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Protocol for fish disease assessment in marine environmental monitoring using common sole (*Solea solea*, Linnaeus 1758) as sentinel organism: identification of externally visible diseases and liver histopathology

Izaskun Zorita[1*], Nagore Cuevas[2]

Resumen

El seguimiento de la cuantificación de enfermedades de peces es una herramienta apropiada y muy utilizada para evaluar el efecto de los contaminantes en el medio marino. Sin embargo, hay muy poca información publicada sobre las enfermedades externas visibles y la histopatología de hígado de lenguados (*Solea solea*), una especie centinela prometedora en el suroeste de Europa. Por ello, este trabajo presenta un protocolo que describe las patologías comunes registradas en lenguados de la costa vasca (sureste del Golfo de Bizkaia) y detalla las técnicas utilizadas, desde la obtención de la muestra, hasta la propuesta metodológica para la evaluación de las lesiones hepáticas mediante un único índice histopatológico.

Palabras clave: Protocolo, enfermedades de peces, enfermedades externas visibles, histopatología de hígado, lenguado

Abstract

Monitoring the quantification of fish diseases is a well established and widely used tool to assess pollution effects in marine environments. However, there is sparse published information on externally visible diseases and on liver histopathology of common sole (*Solea solea*), a promising sentinel species in south-western Europe. To address this issue, we report a protocol in which, commonly recorded pathologies in common soles of the Basque coast (south-eastern Europe) are described. This protocol explains in detail the techniques used, from the sample collection to the methodological proposal for the evaluation of liver lesions through a single histopathologic index.

Key Words: Protocol, fish disease, externally visible diseases, liver histopathology, common sole

Introduction

Flatfish have been proposed as suitable sentinel species to study the effects of exposure to environmental stressors, such as pollution (Myers *et al*., 1994; Khan, 2003; Stentiford *et al.,* 2003; Stehr *et al.,* 2003; Dabrowska *et al.,* 2012) due to their benthic behavior and their propensity to develop toxicopathic liver lesions (Myers *et al.,* 1998). Species such as dab (*Limanda limanda*), European flounder (*Platichthys flesus*) and English sole (*Parophrys vetulus*) are the main target species for monitoring purposes in the North Europe and north-western America (Köhler *et al.,* 1992; Vethaak *et al.,* 1996; Myers *et al.,* 2003; Lang *et al.,* 2006).

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Fish disease measured in terms of externally visible diseases, macroscopic neoplasms and liver histopathology has been proposed as a method to assess biological effects of pollution in ICES (Bucke *et al.,* 1996; Feist *et al.,* 2004). Externally visible diseases provide an appropriate indicator of the general health of individuals and populations (Davies and Vethaak, 2012). The presence of macroscopic liver neoplasms and certain types of histopathological liver lesions is a more direct indicator of contaminant effect and has been used for many years in environmental monitoring programmes (Vethaak and Wester, 1996; Stehr *et al.,* 2003). The liver is the main organ involved in the detoxification of xenobiotics, and several categories of hepatocellular pathology are now regarded as reliable biomarkers of toxic injury and representative of biological endpoints of contaminant exposure (Myers *et al.*, 1987, 1992, 1998; Vethaak and Wester, 1996; Stentiford *et al.*, 2003; Feist *et al.*, 2004). However, it is worth noting that the conditions that trigger disease present a multifactorial aetiology indicating that not only anthropogenic contaminants but also other endogenous (i.e. age, size, gender, stage of sexual maturation, condition, population density, occurrence of other diseases, etc.) and exogenous factors (i.e., pathogens, water temperature,

oxygen content, salinity, nutrients…) may contribute to disease development (Davies and Vethaak, 2012).

