Appendix 2

SAMPLING PROTOCOLS FOR THE GBYP BIOLOGICAL SAMPLING

Each partner is responsible to follow all protocols for sampling, labeling, filling the data forms and shipping.

SAMPLING PROTOCOL FOR GENETICS (M or F)

MATERIALS

Product	Catalog Code
100 tubes 5mL with screw cap (SARSTEDT)	60.558.001
Surgical scissors, tweezers, disposable scalpels	
Non-denatured Ethanol 96%	
Gloves	
S-Monovette® racks D17, 50 apertures(SARSTEDT)	93.852

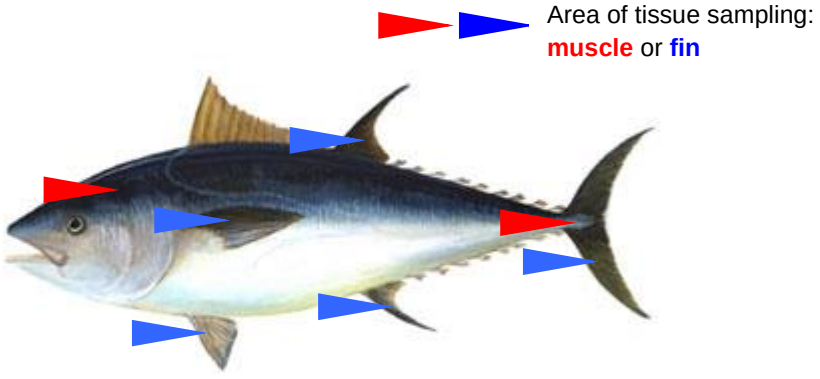
SUMMARY OF SAMPLING:
Sample size: 50 individuals by area and size-class (as defined in the sampling scheme given in the proposal), with two replicates for each individual. The 50 individuals will be collected in at least 5 fishing days spread along the fishing season, with a target of no more than 10 (randomly selected) individuals sampled per fishing day.

Type of sampling:

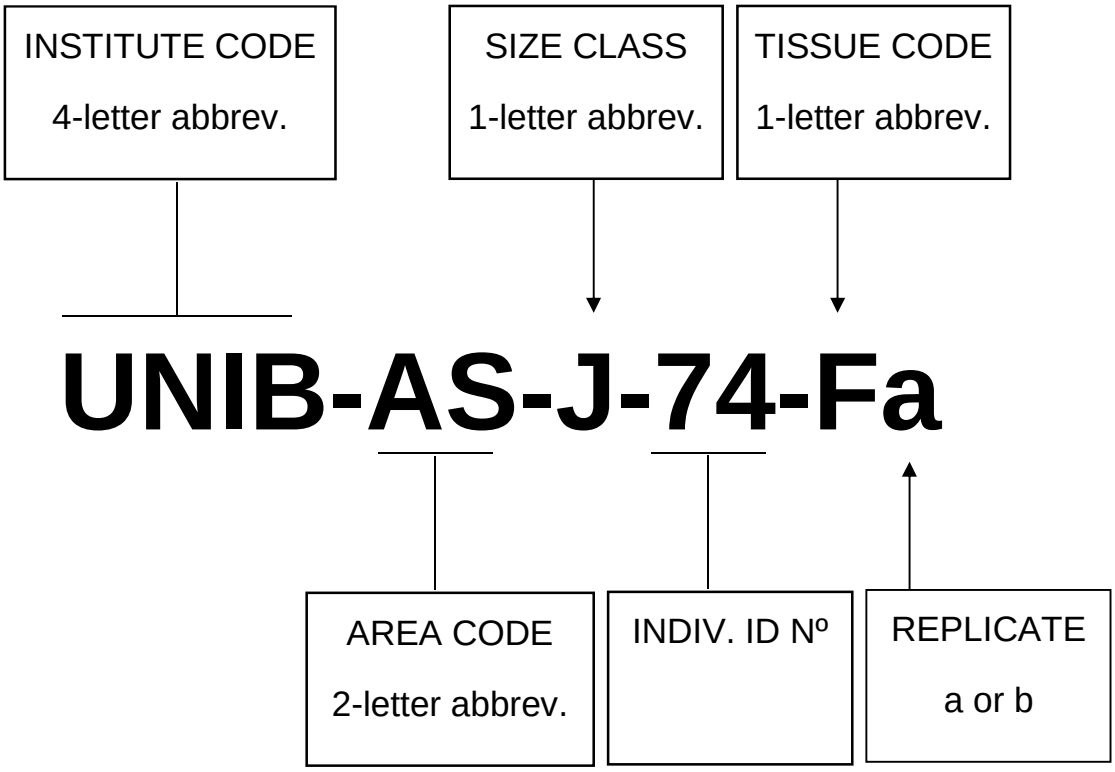
Tissue type: white skeletal muscle near caudal fin when possible. Alternative: other muscle (e.g. from head) or fin clip from pectoral, 2nd dorsal, anal, pelvic or caudal fin.

Volume of the muscle sample: 0.5 cm^3

Size of the fin clip: 1 cm^2



1. Labeling procedure and codes
Each partner is responsible for the correct labeling of the samples. The fish to be sampled must be first classified by size-class (larvae, age 0, juvenile, medium, large), then numbered from 1 to N. We'll use the same code to label all samples. Sample labels must be visible on the tube. Please, refer to the sampling scheme given in the proposal and use the codes in Appendix 2. The following example shows how samples will be labelled.



