Food and Agriculture Organization of the United Nations

COPEMED II

TRANSBORAN Project

'Transboundary population structure of sardine, European hake and blackspot sabream in the Alboran Sea and adjacent waters: a multidisciplinary approach'

Sampling protocol

CONTENT

1. Background and summary of project objectives.

Historically, stock delimitation has followed a top-down approach, in which management stewardships decide the stock boundaries attending mainly to political reasons and, consequently, fisheries and assessment modelers adapt their work to these boundaries. However, there are currently vast research evidences showing that stock delimitation should follow a bottom-up approach in which the stock delimitations should be based in scientific evidences. This inherently requires multidisciplinary and holistic approach.

One of the main topics in the identification of stock structure and boundaries is the strength of interdisciplinary analyses when they are developed across national boundaries. In the last decades, a lot of methods have been developed and proposed as proper ways to deal with stock identification. However, applying different approaches may lead sometimes to competing hypotheses because different methods are sensitive to show differences at contrasting spatial (from local to regional) and temporal scales (from daily to evolutionary scales). However, whilst a general agreement exists on the need to adopt a holistic approach with multiple perspectives to improve information on stock structure for resource management that capture different ecological and structuring processes acting at contrasting scales.

The research project **TRANSBORAN** (*Transboundary population structure of Sardine, European hake and blackspot sabream in the Alboran Sea and adjacent waters: a multidisciplinary approach)* is a two-year project funded by FAO and GFCM. The **main objective** is to describe the spatial structure of populations in the most precise way to improve and to be applied in the regular assessments to inform the management advice for sardine, hake and blackspot seabream in Western Mediterranean. To investigate the spatial population structure and to identify the most plausible stock units of sardine, European hake and blackspot seabream as well as their boundaries in the Alboran Sea, the project will apply a multidisciplinary approach. The **project output** will reveal and check if the current GSA limits boundaries are the appropriate spatial scale of assessment and management for these species.

The following **techniques** will be applied: genetics, muscle and otolith stable isotopes, otoliths microchemistry, shape otoliths, parasite composition, meristic description of body and fish-bone. These techniques will be applied in sardine and hake, while for blackspot sabream only genetics and otoliths microchemistry will be applied.

The present protocol describes the complete biological sampling that will be developed on sardine and hake, along with the specified sampling to be done on seabream. For each of the **17 sampling locations** indicated the Figure 1, **50 sardines** of a size between 14-17 cm (i.e., same age class, and all mature) will be sampled in a period between May-June; and **40** mature **hakes** of a size between 25-30 cm (second year class) in a range of 8 weeks between October-November.