One of the most abundant flatfish found in the Basque estuaries and coast (south-western Europe) is the sole, but especially the common sole (*Solea solea*) although the presence of Senegalese sole (*Solea senegalensis*) is remarkable in estuaries too (Quincoces *et al.,* 2011; Franco *et al.,* 2012). Soles are benthic marine species that are buried under sandy and muddy bottoms. Juveniles are found during the first 2 to 3 years in coastal nurseries (bays and estuaries) before migrating to deeper waters (Koutsikopoulos *et al.,* 1995). Adults feed on worms, mollusks and small crustaceans at night. The species is of ecological and economic importance and also an important aquaculture species (especially *S. senegalensis*) in the southern Europe and the Mediterranean. Several ecotoxicological studies based on bioassays, including laboratory exposure to waterborne or contaminated sediments and directly injected contaminants (Arellano *et al.,* 1999; Riba *et al.,* 2004, 2005; Costa *et al.,* 2008, 2009; Oliva *et al.,* 2009; Prieto-Álamo *et al.,* 2009), with the Senegalese sole have taken advantage of the availability of the fish from aquaculture facilities and its benthic behavior. For all these features, sole seems to be a good candidate to study biological effects of contaminants and this species may achieve the potential in south-western Europe that flounder and dab have been recognized in the North and Baltic Sea (Costa *et al.,* 2011). Nevertheless, as other flatfish sole lacks yet field-based validation for possible implementation in biomonitoring programmes (Gonçalves *et al.*, 2013).

Guidelines for methodologies used for monitoring histopathological liver lesions on flatfish dad and flounder have been developed by ICES and the Biological Effects Quality Assurance in Monitoring Programme (BEQUALM) (Feist *et al.,* 2004; BEQUALM, 2005). The diagnosis of liver histopathology uses a subjective grading and descriptive qualitative criteria. For this reason, different attempts have been made to semi-quantify histopathological features in fish exposed to xenobiotics (Costa *et al*., 2009; 2011; Gonçalves *et al.,* 2011). Among these, weighted indices are of especial relevance since they are based on the premise that the histological changes may not reflect the same impact (biological significance) in the individual. By attributing a numerical value to the relative importance (weight) of the alteration plus a dissemination factor, a histopathologic condition index can be obtained for each individual (Bernet *et al.,* 1999; Costa *et al.,* 2009, 2011).

Compared to dab and flounder, there is sparse published information on liver histopathology in common sole. To address this issue, we report common liver histopathological lesions registered in 650 common soles collected from the Basque coast in the framework of two projects carried out by AZTI-Tecnalia namely, Consorcio de Aguas Bilbao Bizkaia and Itsasteka. In the former, juvenile fish (<2 years old) were collected in autumn in the Ibaizabal estuary during three years (2011-2013). In the later, adult individuals (2-6 years old) were sampled monthly in Basque offshore waters during one year (summer 2012-summer 2013).

Thus, the present study intends essentially to contribute for fish disease assessment in common sole in order to implement the European Marine Strategy Framework Directive (MSFD) in the Basque coast. Specifically it aims to provide a detailed protocol for the identification and quantification of (i) externally visible diseases and (ii) liver histopathology, describing the procedure from the sample collection to the integration of liver lesions into a single histopathologic index.

Protocol for sampling procedure

Sample collection

It is recommended to use the same fish for the assessment of externally visible diseases and for liver histopathology (Stentiford *et al.,* 2009). Additionally, it is desirable to use fish of a certain size group in order to minimize the effect of age. A minimum of 30 fish should be sampled per sampling site in order to provide a 95% confidence of detection of 10% disease prevalence in the population (Feist *et al.,* 2004). The choice of the sampling sites and season depend on the objective of the study. Soles should be caught by bottom trawling using appropriate fishing gear. The number of trawls is influenced by the number of fish sampled but in any case, the duration of individual trawls should be minimized (10-15 min approximately) in order to reduce the effects of stress and the possible mechanical damage that results from net abrasion. As soon as possible, live fish should be transferred to uncovered tanks (≈1 m^3) containing running seawater at room temperature. If running seawater is not available on board, it is desirable to keep live fish on ice in order to avoid tissue degradation. Prior to dissection, common soles should be anesthetized with an overdose of tricaine methanesulfonate. This anesthetic is recommended due to its easy use since it is added to water until oversaturation. Other anesthetics can also be used. Total length and wet weight must be measured for the calculation of condition factor (CF = wet weight [g] / total length^3 [cm] × 100). Be aware of the error that the weighing scale may give due to the movement of the boat.