The first 4 codes (e.g. "UNIB-AS-J-O1") have to be unique for each fish. The last code (e.g. "Fa") indicates the tissue type (F, M or L) and replicate (a or b).

2. Preparation of the material before sampling

- Before sampling, prepare 100 5mL-tubes with screw cap with at least 2.5mL of Non-denatured Ethanol 96%; each microtube must be labeled with Sample ID according to the labeling code reported above (step 2). Label the microtubes with pens containing water-resistant ink, as well as including a waterproof paper inside the tube with the code written with pencil (as ethanol may dissolve the ink).
- Operator has to wear cleaned gloves.

3. Sampling procedure

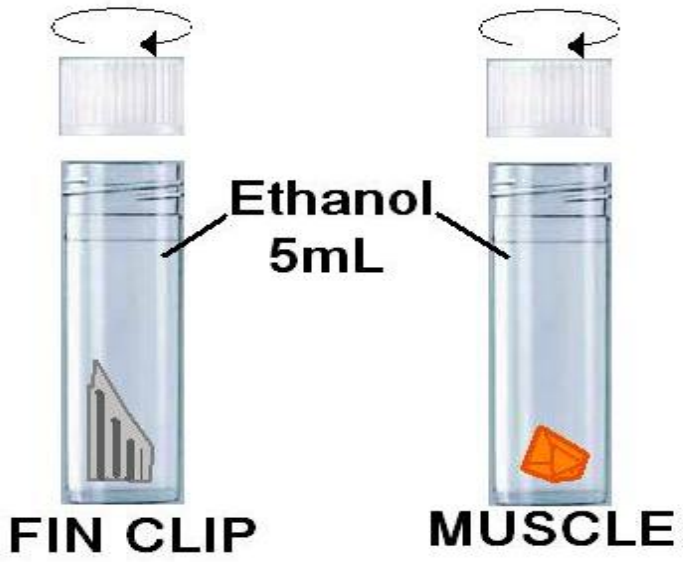
IMPORTANT: Sampling of tissue should be carried out twice from the same individual. Mark the replicates as "a" and "b". Both replicates should be shipped to AZTI following the shipping instructions (see step 5).

- Cut a 0.5 cm^3 muscle sample or a 1 cm^2 fin clip from each individual. In the case of larvae, collect the entire larvae.
- Put the tissue clip into the ID labeled microtube with ethanol 96%. Ensure the tissue volume is no more than 10% of the liquid volume.
- Clean surgical instruments for each sampled animal with water or commercial ethanol and dry it with a new scrip each time.

- Store the microtube containing the tissue at -20°C. If it is not possible make sure that temperature does not exceed 4 ° C.
- 4-5 days after sampling, carefully remove the ethanol from the microtube and replace it by new ethanol. The water contained in the sample may have partly diluted the first ethanol used, so this ethanol replacement will ensure a better long-term conservation of DNA.

If your fish is already frozen:

- Take the sample from each frozen individual and put it directly into the microtube with ethanol 96%.
- The ice contained in the sample may dilute the ethanol. Therefore, change the ethanol after 4-5 days, in order to ensure proper sample preservation.



4. Fill sampling data form

The code of the sample must be clearly visible on the microtube.
- Fill the "GBYPdata.xls" file.
- Email the form to the database supervisors in AZTI as soon as some samples are collected (e.g. on a daily basis): Igaratza Fraile (ifraile@azti.es) and Haritz Arrizabalaga (harri@azti.es). The newly labeled samples will be checked against already existing samples to avoid doubling names. Wait for their confirmation before shipping the samples, and coordinate shipping dates with the above mentioned persons.

5. Shipping procedure

- Put the rack of microtubes with tissue samples in a storage box with the reference "GBYP".
- Ship the box by express courier to:

Igaratza Fraile / Inma Martín
AZTI-Tecnalia Marine Research Division
Herrera kaia portualdea z/g
20110 PASAIA
SPAIN

Ship the samples by Express Courier mail.

Notes

SARSTEDT website : http://www.sarstedt.com

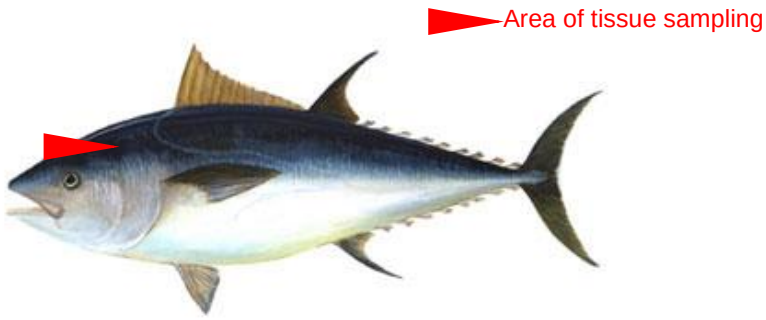
Last revised: 18 October 2016

SAMPLING PROTOCOL FOR OTOLITHS (O)