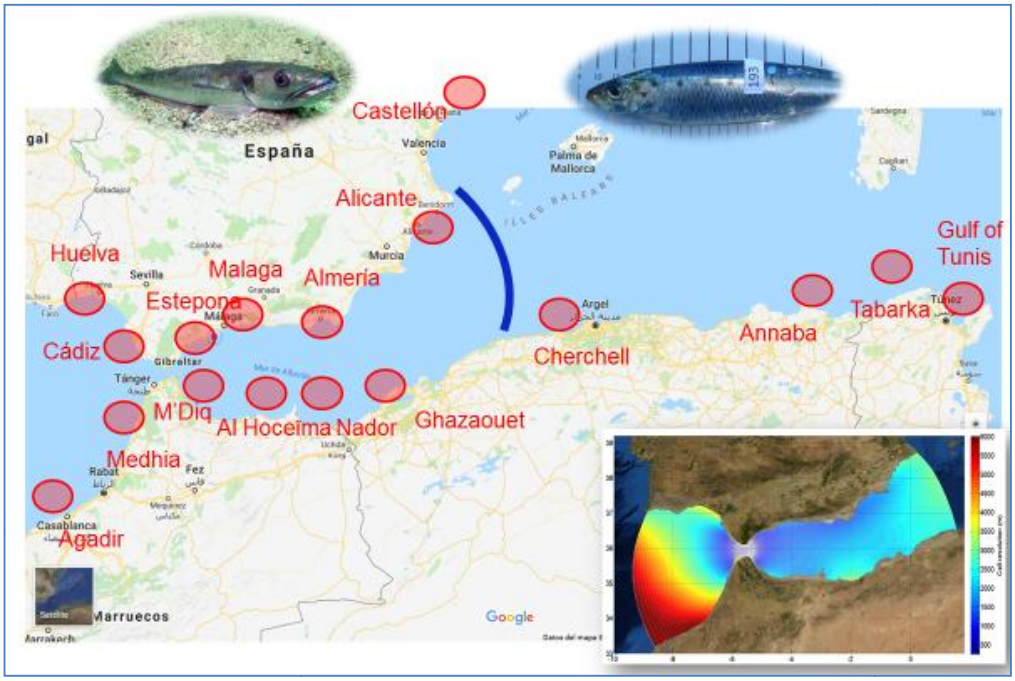

Figure 1. Sampling ports for hake and sardine (blue line is the limit of the hydrodynamic model used in the project as illustrated in the inset map).

In the case of **blackspot seabram**, **30** individuals of size between 30-35 cm will be collected in the locations indicated in the figure 2 between May and June.

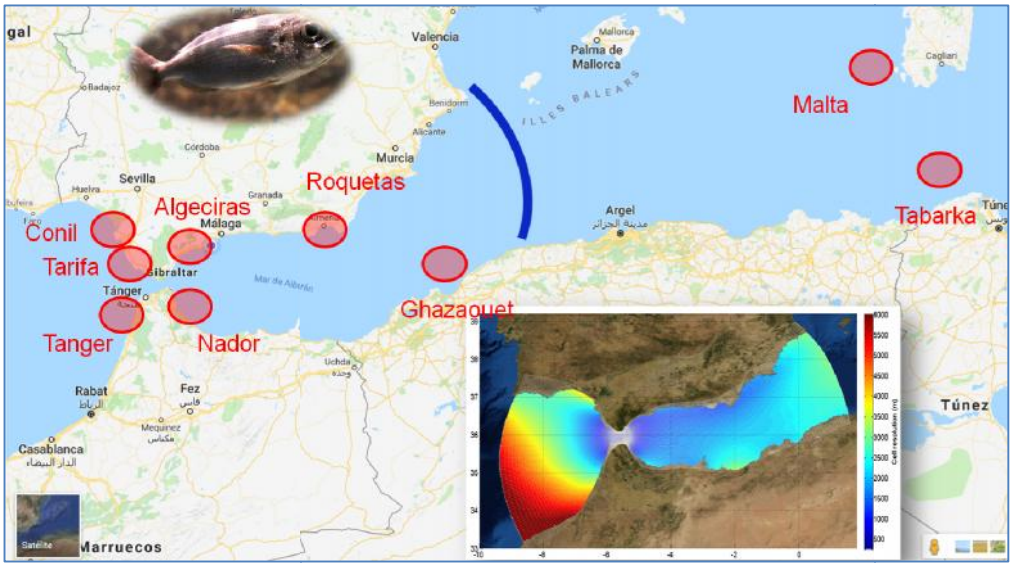

Figure 2. Sampling ports for blackspot seabream (blue line is the limit of the hydrodynamic model used in the project as illustrated in the inset map).

2. Summary of sampling protocol.

For the complete sampling on **hake** and **sardine**, the complete sampling will be divided in **4 days**.

DAY 1

Prepare all the material: vials, liquids, structure and material for the photos, and the most important label all the vials and small papers you are going to use. Please, do not start sampling without having all the material prepared.

DAY 2

Once the fish samples are collected, the following steps have be followed in each specimen identically for each species (see details in **section 3**):

A. Size (in mm) the fish on an ichthyometer and weight it (in g).

B. Take a picture of the whole individual on a calibrated background (contrasted color with plastic ruler, or a mesh cradle with a ruler) using a digital camera positioned in a horizontal tripod camera. Label the code of the individual on a small paper (see section 4) and put it close to the fish to let the code appearing in the photo.

C. Staple a small tracing paper with the code written with pencil to the caudal fin of the fish.

D. Take a piece of white skeletal muscle (c.a. 0.5 cm^3, **green** squares in the figure 3) on the side for stable isotopes, and store in 1.5 mL eppendorfs filled with non-denatured ethanol 96%.

E. Take a piece of white skeletal muscle (c.a. 1 cm^3, **red** squares in the figure 3) on the side of the fish for genetics. Store in two 5 ml tubes with screwcap, filled with non-denatured ethanol 96%.

