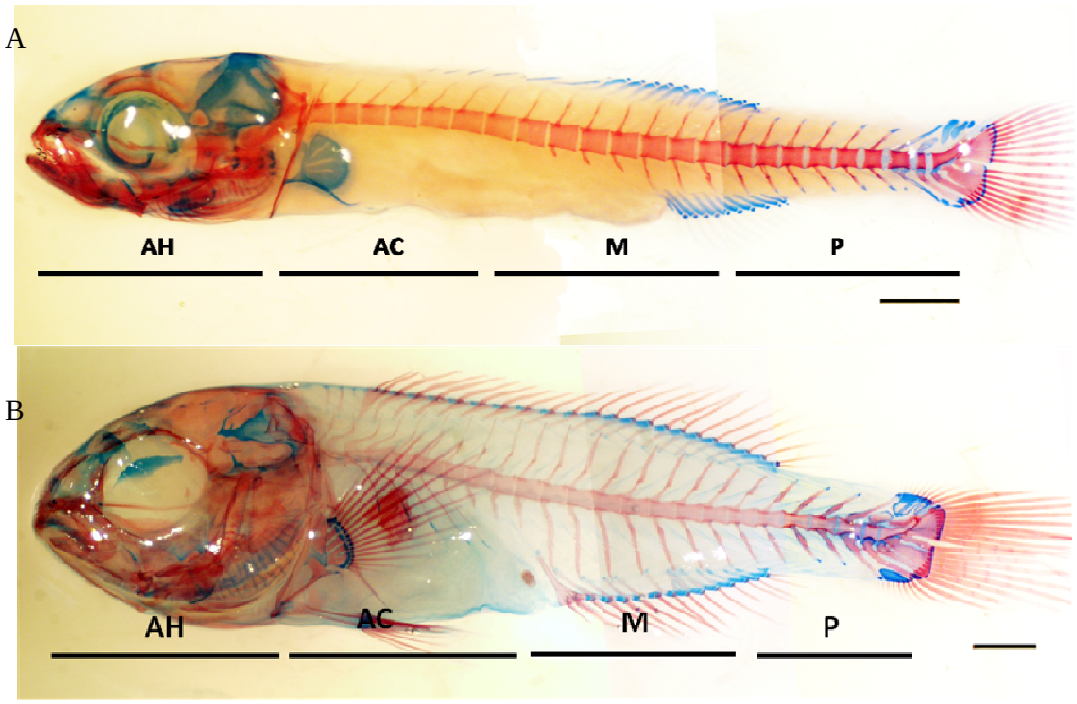

Figure 3 – Photographic presentation of the main divisions of the key regions of the cartilaginous and calcifying skeleton evaluated in the study. Anterior head (AH), anterior column (AC), mid-region (M) and posterior region (P). Panel A) Sea bass larvae 34 dph, and panel B) Sea bream larvae of 62 dph. The blue stained cartilaginous structures have affinity for alcian blue and the red stained bony skeleton for alizarin red. The scale bar corresponds to 1 mm.

The evaluation of abnormalities using the "score card" system can be supplemented by consultation of the on line FINEFISH manual (http://www.finefish.info/finefish/diagnostic_manuals/bassandbream_en.asp) and also schemes of the most common vertebral abnormalities collected during the screening of various thousands of individual sea bream (figure 4) and X-ray plates with images of fish with abnormal vertebral columns have been produced.