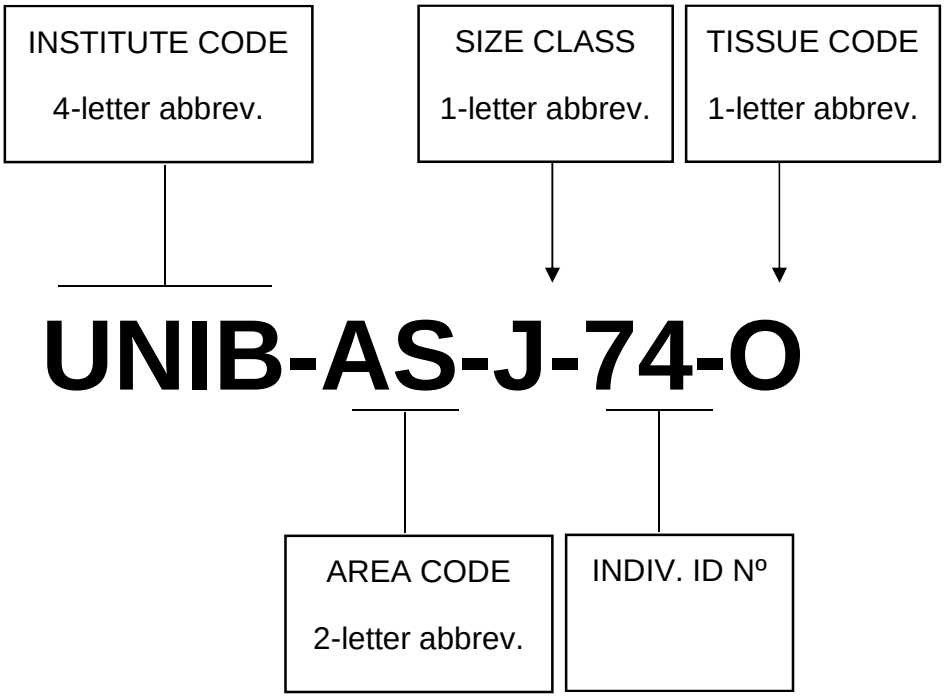
MATERIALS

Product	Catalog Code
Gloves (laboratory gloves)	
Knife, handsaw or circular saw	
Plastic cocktail stick, plastic toothpick	
Non-metallic forceps (plastic or ceramic)	VWR: FORCEPS TEFLON-COAT SHARP (232-0016, -0018, or -0125), or AgarScientific: Ceramic Tweezers (T5150, T5156); Plastic tweezers (T5233)
50 Microtubes 2mL with O-ring cap (SARSTEDT)	72.694.007
Storage boxes (SARSTEDT)	95.64.997

SUMMARY OF SAMPLING:
Sample size: 50 individuals by area and size-class (as defined in the sampling scheme given in the proposal). The 50 individuals will be collected in at least 5 fishing days spread along the fishing season, with a target of no more than 10 (randomly selected) individuals sampled per fishing day.
Tissue type: otoliths.



1. Labeling procedure and codes
Each partner is responsible for the correct labeling of the samples. The fish to be sampled must be first classified by size-class (larvae, age 0, juvenile, medium, large), then numbered from 1 to N. We will use the same labeling procedure used for genetic samples, the tissue code being here O (for otoliths). Labels must be visible on the microtube.



2. Sampling procedure

- The otoliths can be sampled once all other tissues are sampled. Change into clean gloves (disposable lab/examination gloves) for each fish. If tuna heads have been previously frozen, ensure that thawing is complete before starting the extraction. Extracting otoliths from partly frozen canals could break them.

- Cut into the fish head CAREFULLY to make certain that you don't break the otoliths. The easiest way is to cut the head with a large knife in the frontal plane above the supraorbital ridge (Fig.1a). At first attempts, it is better not to cut too close from the eyes, and to do successive small cuts then until the brain appears.

- **It is very important to work carefully because the otolith can easily be damaged at this stage!** CAREFULLY remove the brain (Fig.1b). Otoliths will be located at the back of the brain cavity, inside semi-circular canals (Fig.1c). Gently prospect into the canals. The posterior end of the otolith is the most fragile. Use small forceps to CAREFULLY extract the otolith from the bony capsules and GENTLY remove the membrane surrounding the otolith immediately after extraction (the membrane is harder to remove after it has dried).

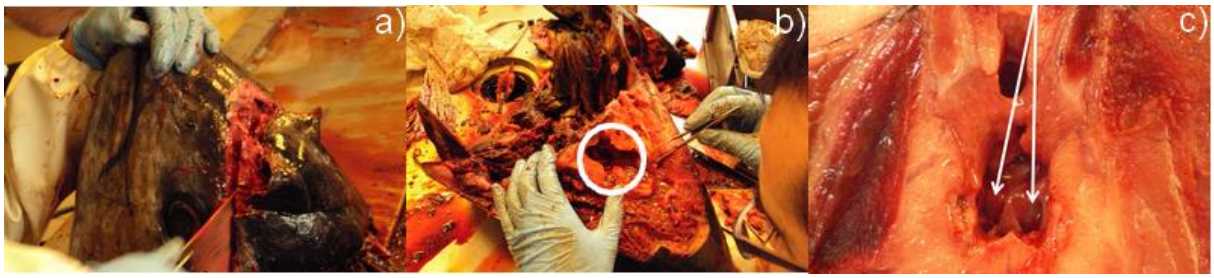


Fig.1: Extraction of sagittal otoliths from a bluefin tuna head

- Clean the otolith with deionized water and store in plastic vials with the corresponding code.