If the sampling will be continued the day after, please store in the fridge, if not freeze the material until the next steps.

DAY 3

F. Sex identification (no maturation stage is needed).

G. Parasite inspection and extraction (see details in the next section). Retain the first gill arch for the next step.

H. Gill rackers sampling (see section 4). For sardine, store the gill arch in a 2 mL eppendorf or vial. For hake, take a picture on black background, label on a small paper the code of the individual and put it close to gill to let the code appearing in the photo (see section 4).

DAY 4

I. Extract both otoliths using plastic forceps, clean twice with distilled (or Milli-Q or distilled water), dried in a laminar flow hood, and store in a 1.5. mL eppendorf decontaminated with nitric acid 1%. Extract all the otoliths before to proceed with the last step.

J. Remove the flesh following details in Section 3 and a photo of the fish bone. Keep the label of the fish and put it close to the fish bone for the photo (see labeling details in section 4).

Figure 3. Body location of genetics (**red** square) and stable isotopes (**green** squares) sampling, for sardine (left) and hake (right).

In the case of **blackspot seabream**, the complete sampling will be divided in **2 days**.

DAY 1

Prepare all the material: vials, liquids, structure and material for the photos, and the most important label all the vials and small papers you are going to use. Please, do not start sampling without having all the material prepared.

DAY 2

Once the fish samples are collected, the following steps have be followed in each specimen identically for each species (see details in **section 3**):

A. Size (in mm) the fish on an ichthyometer and weight it (in g).

B. Take a piece of white skeletal muscle (c.a. 1 cm^3, **red** squares in the figure 4) on the side of the fish for genetics. Store in two 5 ml tubes with screwcap, filled with non-denatured ethanol 96%.

C. Extract both otoliths using plastic forceps, clean twice with distilled (or Milli-Q or double-distilled water), dried in a laminar flow hood, and store in a 1.5. mL eppendorf decontaminated with nitric acid 1%.

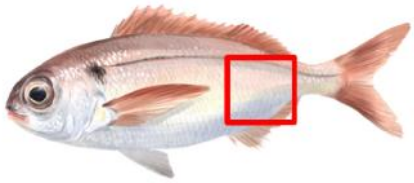

Figure 4. Body location of genetics (**red** square).

3. Additional explanations for each step and technique.

Specific detailed for each step of the biological sampling indicated in the section 2 are here below expanded.

3.1 Photo for body morphometric analyses.

3.1.1. Preparation

Poor-quality specimens or distorted digital images of subjects can bias the morphometric analyses. Use a digital single-lens reflex (DSLR) camera for capturing images of body shapes. Position the camera on a horizontal tripod or Kaiser Repro table directly above the specimen (Figure 5). The tripod should be set up so that the camera lens is far enough away from the subject (i.e., focal distance) such that the largest specimen in the sample fills the entire camera frame.

Figure 5. Equipment setup for capturing digital images of fish for morphometrics analysis: frontal view of Kaiser Repro table, side view of horizontal tripod with camera, and mesh cradle with ruler for displaying fish.

3.1.2. Digitalization of samples

The specimens have to be placed over the right side and photographed in left horizontal lateral position keeping a same distance between subject and camera. Position the fish as following:

- The longitudinal axis from the tip of the snout to the center of the caudal fin along the frontal plane (often parallel to the lateral line) is the most obvious axis that can be used to orient the individuals. To help achieve consistently straight alignment, a ruler can be placed along the longitudinal axis of the fish.
- The mouth and opercle should be fully closed. The mouth can be pinned shut; the opercle, if flared, can be held down with a dissecting needle. Failure to secure the jaws and opercles in the standard closed position will result in changes to the shape of the head.
- Paired fins should be folded against the body, and the unpaired fins (e.g., dorsal and ventral) should be fully extended and can be secured in this position by using dissecting pins. In the case of hake, there are two dorsal fins; distance between dorsal fins must appear in image.

As images are translated from the camera to the computer and among software programs, a series of compressions and decompressions occurs. To counteract the degradation of image quality, the tagged image raw file format (RAW) or TIFF is desirable because no image compression occurs and the full spectrum of brightness levels is recorded by the camera.

