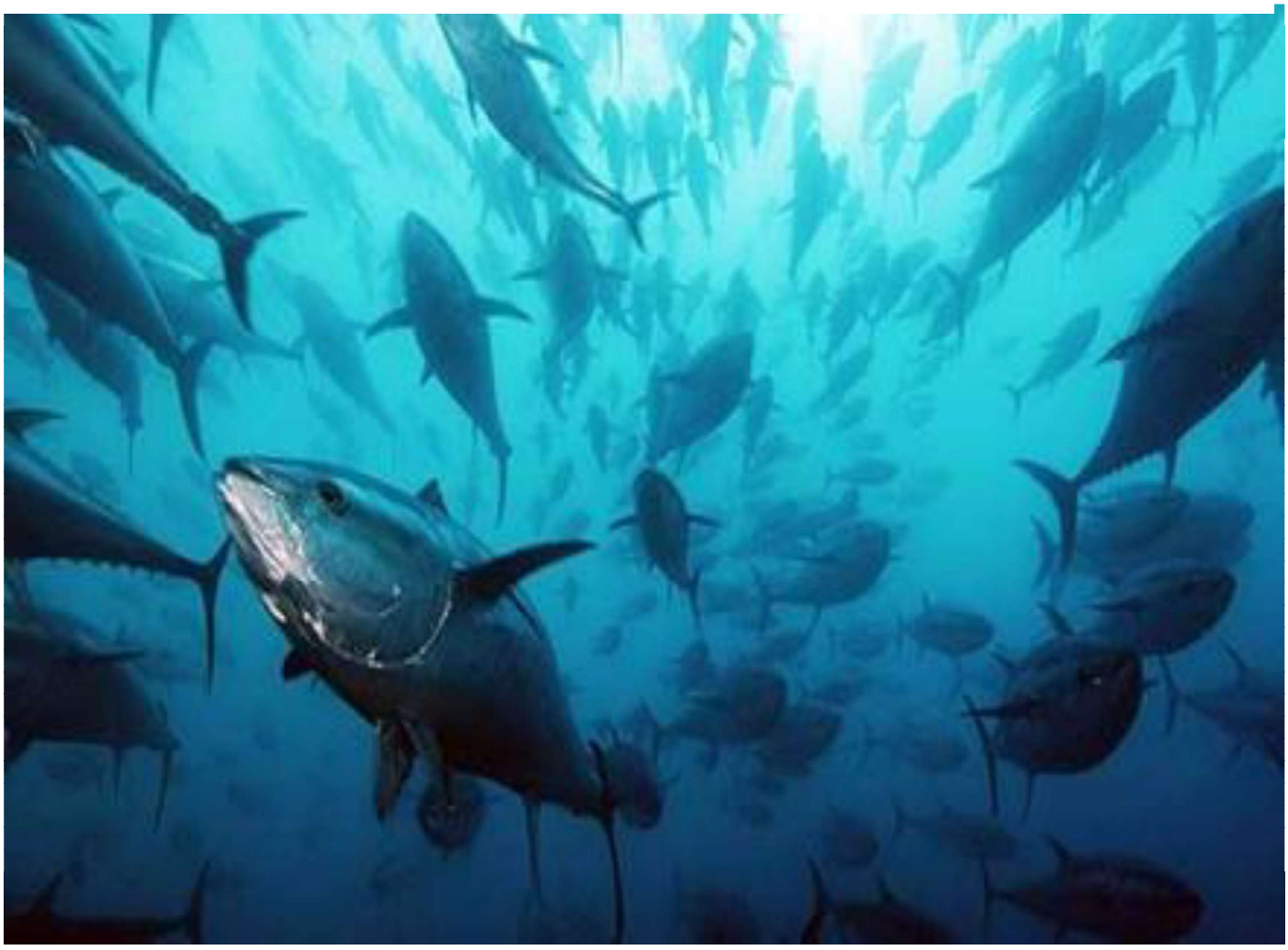
PROTOCOL FOR THE GBYP BIOLOGICAL SAMPLING



Last updated: March 2022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COMMITMENT AGREEMENT