3. Decontamination and storing procedure

Otoliths must be carefully cleaned prior to processing for microchemical analyses. Decontamination procedure suggested by Rooker et al. (2008) is recommended.

- Immerse the otoliths into deionized water to hydrate biological residues adhering to the otolith surface. Remove these residues using small forceps.

- Then, immerse the otoliths into 0.1% nitric acid for 5 min. to remove surface contamination.

- Clean the otoliths again with deionized water to remove the remaining nitric acid.

- Finally, dry otoliths in a fume hood during 24 hours and store them by pairs in plastic vials with their corresponding labels. Cleaning otoliths immediately after collection reduces the amount of time required to prepare them for sectioning.

4. Fill sampling data form

The code of the sample must be visible on the microtube.

- Fill the "GBYPdata.xls" file.
- Email the form to the database supervisors in AZTI as soon as some samples are collected (e.g. on a daily basis): Igaratza Fraile (ifraile@azti.es) and Haritz Arrizabalaga (harri@azti.es). The newly labeled samples will be checked against already existing samples to avoid doubling names. Wait for their confirmation before shipping the samples, and coordinate shipping dates with the above mentioned persons.

5. Shipping procedure

- Put the vials in a storage box with the reference "GBYP".
- Ship the box by Express Courier to:

Igaratza Fraile / Inma Martín
AZTI-Tecnalia Marine Research Division
Herrera kaia portualdea z/g
20110 PASAIA
SPAIN

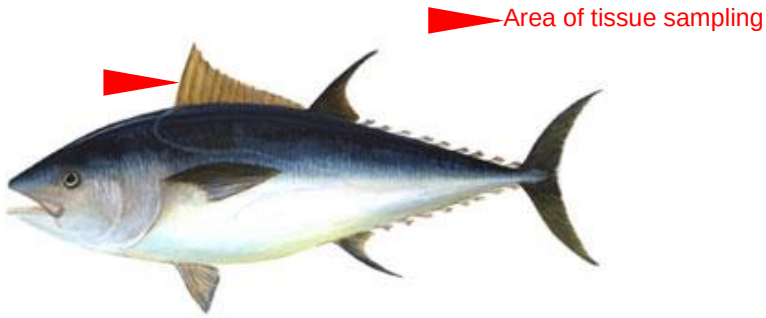
Last revised: 18 October 2016

SAMPLING PROTOCOL FOR FIRST DORSAL FIN RAYS, SPINES (S)

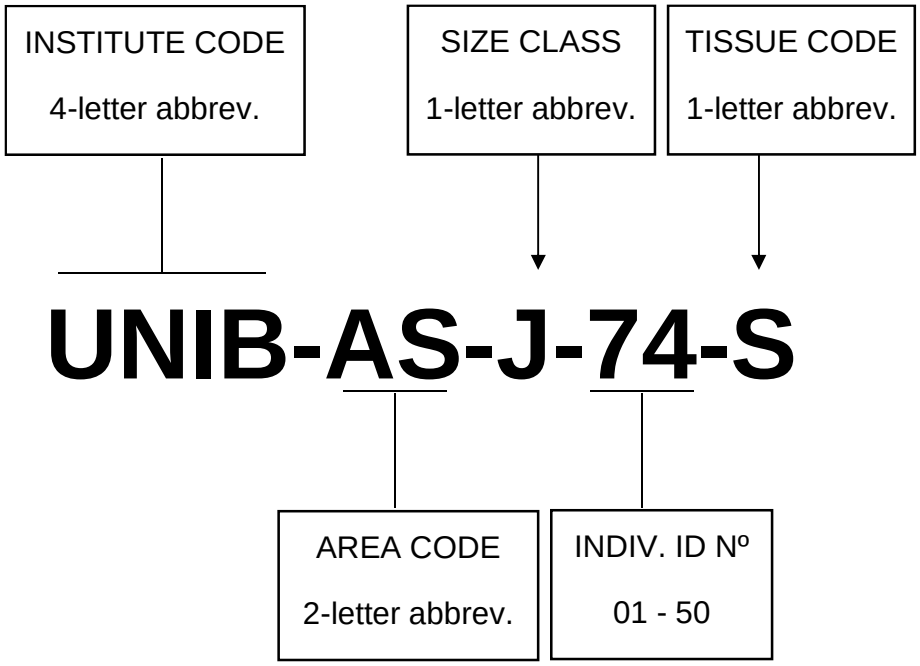
MATERIALS

Product	Catalog Code
Gloves (laboratory gloves)	
Knife, forceps	
Paper envelopes	

SUMMARY OF SAMPLING:
Sample size: 50 individuals by area and size-class (as defined in the sampling scheme given in the proposal). The 50 individuals will be collected in at least 5 fishing days spread along the fishing season, with a target of no more than 10 (randomly selected) individuals sampled per fishing day.
Tissue type: first dorsal fin ray.



1. Labeling procedure and codes
Each partner is responsible for the correct labeling of the samples. The fish to be sampled must be first classified by size-class (larvae, age 0, juvenile, medium, large), then numbered from 1 to N. We will use the same labeling procedure used for other tissues, the tissue code being “S” (for spines). The label must be visible on the envelope.