Appropriate lighting should be used. Soft and homogeneous light, such as that occurring on an overcast day, is diffuse and provides even illumination of the subject.
If the specimens of hake are bloated with gas in the swim bladder, an incision can be made on the right side of the fish immediately below the lateral line to expel air from the swim bladder to orienting the specimen. Alternatively, it can be pinched with a needle or a dissecting pin.

The shape of the body should be visible, landmark must be appeared in images are (Figure 6):

- anterior tip of snout;
- posterior tip of premaxilla;
- center of eye;
- top of cranium directly above the eye;
- posterior of neurocranium;
- anterior insertion of dorsal fin;
- posterior insertion of dorsal fin;
- dorsal insertion of caudal fin;
- midpoint of hypural plate;
- ventral insertion of caudal fin;
- posterior insertion of anal fin;
- anterior insertion of anal fin;
- anterior insertion of pelvic fin;
- anterior insertion of pectoral fin;
- isthmus between gill covers.

Take a picture of the whole individual on a calibrated background (contrasted color with plastic ruler, or a mesh cradle with a ruler) using the digital camera positioned in a horizontal tripod camera. Label the code of the individual on a small paper (see section 4) and put it on the calibrated background to let the code appearing in the photo (section 4). The sequence of digital images (the file) must be codified the same way.

Figure 6 shows examples of valid and invalid photos for the two species.

Figure 6. Valid photos for hake and sardine above, and invalid photos with shadows below.

3.2 Stable isotopes in muscle.

The sampling procedure used for stable isotopes is the same as used for genetics (see below). A piece of white skeletal muscle (c.a. 0.5 cm^3, **green** squares in the figure 2) on the side for stable isotopes, and store in 1.5 mL eppendorfs filled with no denaturalized ethanol 96% and stored in an eppendorf box.

3.3 Genetics

3.3.1. Preparation of the material before sampling

Before sampling, prepare 5 mL tubes with screw cap (vials) with at least 4 mL of non-denatured Ethanol 96%. Ethanol can be poured in the tubes using a wash bottle filled with ethanol. Each vial must be labeled with Sample ID according to the labeling code reported in the section 4. Print the label of the vials and stick it to the side of the vial with scotch tape to prevent label erasing due to probable ethanol escapes. Write also the same code (label) on the cap of the vial.

Operator has to wear cleaned gloves.

3.3.2. Sampling procedure

IMPORTANT: Samples can be taken from fresh or frozen specimens.

Cut with surgical instruments a 1 cm^3 muscle sample from each individual in the **red** region indicated in the figure 2. Note that larger pieces are not needed and can result in bad DNA quality due to low ethanol/tissue ratio.

Put the tissue clip into the ID-labeled vial with ethanol 96%. Fill the tube with ethanol using a wash bottled containing the ethanol. Ensure the tissue volume is no more than 10-20% of the liquid volume and tightly close the cap (Figure 7).

Figure 7. Example showing ratio of tissue/ethanol and sample size.

Clean surgical instruments for each sampled animal with water and commercial ethanol and dry it with a new scrip paper each time.

Store the vial containing the tissue at -20°C; if it is not possible, make sure that temperature does not exceed 4°C.

3.4 Sampling of otolihs

3.4.1. Decontamination of eppendorf with nitric acid.

To decontaminate the tube, fill a beaker with 1/3 of nitric acid (HNO_3) 10% and 2/3 H_2O MilliQ. The tubes will be left to soak for two hours in the beaker. Rinse the tubes three times with MilliQ water then left dry under a laminar flow hood

3.4.2. Otolith extraction.

IMPORTANT: Use **plastic or ceramic forcerps**.

Cut over the fish head CAREFULLY to make certain that you do not break the otoliths. There are 2 main extraction methods, and these can be applied to the 2 species as shown in the training course.

Gently probe into the otolith chambers, using a plastic stick or plastic forceps to expose the otoliths. Please, work carefully since otolith can easily be damaged at this stage - the posterior end of the otolith is the most fragile.

Using plastic forceps, remove the otolith (Sagittae), taking care not to damage the rostrum or edges. If there is any tissue adhering to the otolith, clean each one by gently wiping it on a clean part of your glove on the back of your hand.

Afterwards, clean twice with Milli-Q or distilled water, dried in a laminar flow hood, and store in a 1.5. mL eppendorf decontaminated with nitric acid. Store it an eppendorf box.