You have been selected to collect biological samples for the Atlantic-Wide Research Programme for Bluefin Tuna under the Grand Bluefin Tuna Year Programme (**GBYP**). Please read carefully the steps described in this manual. Each partner is responsible for storing good quality samples and follow the standard procedures regarding the labelling, data form filling and shipping. Following the proposed steps is essential for the standardisation of sampling across locations and across sampling teams. Please note that failure to do so may result in the samples/information being returned for correction. Altering or falsifying information is more serious than not collecting data at all, as it can lead to inaccurate estimates of biological parameters. AZTI-BRTA is responsible of receiving and storing the biological samples, as well as keeping the tissue bank updated. Please contact the coordinator Igaratza Fraile (ifraile@azti.es) and Iraide Artetxe (iraide.artetxe@azti.es) if you have any doubts about how to proceed. Thank you very much for your cooperation.

SAMPLING MATERIAL

General tool kit	
	Printed Sample Data Sheets
	Office material (pencils, rulers, indelible marker pens…)
	Disposable latex gloves
	Tape measure or/and caliper
	Sharp knife
	Surgical scissors
	Tweezers
	Disposable scalpels with solid blade
	Waterproof paper
	Deionized water
	Cool box (with ice, for cool storage of muscle vials and gonads)
	Crioboxes and microtube storage racks
Specific for genetics (M or F)	
	2 to 5 mL tubes with screw cap
	Non-denatured Ethanol 96%
Specific for otoliths (O)	
	1.5 to 2mL microtubes
Specific for dorsal fin spines (S)	
	Paper envelopes
Specific material for gonads (G)	
	50-100 mL plastic containers assembled with screw cap
	Fixator (Bouin or Hollande)
	Parafilm

1. Fish sampling

When the characteristics of the fishery allow it, an effort will be made to collect the fish spread throughout the whole fishing season to be as representative as possible of the area and time of year. Individuals will be measure (preferably in straight fork length, SFL) to the nearest centimetre (Figure 1), and weighted to the nearest 0.1 kg. When it is not possible to take these measures, alternative measures are taken, such as Curved fork length (CFL), First dorsal length (LD1), or head length (LHead), will be taken. Always describe the method used.

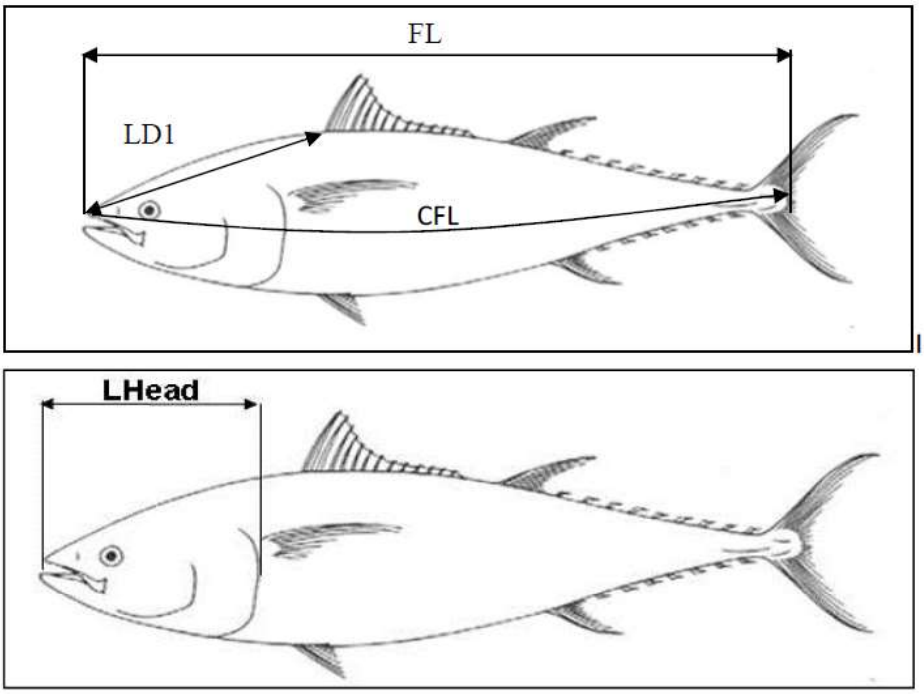


Figure 1. Types of length measurements. Straight fork length (SFL), First dorsal length (LD1), Curved fork length (CFL) and head length (LHead).