Protocol for macroscopic examination: externally visible diseases

Examination of externally visible diseases

The examination for externally visible diseases should be carried out in each individual fish. The fish should be examined whilst fresh, i.e., shortly after they have been landed on the ship or taken from nets (not frozen or refrigerated). An area for working should be cleared, preferably a bench or table at standing height, with good lighting and running water. For a correct inspection of externally visible diseases the following parameters should be taken into account:

- intensity of the skin color
- state of pigment
- total amount of mucus on the body

- state of squama and oral cavity
- circulatory disturbances (hyperemia, hemorrhages, sores or hydremia)
- deformation of skull and skeleton bones
- state of eye crystalline lens and cornea
- presence of macroscopic parasites in the whole body
- traumatisms by extraction

Diagnosis of externally visible diseases

Externally visible diseases are assessed according to the training guide for identification of common disease and parasites of fish from the North Atlantic (Bucke *et al.,* 1996) but with some modifications for common sole. The eyed side of the common sole is characterized by its greyish brown to reddish brown skin color with diffuse dark spots (Figure 1a). The color variability of the eyed side depends on the substratum color since flatfish are able to camouflage (Ramachandan *et al.,* 1996). The pectoral fin of the eyed side shows a black blotch restricted to the distal end of the fin. The blind side is whitish (Figure 1b).

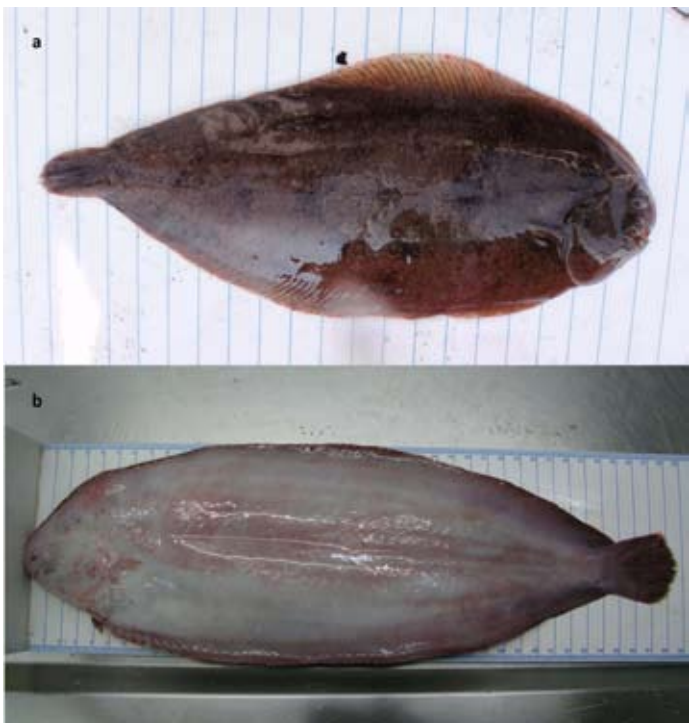

Figure 1. Normal common sole (a) eyed side and (b) blind side.

The externally visible diseases described below are the most common diseases found in 650 common soles from the Basque coast but new undescribed lesions should be also recorded.

a. Tumour formation

Tumours appear most often as rounded, well-defined (benign) formations arising from the subcutaneous connective tissue (Figure 2). The cause is unknown.

Figure 2. Tumour in the blind side of a common sole

b. Skin ulcerations

Ulcerations are easily recognized at the acute stage, being rounded, haemorrhagic centers with white peripheries (Figure 3). Healing stages are partly open with more white and brown pigmentation forming scar tissue. Healed ulcers show complete closure of the lesion. Scale loss (or scale malformation) is evident in old lesions. The causes of ulcerations have been attributed to bacterial infection but can arise as the result of parasite infestation, scale loss, traumatic injury, and debilitation associated with poor nutrition, salinity fluctuations and other adverse environmental factors (Bucke *et al.*, 1996; Iglesias *et al.,* 2001).

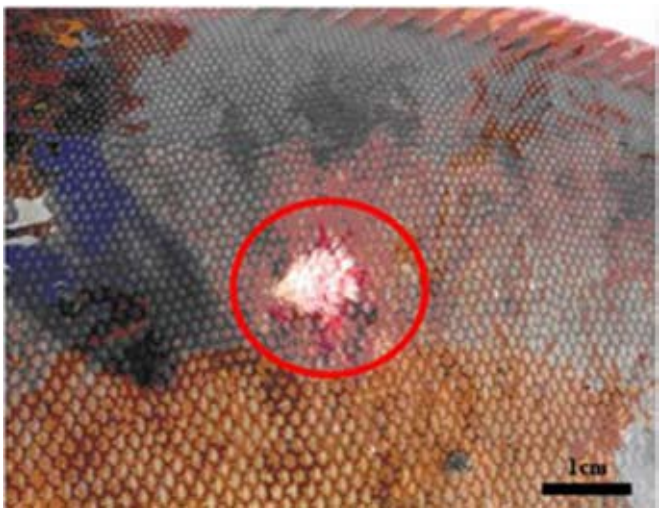

Figure 3. Skin ulceration in a common sole.