2. Sampling procedure

Spine extraction

The spine used for ageing purposes is the first ray of the first dorsal fin. It is important to extract a complete spine from the base including the condyle where the spine inserts in the fish avoiding any damage of the spine base as it is an essential part for ageing. It is not difficult to remove the dorsal fin first ray from juveniles but for larger specimens it is recommended to use a sharp knife or scalpel to cut carefully the strong ligaments that support the spine base deep in the fins insertion in the body depression.

Spread out the first dorsal fin and cut the membrane joining the two first dorsal rays by using a knife (Figure 2). Then, push the spine forward and down progressively (Figure 3B), then cut and turn it alternately to the right and to the left until the ligament breaks (Figure 3C). Finally, the spine must be twisted and pulled out (Figure 3D). Care should be taken in order not to twist the spine in its base.

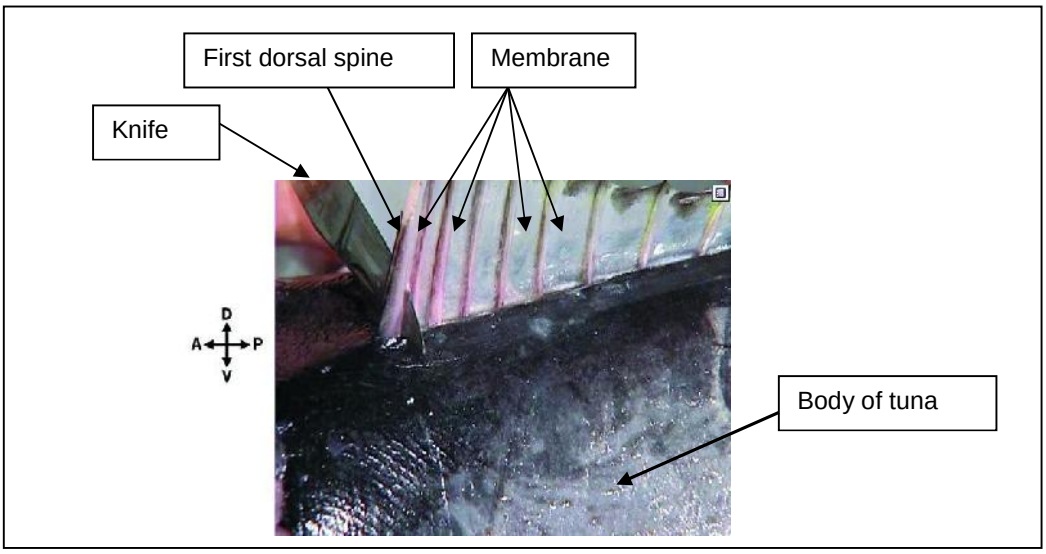


Figure 2. Insertion of the knife into the membrane separating the first two spines of the 1st dorsal fin (figure taken from Panfili *et al*., 2002).

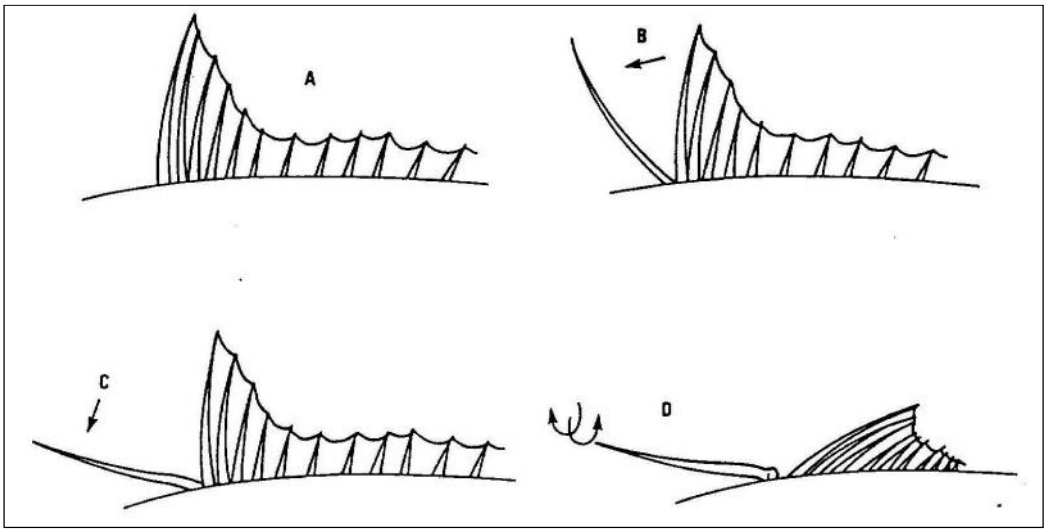


Figure 3. Technique of extraction of the first spine of the bluefin tuna dorsal fin. (Figures taken from Compeán-Jiménez, 1980).

3. Cleaning and storing procedure

It is recommended to carry out the whole cleaning step just after the spine has been extracted, as the connective tissue is still fresh and can be removed easily. It is recommended to remove carefully the remainder connective tissue with a scalpel and tweezers, avoiding causing any damage to the surface of the base of the spine. Moreover, the epidermis covering the spine must be also removed before carrying out the procedure of the spine preparation.

Before storing the sample, it is advisable to remove all tissue remains and dry the spine out on blotting paper. Spines are ideally stored dry in a paper envelope, which should be kept in a cool place (refrigerated) if they are not going to be cut immediately. NOTE: Do not use plastic bags for preserving them.