Operator must wear cleaned gloves during the whole sampling operation, changing them as many times as necessary to avoid cross-contamination. All the biological information should be recorded in a standardized format, as described in *Fill sampling data form* section.

2. Labelling

The same **unique code** will be used to label each tissue sample collected from a single specimen. Sample labels must be visible on the tube, container, or envelope. For additional safety, when collecting gonads, use submersible (waterproof) paper tags labelled with pencil and put them into the plastic container. Each partner is responsible for the correct labelling of the samples with a unique code (Figure 2):

1) The first four digits correspond to the sampling institute. Check "Partner code" abbreviations in *Fill sampling data form* section to verify the code for each institute.

2) The next two digits correspond to the sampling area. Check "AREA" abbreviations in *Fill sampling data form* section to verify the code for each area.

3) Then, add a single letter code after classifying the fish by size-class (i.e., larvae, age 0, juvenile, medium, large). Check "Size class code" abbreviations in *Fill sampling data form* section to verify the code and the classification criteria for each size class code.

4) Give a number to the fish. This number is a serial numbering that is correlative to the last sample with the same codes for the previous classifications. E.g., imagine that in the previous batch the last sampled fish was AZTI-BB-J-73 for juveniles, and AZTI-BB-L-1000 for large individuals. Then in this new batch, I should start labelling the new collected fish as AZTI-BB-J-74 and AZTI-BB-L-1001 respectively.

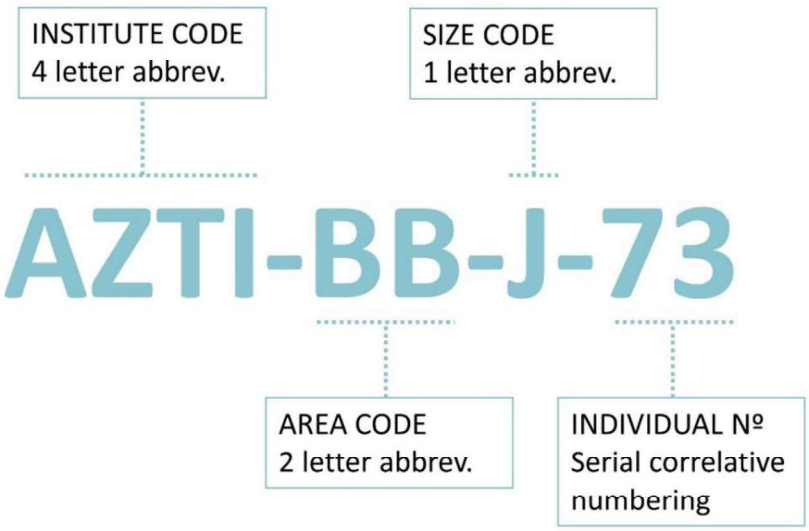


Figure 2. Example of unique code generation for GBYP sample labelling.

The responsibility to check which number to start with is up to the sampler, but in case of doubt always contact Igaratza Fraile (ifraile@azti.es) and Iraide Artetxe (iraide.artetxe@azti.es) that will tell you which number should you start from. **This step is very important** because it allows us to have a traceability of the samples, avoid duplicates or generate extra work when samples arrive mislabelled at AZTI.

3. Sampling procedure for genetics (M and F)

Where possible, white muscle will be sampled for genetics. When this option is not available for sampling (e.g., we cannot damage a fish from the market), a small piece from any fin clip will be collected (Figure 3). When the fish is frozen, do not wait for it to thaw before sampling.

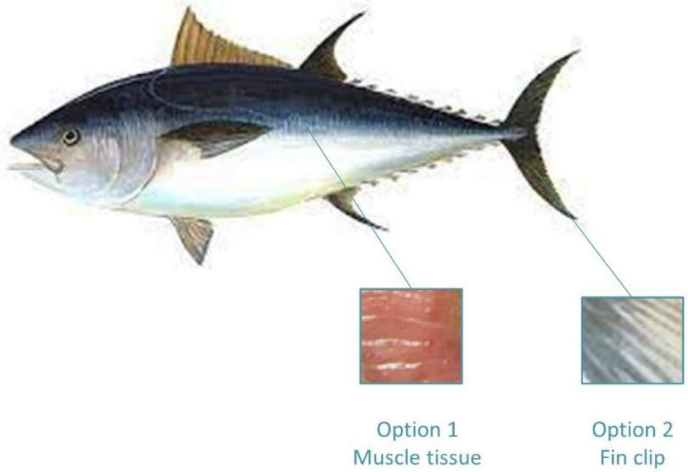


Figure 3. Sampling for genetics where desired muscle (option 1) or fin (option 2) extraction examples are indicated.