c. Pigment anomalies

Pigment anomalies in the form of hyper-melanization appear as dark brownish colorations (Figure 4) in the depigmented side or white areas on the pigmented side. The cause of hyperpigmentation is still unknown but there is indication that the condition may be linked to nutritional factors and/or effects of UV-B radiation on early life stages (Grütjen *et al.,* 2013). Other pigment anomalies may be of genetic origin (Bucke *et al.,* 1996; Venizelos and Benetti, 1999).

Figure 4. Hyperpigmentation in the blind side of the tail of a common sole.

d. Fin rot

Initial stages of fin rot appear as thickening of the epithelium in the marginal edge of the fins. Further development results in necrosis of the soft tissue and fraying of the fin edges, followed by exposure of the fin rays, gaps in the fins (Figure 5) and often ending with total necrosis of the fin. Among the causes, it is difficult to conclude whether the primary cause of fin rot is the bacterial infection or if the bacteria are secondary to traumatic damage of the fins, i.e., gear damage (Bucke *et al.,* 1996).

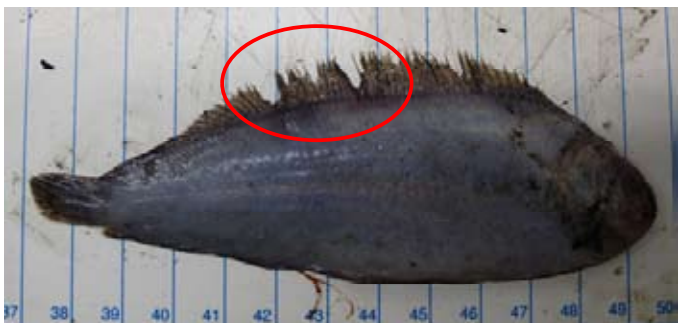

Figure 5. Fin rot in a common sole

e. Skeletal deformities

The vertebral column may exhibit upward and/or downward curvatures, sidewards curvatures or compressions (Figure 6). Deformities of the fin rays may also be observed, but in this context gear damage has to be considered. The cause of the deformities may be due to parasitic infestation, genetic background, vitamin deficiencies or environmental factors such as low water temperature or pollutants exposure during early life stages (Bucke *et al.,* 1996).

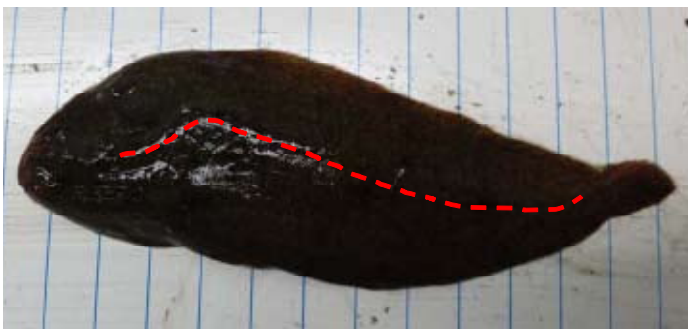

Figure 6. Skeletal deformity in a common sole

f. Parasites

Numerous internal and external parasites can be found in all species of fish and therefore their presence should be registered as a supporter parameter of the histopathological diagnosis. In our studies, no macroscopic parasites have been noticed in common soles.

g. Gear damage

Net injuries normally affect the fin rays. Damage appears often as curvatures of the affected fin rays or possibly the loss of soft tissue resulting in a U-shaped scar formation (Figure 7). Especially the "spine" behind the anal region is often broken. Damage may result in scrapings of the epithelium or pinpoint haemorrhages in the skin produced during the trawl movements over the bottom, especially when the codend is packed with fish. Fish also escape through net meshes by wriggling through, thus injuring themselves.

Figure 7. Common sole showing signs of gear damage.

The presence of parasites and the signs of gear damage identify possible confounding factors contributing in the development of externally visible diseases.