3.5 Parasites

3.5.1. Inspection of fish for parasites and their extraction.

Hake and sardine samples should be examined as soon as possible after their caught, otherwise the samples must be stored in frozen (-30 °C) until their examination. Parasitological examination consists in the necroscopy of each individual host as following:

1. Defrost the fish if this is the case.

2. Scan fish under dissecting microscope for ectoparasites, such as copepods and monogeneans.

3. Remove gill archs and scan them under dissecting microscope, for monogeneans and crustacean ectoparasites (should you find parasites, please follow the **3.5.2**).

Important: Keep gill archs until meristic information has been taken (see section 3.5).

4. Cut (dissect) longitudinally the fish from the anal opening to pectoral fins.

6. Look carefully to the internal viscera cavity and surface of the internal organs (gonads, stomach, intestine, etc) for the detection of larval nematodes (should you find larval nematodes, figure 8, please follow point **3.5.3**).

7. Pick out ALL the larval nematodes (should you find parasites, please follow point **3.5.3**) visible at eye.

8. Fillet fish should be analyzed by UV-press system. Alternatively, press the fillets and examine them under a trans-illuminator light or a dissecting microscope (Figure 9).

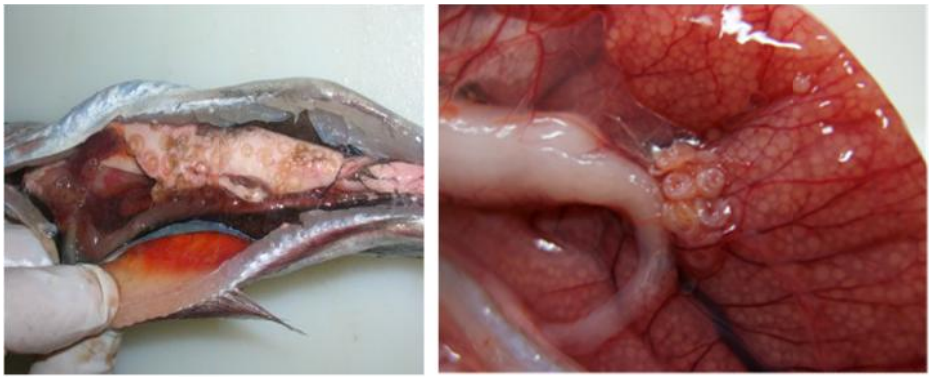

Figure 8. Larval nematodes encysted in the liver (left) and outside the organs (gonads)

Figure 9. Preparing of fish fillets (left) for larval parasites detection, under a trans-illuminator light (right, use dissecting microscope if this is not possible).

3.5.2. Collection and storing of helminth parasites.

Wash the removed parasites in saline solution; then in distilled water, and, finally, store them in alcohol 70°. This is not mandatory but is it optional if these parasites appear.

3.5.2. Collection and storing of larval and adult nematodes.

This is obligatory. Larval and adult nematodes of all internal organs, and muscle (filet) have to be counted and stores separately.

First, collect all larval and adult nematodes of all internal organs, wash larval and adult nematodes removed in saline solution, then in distilled water; count them, and finally, store them them in alchool 96°. Please use eppendorf tubes with caps, including at least 50 worms per eppendorf, or less, in case of low infection. It is necessary to collect all the worms from each infected. At the end, please close the cap also by using parafilm. If more than one vial is need label them and include this information on the table (see section 4) (e.g. 1/3, 2/3 and 3/3 if three vials were needed). Label each eppendorf of the collected nematodes as indicated in **section 4** and stored.

Second, follow the same procedure for the larval and adult nematodes appear in the fillet. Check the ventral side of the fish musculature (Figure 10) for the occurrence of the larval parasites (Anisakis spp.) visible "at eye". After their removal from the fillets, please follow the same procedure as indicated above for the larval and adult nematodes. Eppendorfs labels of samples of filets must include an 'F'.

Finally, all the eppendorf will be included in the same box, indicating, on its top, the host (i.e. hake, or sardine) and the sampling location (code of the sampling area). In the excell file, use extra columns to report the recovered and stored parasites (see **section 4**).

Figure 10. Body area were filets must be analyzed (**red square**).

3.6 Meristics

3.6.1 Gill rakers counts

Place each individual on the right side, open the operculum and remove the first branchial arch (Figure 11). All the rakers count will be made from the first gill arch of the left side, if the left side is damaged so the right side will be used.