- Before sampling, prepare 5mL-tubes with screw cap with **at least 2.5mL of Non-denatured Ethanol 96%**; each microtube must be labelled with Sample ID according to the labelling code reported above (step 1). 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).
- 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. Ensure that the sample area you pick up has not had contact with other surfaces.
- **IMPORTANT: Sampling of genetic 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**.
- Put the genetic tissue sample into the ID labelled microtube with **ethanol 96%** and push the sample to the bottom of the vial so that it is completely submerged, avoiding air or bubbles under or over the sample. Ensure the tissue volume is no more than 10% of the liquid volume (Figure 4).

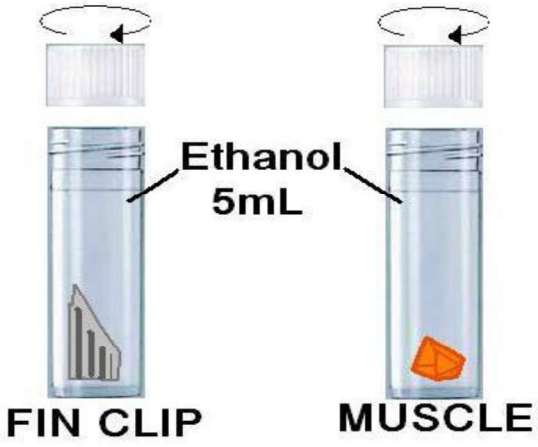


Figure 4. Illustration of genetic sampling preparation

- **Clean surgical instruments for each sampled fish** with water or commercial ethanol and dry it with a new paper tissue moistened in ultrapure water each time. This step is very important to avoid cross-contamination of the samples.

- **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.

4. Sampling procedure for spines (S)

The spine used for ageing purposes is the first ray of the first dorsal fin (Figure 6). 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.

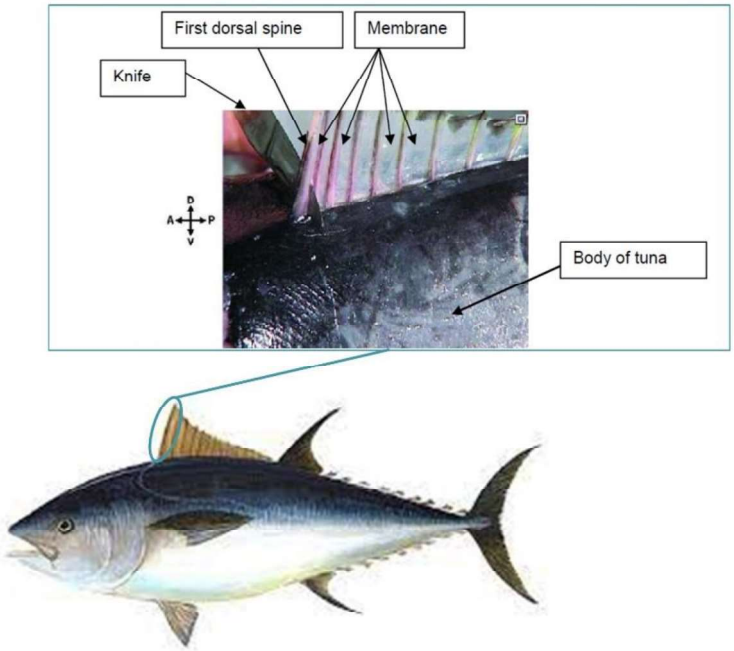


Figure 5. Sampling for first dorsal spine.

- Spread out the first dorsal fin and **cut the membrane joining the two first dorsal rays** by using a knife.

- Then, push the spine forward and down progressively (Figure 6B), then cut and turn it alternately to the right and to the left until the ligament breaks (Figure 6C). Finally, the spine must be twisted and pulled out (Figure 6D). **Care should be taken in order not to twist the spine in its base**. 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.