Quantification of externally visible diseases

a. Calculation of prevalence

Since serious externally visible diseases have not been registered in this species, there is no need to classify diseases according to degree of severity. Thus, calculation of prevalence is the method recommended for externally visible diseases assessment in common sole. Prevalence describes the proportion of individuals in the population that are affected by specific lesion or pathology and is calculated as prevalence = [(number of fish with pathology) / (number of fish analyzed)] x 100.

Protocol for microscopic examination: liver histopathology

Histologic sample preparation

a. Dissection method

Fish should be sacrificed by a blow to the head. The head should be stored in the refrigerator for otolith's extraction and subsequent age determination (see Annex 1). Once the body cavity has been opened and viscera exposed, the liver should first be examined *in situ* in order to record any macroscopically visible nodules, parasites or other lesions (Figure 8). Additionally, the sex of the fish should be noted. Afterwards, the liver should be dissected and removed from the fish for examination. If data on hepatosomatic index (HSI) is required, both the fish (before dissection) and the liver have to be weighted and HSI calculated as follows: (HSI = liver wet weight [g] / total wet weight [g] × 100).

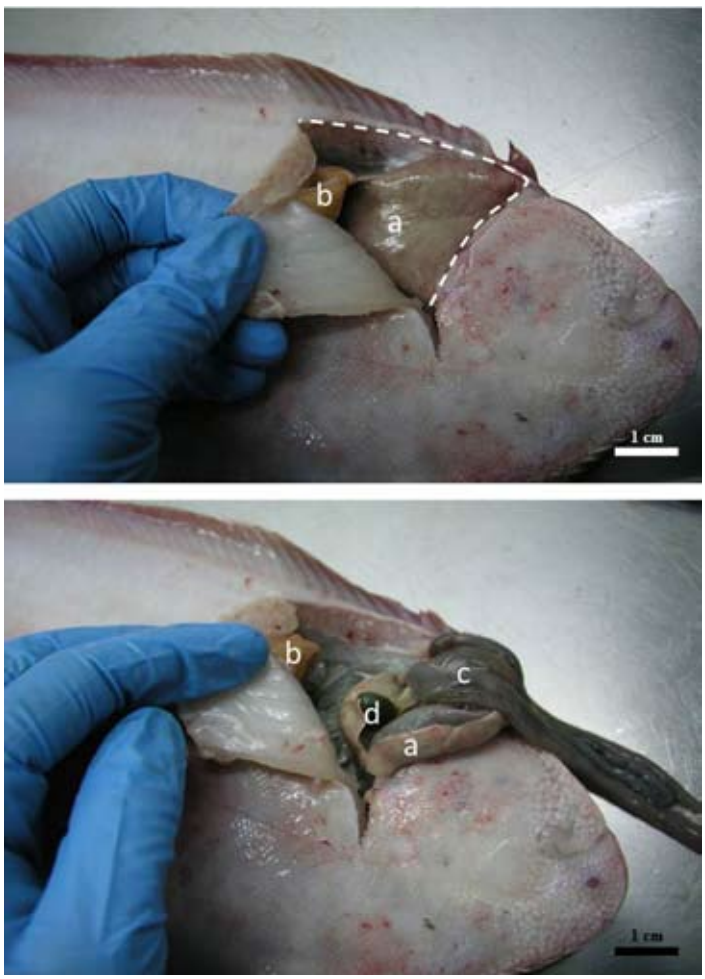

Figure 8. Dissection of the organs of a female common sole through an incision into the body cavity (a) liver; (b) female gonad; (c) stomach and digestive tract; (d) bile.

b. Tissue fixation

Once the liver is dissected out, a 5 mm slice has to be cut longitudinally through the central axis of the liver using a sharp blade. The central axis of the liver is generally selected to avoid tissue damage associated with the borders. Hence, it is essential that care be taken to avoid crushing or ripping the tissue with forceps or other dissection instruments. The piece of liver has to be placed into a pre-labelled histological processing cassette and immersed into a container of fixative. Different solutions were tested for liver fixation and the best results for routine liver histopathology analysis were obtained with Davidson's fixative (see Annex 2).

c. Processing liver samples for embedding

Fixed liver samples are dehydrated through a progressive series of alcohol, cleared and embedded in resin (Technovit 7100) prior to their placement into resin blocks. Better resolutions of histopathological features are obtained with resin than other embedding medium because thinner sections can be cut. Tissues should be preferably processed using commercial automatic tissue processors instead of manually because vacuum conditions improve the quality of histologic samples (Annex 3).