If the spine is too large to fit in the envelope, it can be cut in half and both pieces kept in the envelope, remembering that the piece forming the base of the spine is the most important since it is the part used for age interpretation.

4. Fill sampling data form

- Fill the "GBYPdata.xls" file.
- Email the form to the database supervisors in AZTI as soon as some samples are collected (e.g. on a daily basis): Igaratza Fraile (ifraile@azti.es) and Haritz Arrizabalaga (harri@azti.es). The newly labeled samples will be checked against already existing samples to avoid doubling names. Wait for their confirmation before shipping the samples, and coordinate shipping dates with the above mentioned persons.

5. Shipping procedure

- Put the envelopes in box with the reference "GBYP".
- Ship the box by Express Courier mail to:

Igaratza Fraile / Inma Martín
AZTI-Tecnalia Marine Research Division
Herrera kaia portualdea z/g
20110 PASAIA
SPAIN

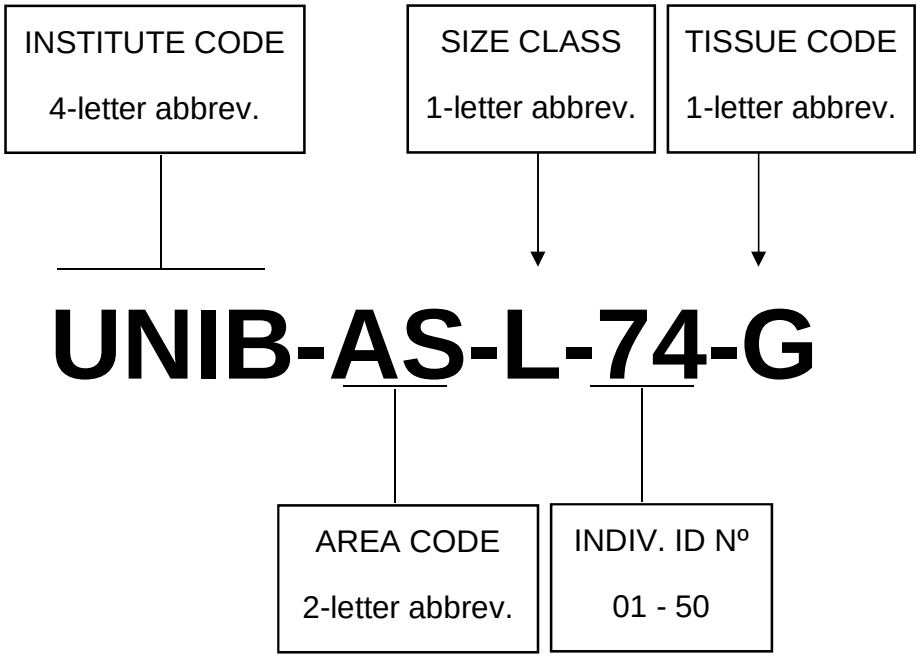
SAMPLING PROTOCOL FOR GONADS

MATERIALS

Product	Catalog Code
50 containers 70 mL assembled with yellow screw cap (SARSTEDT)	75.9922.744 (www.sarstedt.com)
Surgical scissors, tweezers, scalpel blades	
Fixator (Bouin, Hollande or 4% Formaldehyd)	
Gloves	
Parafilm	

SUMMARY OF SAMPLING:
Sample size: 50 individuals by region and size-class (medium and large individuals only, as defined in the sampling scheme given in the proposal). The 50 individuals will be collected in at least 5 fishing days spread along the fishing season, with a target of no more than 10 (randomly selected) individuals sampled per fishing day.
Tissue type: gonad.
Size of the tissue sample: tissue sample length must be approximately half its width.

1. Labeling procedure and codes
Each partner is responsible for the correct labeling of the samples. The fish to be sampled must be first classified by size-class (larvae, age 0, juvenile, medium, large), then numbered from 1 to N. We will use the same labeling as in previous procedures, the tissue code being "G" (for gonads). The label must be visible on the container.



2. Preparation of the material before sampling

- Bouin or Hollande fixator will be used for storing the gonad samples

- In case of not disposing of Bouin or Hollade fixator, a 4% formaldehyd solution will be used. To prepare 1L of 4% formaldehyd solution, use the following components:
  - → 9.4g of dibasic phosphate
  - → 4.7 g of monobasic phosphate
  - → 100 mL of 40% formaldehyd (usual concentration of commercial formaldehyd)
  - → non-salted water

Dilute the phosphate progressively in non-salted water, then add water up to 900 mL, then complete with 100 mL of 40% formaldehyde.

- Before sampling, each container must be labeled using formalin-resistant ink. For additional safety, we will also use submersible paper tags labelled with pencil, and put them into the containers.

- The operator has to wear cleaned gloves.

3. Sampling procedure

- Extract the gonads from the peritoneal cavity and weight them.

- Cut a slice from one of the gonads. The length of the slice must be approximately half of its width. Then cut a portion of 1/8 of the slice and weight it. This will be the gonad sample.

- Put the gonad sample into the labeled container, add fixator and cap the container.

- In case of using Bouin fixator, remove the fixator after 12 hours and fill the container with 70% ethanol.

- Check that the containers are properly closed and ensure their closure with parafilm.

- Clean surgical instruments for each sampled animal.

- Store the tubes at ambient temperature.