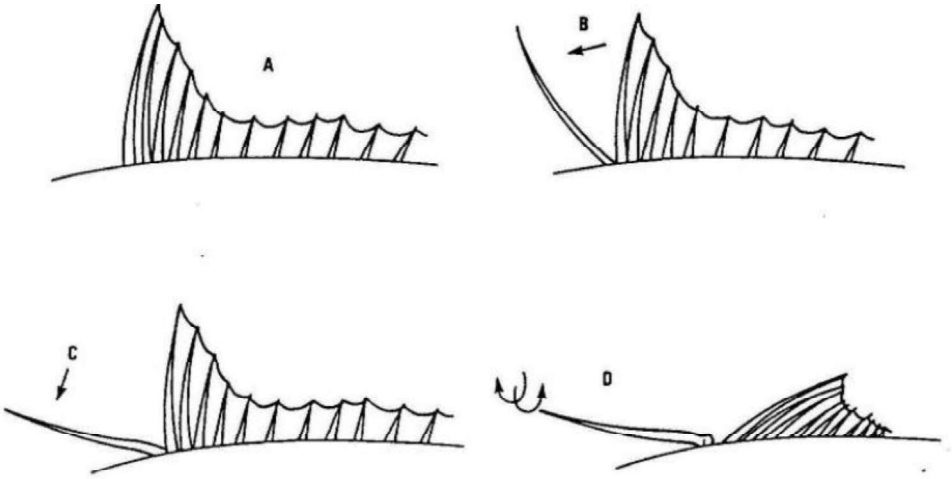


Figure 6. Technique of extraction of the first spine of the bluefin tuna dorsal fin. (Figures taken from Compeán-Jiménez, 1980).

- 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.

- Before storing the sample, it is advisable to remove all remaining tissue and **dry the spine out on blotting paper**.

- **Spines are ideally stored dry in a <u>paper envelope</u>, 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.

5. Sampling procedure for gonads (G)

Gonads should be extracted from the abdominal cavity (Figure 7). Gonads are paired, elongated organs that allow us to better understand the reproductive biology of Atlantic bluefin tuna.

Figure 7. Gonad extraction from the peritoneal cavity (www.bdoutdoors.com).

- Before gonads extraction the **preservation liquid for sample storage must be prepared**. Ideally, Bouin or Hollande fixator will be used for storing the gonad samples, but in case of not disposing of Bouin or Hollade fixator, a 4% formaldehyd solution will be used.

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

- To extract the gonads from the peritoneal cavity, **make a shallow (approx. 1-2 cm) cut with a knife along the midline of the underbelly of the fish**. Start approximately midway along the ventral surface of the fish and cut all the way to the vent. The gonads can be found near the small intestine towards the posterior dorsal wall of the body cavity (just below the fish's backbone).

- Cut the connective tissue to separate the gonad from the intestine and **remove the entire gonad** by pulling it away from the dorsal wall of the body cavity. NOTE: in some cases, the gonads will be removed along with other internal organs by the fishing vessel crew. However, the gonads may remain in the gut cavity and will need to be removed by hand.

- **Weight the whole gonads** after extraction and **note the sex of the fish**. Ovaries in females are generally pinkish orange in colour and have a circular cross-section with a lumen (hole) in the centre. Testes in males are firm, white/grey in colour and have a triangular cross-section (Figure 8).

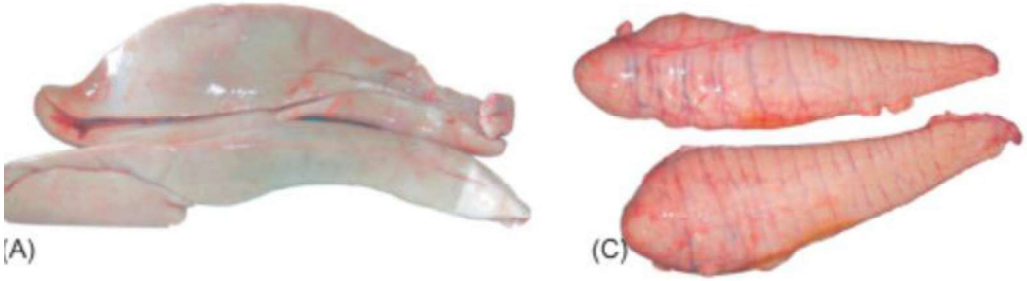


Figure 8. Atlantic bluefin tuna male (A) and female (C) gonad pairs (Zohar et al. 2016).