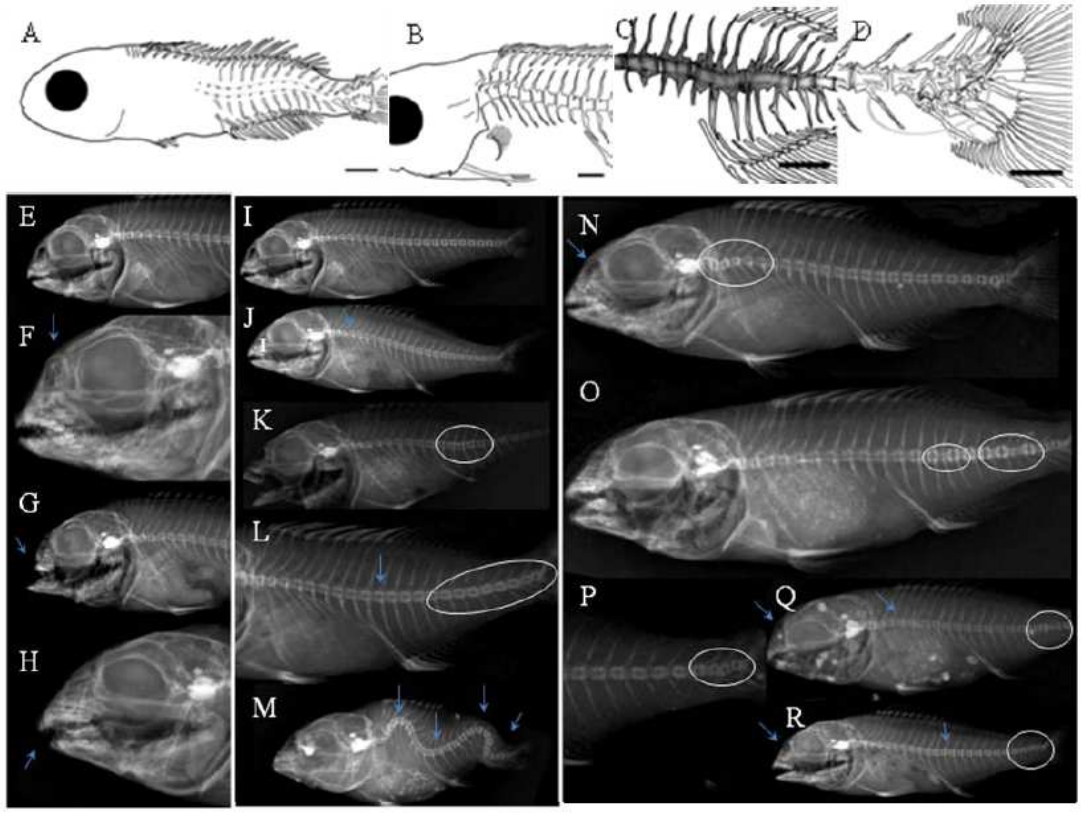

Figure 4 – Examples of common vertebral column deformities detected by whole mount staining or X-rays. A) Undulating vertebral column, presenting lordosis and kyphosis; B) Vertebral column with light kyphosis; C) vertebral column with fused vertebrae, note that multiple neural and haemal arches emerge from the same vertebrae; D) Triangular vertebra in the caudal region – incomplete. The scale bars correspond to 1 mm. (Schemes prepared by *Isabel Penisga*); E) Normal head; F) Fusiform head shape (→); G) Specimen with severe pughead (→); H) Constricted head (→); I) Normal body shape; J) Specimen with slight kyphosis (→); K) Specimen presenting light lordosis (○); L Vertebral column with light lordosis (→), and reduced intervertebral space (○); M) vertebral column presenting a high severity of kyphosis and lordodis compromising

the body shape (→); N) Specimen with different head shape (→), and a vertebral column with a light kyphosis (○); O) Fused vertebra (○); Vertebral column with an irregular disposition, and fused vertebra (○);Q) Specimen with a shortened frontal (→). Vertebral column with a slightly kyphotic shape, and fused vertebra in the caudal region (○); R) The frontal in the cranium is extended and projects outwards (→). The second arrow on the same scheme highlights the irregular disposition of vertebrae (→). In the caudal region fused vertebra are evident (○).

Artifacts

Poor anesthesia, fixation or sample storage can give rise to a range of artifacts which severely reduce confidence in the analysis of abnormalities. For examples if larvae are fixed with inappropriate anesthesia they tend to gasp resulting in specimens with the mouth strained open and also leading to changes in the presentation of the gills, operculum, branquiostegal and possibly hyoid. Inappropriate anesthesia also causes fish to contort and writhe which can cause extreme curvature and even breakage of the vertebral column which makes analysis of malformations impossible. Poor fixation may also compromise body shape and sample integrity reducing the usefulness of samples for analysis.