4. Fill sampling data form

- Fill the “GBYPdata.xls” file.
- Email the form to the database supervisors in AZTI as soon as some samples are collected (e.g. on a daily basis): Igaratza Fraile (ifraile@azti.es) and Haritz Arrizabalaga (harri@azti.es). The newly labeled samples will be checked against already existing samples to avoid doubling names. Wait for their confirmation before shipping the samples, and coordinate shipping dates with the above mentioned persons.

5. Shipping procedure

- Put the containers with tissue samples in a storage box.
- Ship the box to:

  Igaratza Fraile / Inma Martín
  AZTI-Tecnalia Marine Research Division
  Herrera kaia portualdea z/g
  20110 PASAIA
  SPAIN

Ship the samples by Express Courier mail.

Last revised: 18 October 2016

Filling the GBYP SAMPLING DATA Template:

Below is an extract of the "GBYP Sampling Data template.xls" file:

INDIVIDUAL FISH CODE							TISSUE COLLECTED				
SAMPLE ID	**PARTNER CODE**	**GrAREA**	**AREA**	**FISHING GEAR**	**INDIVIDUAL ID NUMBER**	**SIZE CLASS CODE**	**TISSUE otoliths**	**Nº Otoliths**	**TISSUE Spine**	**TISSUE Gonads**	**TISSUE Muscle/Fin**
AZTI-BB-J-1	AZTI	NEATL	BB	TROL	1	J	O	2	S	G	M

SAMPLING DATA							
Harvest Date [dd/mm/yyyy]	**Catch Date [dd/mm/yy]**	**Sampling date [dd/mm/yy]**	**Latitude**	**Longitude**	**Length [cm]**	**Type of length**	**Estimated Straight Fork Length [cm]**
[for farmed fish]	23/07/2015	23/08/2015	44,24	-3,67	104	SFL	

SAMPLING DATA						
Weight [kg]	**Type of weight**	**Estimated Round weight [kg]**	**Notes for length and weight**	**Sex**	**Collector**	**Notes other matter**
22,5	TW		*[if estimated or converted, provide reference equations etc.]*	M	Igaratza Fraile	

Only direct measurements of length and weight are noted under "length", "type of length", "weight" and "type of weight". Whenever length or weight are estimated (e.g. using conversion factors), this is noted under "estimated straight fork length" or "estimated straight fork weight", and the specific conversion factors that were used are noted under "notes for length and weight".

And below are the alternative categories and codes that are needed to fill in the template:

Main body measurements:

- Fork length (SFL): this is the straight line from the end of the upper jaw (end of the snout) to the posterior of the shortest caudal ray (fork of the caudal fin) (Figure 1). This can best be measured using a **caliper** or alternatively with a tape measure, although it must be kept straight while measuring. The fish should be placed on a flat surface in a horizontal position. In the case of very large specimens in which this measurement is very difficult to make, one of these other two lengths may be used to substitute it:

- First dorsal length (LD1): this is the straight line from the end of the upper jaw (end of the snout) to the base of the first dorsal spine (the start of the first dorsal fin) (Figure 1).

- Curved fork length (CFL): this is the length from the upper jaw (end of the snout) to the fork by an imaginary longitudinal line, with the corresponding fish curvature (Figure 1).

- **Head length (LHead):** This is the straight line from the end of the upper jaw (end of the snout) to the posterior border of the operculum. (Figure 1)

The type of measurement being used must be clearly specified and the measurement unit (e.g. cm). SFL and CFL are measured to the lower centimeter (a specimen of 70,8 cm or 70,2 cm would correspond to the 70 cm range), LD1 is measured to the lower half centimeter (a specimen of 30,4 cm measures as 30 cm and one of 30,7 cm corresponds to 30,5 cm).

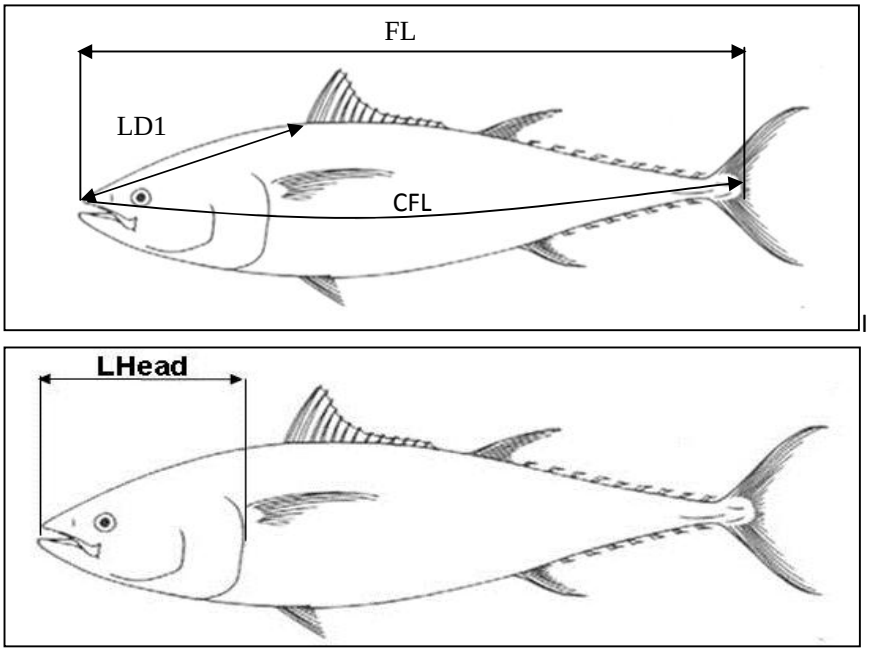


Figure 1. Types of measurements of bluefin tuna: Strait fork length (SFL), First dorsal length (LD1), Curved fork length (CFL) and head length (LHead).