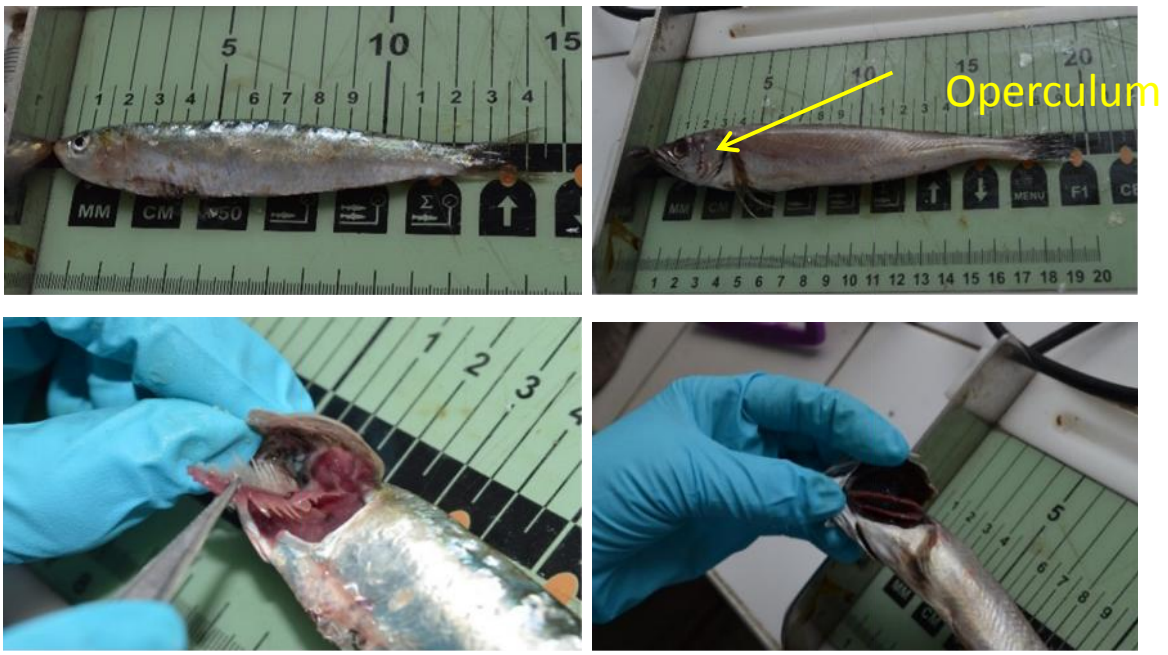

Figure 11. Procedure to remove the first branchial arch to count gill rakers of sardine (left) and hake (right).

Place on absorbent paper to remove impurities and dry the gill arch (Figure 12).

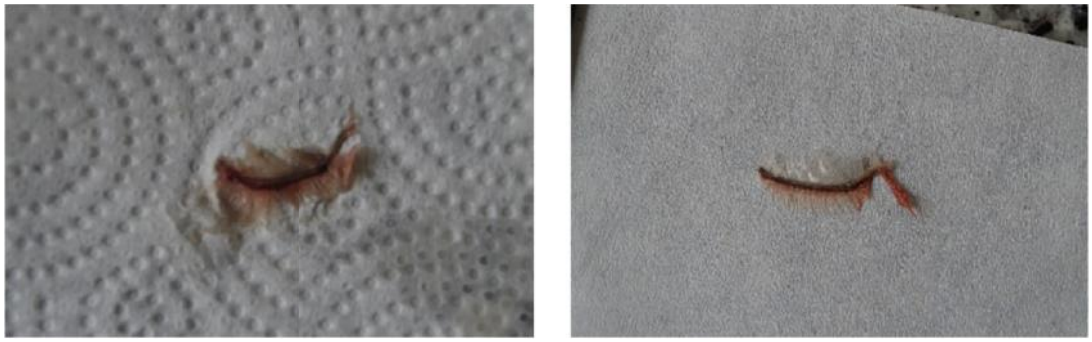

Figure 12. Gill rakers of sardine (left) and hake (right) over an absorbent paper

For **sardine**, take the brachial arch and store in a 2 mL labeled eppendorf or similar vial filled with 70% ethanol.