- 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 this slice too**. This will be the gonad sample.

- Put the gonad sample into the labelled 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% ethano**l.

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

- Store the tubes at **ambient temperature**.

6. Sampling procedure for otoliths (O)

The otoliths can be sampled once all other tissues are sampled (Figure 9). Tunas have fragile, thin, and elliptic otoliths that require particular care during extraction and posterior preparation. You can check a nice video "How to Extract a Bluefin Tuna Otolith" in https://www.youtube.com/watch?v=EuBIHg3n2_s if needed.

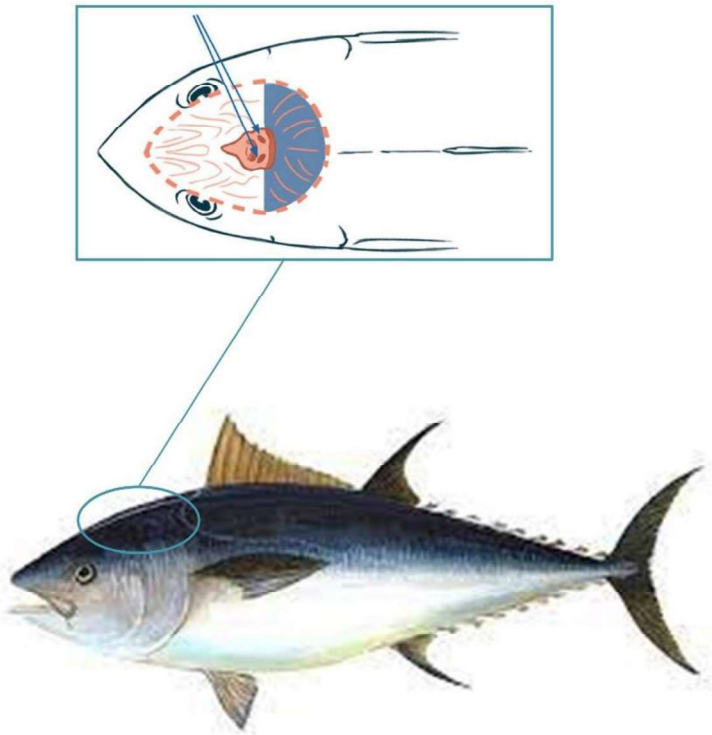


Figure 9. Sampling for otoliths. Otolith diagram from ©PlanetTunaIEO

- 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 (Figure 10a). 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 (Figure 10b). Otoliths will be located at the back of the brain cavity, inside semi-circular canals (Figure 10c). Gently prospect into the canals. The posterior end of the otolith is the most fragile. Use small tweezers to CAREFULLY extract the otolith from the bony capsules.

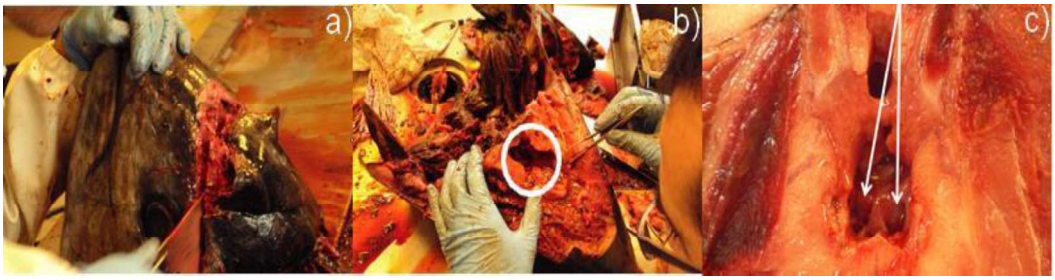


Figure 10: Extraction of sagittal otoliths from a bluefin tuna head

- GENTLY **remove the membrane surrounding the otolith** immediately after extraction (the membrane is harder to remove after it has dried).

- Clean the otolith with deionized water, **until it is certain that there is no biological material adhering to the otolith**.

- Store in plastic vials with the corresponding code, and **let the otoliths dry with the plastic vial lid open** for at least 24 hours at ambient temperature.