Institute code	
AZTI	AZTI-Tecnalia
FMAP	Federation of Maltese Aquaculture Producers
HCMR	Hellenic Centre for Marine Research
IEO	Intituto Español de Oceanografía
IFRE	French Research Institute for Exploration of the Sea (IFREMER)
INRH	Institut National de la Recherche Halieutique
IPIM	Instituto de Investigação das Pescas e do Mar (IPIMAR)
ISTA	Istambul University

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CYPR	Cyprus Marine Science Foundation
NECT	Necton Marine Research Society
NRIF	National Research Institute of Far Seas Fisheries (NRIFSF)
UNIB	University of Bologna
UNIC	University of Cagliari
UNIG	University of Genova
UCA	University of Cadiz
TAMU	Texas A&M University
CROD	Centre de Recherches Oceanographiques de Dakar
BYP	Bluefin Year Program (ICCAT)
CSIC	Centro Superior de Investigaciones Científicas
BALFEGO	Grup Balfego
UNIM	UNIMAR
IMR	Institute of Marine Research
DFO	Fisheries and Oceans Canada
NOAA	National Oceanic and Atmospheric Administration

GrArea	
BS	Black Sea
CMED	Central Mediterranean
CNATL	Central North Atlantic
EATL	East Atlantic - West African coast
EMED	Eastern Mediterranean
GI	Strait of Gibraltar
GM	Gulf of Mexico & Caribean
NEATL	Northeast Atlantic

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NoS	North Sea
NWATL	North-Western Atlantic
SATL	South Atlantic
WMED	Western Mediterranean

Area	
AE	Aegean Sea
AS	Adriatic Sea
AZ	Azores
BA	Balearic
BB	Bay of Biscay
BS	Black Sea
CA	Central and North Atlantic
CR	Crete
CY	Cyprus
DA	Strait of Dardanelles - Bosphorus - Marmara Sea
EG	North Egypt coast
ESA	South Atlantic - Eastern
GI	Gibraltar
GL	Gulf of Lion, Catalan
GM	Gulf of Mexico, Caribbean Sea
GSL	Canada (Gulf Saint Lawrence)
IC	Iceland
LI	Ligurian: Italian artisanal fleet
LS	Levantine Sea (North)
MA	Malta

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MC	Madeira, Canary Islands
MO	Morocco
MS	Mauritania
NA	North African Coast
NL	Canada (Newfoundland-Labrador)
NS	Canada (Nova Scotia)
NW	Norway
PO	Portugal
SA	Sardinia
SE	Senegal
SI	South Sicily, Ionian Sea
SIE	Sicily (East Sicily and Ionian Sea)
SIS	South Sicily, Strait of Sicily
SS	Southern Spain
SY	Gulf of Syrta
TU	Gulf of Gabes
TY	Tyrrhenian Sea
UI	UK, Ireland
US	US Coast
WSA	South Atlantic - Western

fishing gear	
BB	**baitboat**
FAD	**fish aggregating device**
GN	**gillnet**
HL	**handline**
LA	**larval survey**

Last revised: 18 October 2016

LL	**longline**
MWT	**pelagic midwater trawl**
PS	**purse-seine**
PSS	**purse seine, small scale**
RR	**rod-and-reel**
TN	**trammel net**
TRAP	**trap**
TROL	**Trolling lines**
UNCL	**unclassified**

Size class		
V	**Larvae**	
0	**Age 0**	**<=3 kg**
J	**Juveniles**	**>3 & <=25 kg**
M	**Medium**	**>25 & <=100 kg**
L	**Large**	**>100 kg**

Tissue code	
O	otoliths
S	spines
G	gonad
F	fin
M	muscle

Length	

Last revised: 18 October 2016

Type	
FL	straight fork length
CFL	curved fork length
LHead	head length
LD1	first dorsal length
TL	total length (larvae)
L2	preoperculum length
BL	body length (larvae)
L1	second operculum length

Weight Type	
TW	total weight
GGT	gilled/gutted
GW	gutted
DT	dressed weight
TW(lbs)	total weight in lbs

Maturation stage	
I (IMMATURE)	**Small ovaries with no visible ovocytes. Translucid pink color.**
IM (INICIAL MATURATION)	**Ovaries occupy 1/4 to 3/4 of the peritoneal cavity. Pale yellow color.**
M (MATURE)	**Ovaries occupy 3/4 of the peritoneal cavity. Visible vascularization. Yellow/orange color. Opaque and visible oocytes.**
S (SPAWNING)	**Ovaries in maximal development stage. Orange color. Very developped vascularization. Translucid ovocytes visible through the gonad surface.**

R (RESTING)	**Flaccid ovaries. Purple color. Occasionally visible translucid ovocytes and opaque ovocytes in advanced development stage, corresponding to the next spawning.**
PS (POST SPAWNING)	**Flaccid ovaries. Purple color. Gonadal wall thick and very vascularized (very visible capillaries). No ovocytes in advanced development stage.**

INDIVIDUAL ID NUMBER
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