For **hake**, place gill arch on a black background and take one photo with a digital camera. Use a label paper with the code to identify each individual (Figure 13).

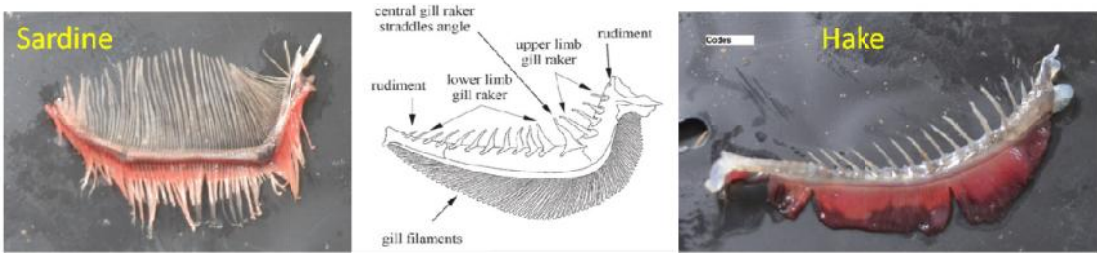

Figure 13. Gill rakers of sardine (left) and hake (right), and schematic drawing (center).

3.6.2 Number of vertebrae

To extract the vertebrates, the individuals will be immersed in boiling water (five for hake and eight minutes for sardine) and then the tissue will be carefully detached from the skeleton without altering the structure of the vertebrae before counting (Figure 14). Leave to dry in the air, it whitens and the intervals between the vertebrae become more apparent.

Figure 14. Procedure to extract the vertebrates.

Place the skeleton on a black background and take a photo using digital camera. A label paper will be used write the code and identify the individual (Figure 14). The vertebrae will be counted from the occipital condyle (not counted) to the urostyle, included (Figure 15).

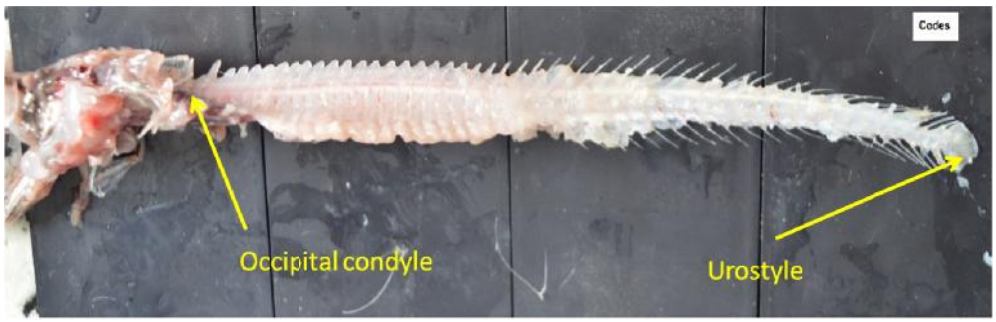

Figure 15. Picture to be taken and location of origin and end of vertebrae counting.

4. Codification of samples and information to compile.

All vials (eppedorfs and tubes) must be coded twice (side and cap) using the same code as used for files of photos. This code for each individual consists of:

- Three alpha code for the name of the species (Table 1).
- Three alpha code for the name of the port (Table 1).
- Number of the specimen within each port (01-40 for hake, 01-50 for sardine, 01-30 for seabream).

Table 1. List of codes

CODES		
Species	*M. merluccius*	**HKE**
	S. pilchardus	**PIL**
	P. bogaraveo	**SBR**
Ports	Agadir	**AGA**
	Medhia	**MDH**
	Huelva	**HUE**
	Cádiz	**CDZ**
	Tanger	**TNG**
	Conil	**COL**
	Tarifa	**TRF**
	Algeciras	**ALG**
	Estepona	**ETP**
	Málaga	**MLG**
	Almeria	**AMR**
	Roquetas	**RQT**
	M´diqu	**MDQ**
	AlHoceima	**ALH**
	Nador	**NDR**
	Ghazaouet	**GHZ**
	Cherchell	**CHE**
	Annaba	**ANB**
	Tabarka	**TBK**
	G. of Tunis	**GTU**
	Malta	**MLT**
Survey	Name	**Name and # of haul**
Date	Day/Montth/Year	
Size	**Total length in mm**	

As an example of two hakes sampled in the port of Agadir and the resulting codes

Species	Port/Survey	Number of individual	Code
HKE	AGA	1	HKE-AGA- #1
HKE	AGA	2	HKE-AGA- #2

Print sheets to write the following information, and afterward transfer to excel files:

Code	Date	Length (mm)	Weigth (g)	Sex	# Nematodes (# vials) Viscera	# Nematodes (# vials) Muscle	# Other parasites	Observ.