FILL SAMPLING DATA FORM

- Fill the database form "*GBYP sampling sheet.xls*" following the described procedure:
  - **Fish identification data**

general ID	AAAA-BB-C-X format, where; A: Partner code B: Area code C: Size code X: Individual ID number. Remember that numbers are correlative and should start following the last number of the individual from the same institute, area and size.	
PARTNER 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
	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
	BALF	Grup Balfego
	UNIM	UNIMAR
	IMR	Institute of Marine Research
	DFO	Fisheries and Oceans Canada
	NOAA	National Oceanic and Atmospheric Administration
	ABTL	AquaBioTech Ltd
	TAXO	Taxon Estudios Ambientales SL
	NGBFT	NextGeneration Bluefin Tuna partnership
	MI	Marine Institute, Ireland
	ROP	REGIONAL OBSERVER PROGRAM
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
	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
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
	NoS	North Sea
	NWATL	North-Western Atlantic
	SATL	South Atlantic
	WMED	Western Mediterranean
FISHING GEAR	**BB**	baitboat
	FAD	fish aggregating device
	GN	gillnet
	GS	Greenstick
	HL	handline
	LA	larval survey
	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
INDIVIDUAL ID NUMBER	Number given to the fish. The numbers are correlative. Remember to start numbering after the last numbering of the individual with the same institute, area and size.	
SIZE CLASS CODE	**V**	Larvae
	0	Age 0
	J	Juveniles
	M	Medium
	L	Large

- **Sample availability**

TISSUE otoliths	"O" if otolith was collected for this individual
Nº OTO	If TISSUE otoliths = O, Then 1 or 2.
TISSUE Spine	"S" if spine was collected for this individual
TISSUE Gonads	"G" if gonads were collected for this individual
TISSUE Muscle/Fin	"M" in muscle was collected or "F" if fin was collected for this individual

- **Sampling info**

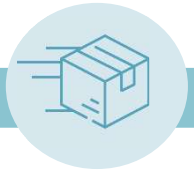
Harvest Date [dd/mm/yyyy]	*[for farmed fish only]* Date of fish death
Catch Date [dd/mm/yy]	Date when the fish was caugth
Latitude	Latitude in decimal degrees
Longitude	Longitude in decimal degrees
Collector	Name of the person who sampled the fish

- **Biological data**

Length [cm]	Only direct measurements of length are noted, in cm. Whenever length is estimated this is noted in "notes for length and weight" section, and the specific conversion factors are described.	
Type of length	**SFL**	Straigth fork length
	LD1	First dorsal length
	CFL	Curved fork length
	LHead	Head length
	TL	[*larvae only*] Total length
Weight [kg]	Only direct measurements of weight are noted, in kg. Whenever weight is estimated this is noted in "notes for length and weight" section, and the specific conversion factors are described.	
Type of weight	**TW**	total weight
	GGT	gilled/gutted weight
	GW	Gutted weight
	DT	Ddressed weight
	TW (lbs)	total weight in lbs
Notes for length and weight	*[if estimated or converted, provide reference equations etc.]*	

Sex	**F**	Female
	M	Male
	I	Indeterminate
Reproductive Stage	**I**	Immature: Small ovaries with no visible ovocytes. Translucid pink color.
	IM	Initial 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.
Gonad weight [kg]	*[if gonads where collected provide their weight.]*	

- **Email the form to the database supervisors in AZTI as soon as some samples are collected**: Igaratza Fraile (ifraile@azti.es) and Iraide Artetxe (iraide.artetxe@azti.es). A reminder will be sent 15 days prior to the delivery date of each accorded deliverable. The newly labelled samples will be checked against already existing samples to avoid doubling names.



SHIPPING PROCEDURE

- When samples are ready to be shipped contact Igaratza Fraile (ifraile@azti.es) and Iraide Artetxe (iraide.artetxe@azti.es) and **wait for their confirmation** to coordinate and arrange shipping dates. This is to ensure that samples can be managed as soon as they arrive at AZTI.

- Put the samples in a storage box with the reference "GBYP" and the partner's name. Ship the box by Express Courier mail to the following address:

Igaratza Fraile / Iraide Artetxe
AZTI Marine Research Division
Herrera kaia portualdea z/g
20110 PASAIA SPAIN