5. Shipping procedures.

Samples must be shipped (paid by each institution) via currier to the coordinator of each technique as following:

A. **Body morphometrics**:
Photos of all fishes must be sent to Dr. Tahar FILALI
filalitahar@gmail.com
Or alternatively stores in the Share-Point of the project.

B. **Muscle samples for isotopes and otoliths** must be shipped to:
Dr. Raúl Laiz Carrión, Instituto Español de Oceanografía, Centro Oceanográfico de Málaga, Puerto Pesquero, 29640 Fuengirola, Spain.

C. **Muscle samples of sardine for genetics** must be shipped to:
Dr. Monste Pérez Rodriguez, Instituto Español de Oceanografía, Centro Oceanográfico de Vigo, Cabo Estay, Canido. Apdo. 1552, 36200 Vigo, Spain.

D. **Muscle samples of hake for genetics** must be shipped to:
Dr. Kenza Mokhtar Jamaï, Institut National de Recherche Halieutique, Département Pêche, Boulevard Sidi Abderrahmane, Ain Diab 20100 Casablanca, Morocco.

E. **Muscle samples of seabream for genetics** must be shipped to:
Dr. Alessia Cariani, Laboratory of Genetics & Genomics of Marine Resources and Environment (Lab GenoDREAM)Dept. Biological, Geological & Environmental Sciences (BiGeA), University of Bologna, Campus of Ravenna via Sant'Alberto, 163 - 48123 Ravenna - Italy.

F. **Parasites samples** must be shipped to:
Prof. Simonetta Mattiucci, Department of Public Health and Infectious

Diseases, Section of Parasitology. La Sapienza - University of Rome, P.le Aldo Moro, 5, 00185 Rome, Italy.
IMPORTANT: *Use the declaration letter provided by Simonetta Mattiucci.*

G. **Meristics**:
Gill archs of sardine must be shipped to:
Dr. Fatima Wahbi, Institut National de Recherche Halieutique, Département Pêche, Boulevard Sidi Abderrahmane, Ain Diab 20100 Casablanca, Morocco

Photos of all fish-bones and hake gill archs, along with excel files must be sent to Dr. Fatima Wahbi: *fatimawahbi67@gmail.com*
Or alternatively stores in the Share-Point of the project.

ALL EXCELL FILES must be sent to Manuel Hidalgo (jm.hidalgo@ioe.es) and will be stored in the Share-Point of the project.

IMPORTANT: In all the hipped material used a declaration letter of the content material as provided here in the annex. In the case of parasites, use the template provided by Simonetta Mattiucci.

6. Material to be made available for each institution.

For EACH sardine and hake sampling location (50 sardines and 40 hakes), sampling needs:

- **360** 1.5-mL eppendorfs (muscle isotopes, otoliths, nematods, larval and adult nematodes, helminth).
- **90** 5-mL tubes with screwcap (genetics).
- **50** 2-mL eppendorfs or similar vials (sardine gill arch).
- Surgical scissors, tweezers, disposable scalpels
- Ethanol 70% (to clean surgical instruments and store gill archs)
- Non-denatured Ethanol 96% (DO NOT USE 99-100% ABSOLUTE ETHANOL OR PARTIALLY DENATURED ETHANOL)
- Racks with 50 apertures for tubes and boxes for eppenderfs.
- Formaldehyde.
- Nitric acid 1%.
- Permanent water-resistant ink marker pens.
- Gloves
- Paper scrips
- Scotch tape.
- Waterproof/tracing paper and pencil for labeling.
- Non-metallic (plastic or ceramic) forceps.

Each institution has to acquire the total amount of material **attending to the number of locations** to sample.

For **blackspot seabrem**, for each location (30 individuals) sampling additionally needs:

- **30** 1.5-mL eppendorfs (otoliths).
- **30** 5-mL tubes with screwcap (genetics).