d. Sectioning and staining

For conventional histology, sections are cut at 3-5 µm using a rotary microtome. It is important to ensure that the liver is trimmed sufficiently to allow sectioning of the complete area of interest within the sample. Two to three sections should be taken throughout the tissue block. Sections are floated out in a water bath full of distilled water at a temperature of 50º C and then sections are picked up on pre-labelled glass slides. Two to three glass slides are prepared per sample and used as replicates. In order to assure tissue adhesion and expansion, glass slides are dried in a dust-free environment on a hotplate at 50ºC. Replicates are used for different purposes but in order to determine the structural morphology of the liver, haematoxylin and eosin (H&E) stain is carried out in one replicate (Annex 4). The other replicates are set aside for other uses such as the application of other histochemical techniques, the analysis of artefacts effect or the determination of the extent of a lesion in the tissue.

Examination of liver sections

The examination of each section should take into account any gross observations made at *post mortem* (or necropsy). It is also important that the reading of slides is conducted in a consistent manner and blindly, without knowing the origin of the sample. As a general rule, it is better to examine slides using the lowest power objective lens first in order to take an overall view of the liver and then, change consecutively to higher power objective lens to observe slides more in detail. For a correct examination of liver sections the following parameters should be taken into account:

- Shape, morphology and homogeneity of hepatocytes

- Shape and morphology of sinusoids
- Size of nuclei
- Homogeneity of hepatic parenchyma
- Presence of inflammatory or immune response cells
- Presence of parasites
- Morphology of bile ducts
- Coloration of the section
- Histologic artefacts as confounding factors

Diagnosis of liver histopathology

Since histopathologic diagnosis depends on the expertise of the pathologist, there are two different methods to standardize the diagnosis criteria. The first standardization method consists of a blind check of 10-15% of previously diagnosed samples, while in the second method different pathologists read the same liver sections. The percentage of agreement or disagreement is evaluated according to Chi square test ($p<0.05$). In case of disagreement or doubt, it is advised that additional sections are examined carefully in order to establish the correct diagnosis. In the following section we describe the most common hepatic lesions observed up to now in 650 common soles collected from the Basque coast.

a. Histological appearance of normal liver

The common sole liver is similar to that of other fish species in that it is traversed by capillaries branched from the portal vein which transports blood from stomach and intestine. These small capillaries (sinusoids) separate the basic structural unit, the tubule or trabecule. The liver in common sole is characterized by the presence of exocrine pancreatic tissue and the organ is often referred as "hepatopancreas" (Figure 9). Other tissue elements include bile ducts that are lined with a cuboidal epithelium surrounded by connective tissue (see Figure 18).

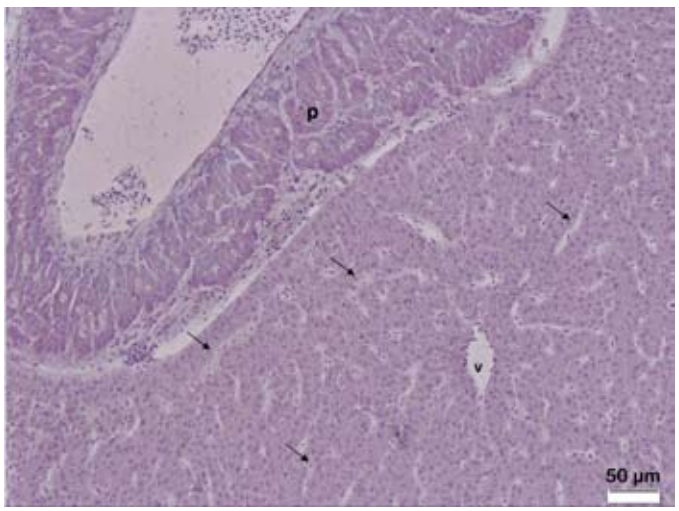

Figure 9. Section of a common sole liver showing exocrine pancreatic tissue (p) and normal hepatic tissue. Narrow arrows: sinusoids; v:vessel (H&E).

The histological structure of the normal common sole liver shows regular hepatocytes, more or less polyedric in shape, with a clear cytoplasm, which should indicate good glycogen storage (Costa *et al.,* 2009) and regular-sized nuclei with conspicuous nucleoli. Many sinusoids could be observed branching out of larger blood vessels where a few blood cells (mostly erythrocytes) are observed (Figure 10).

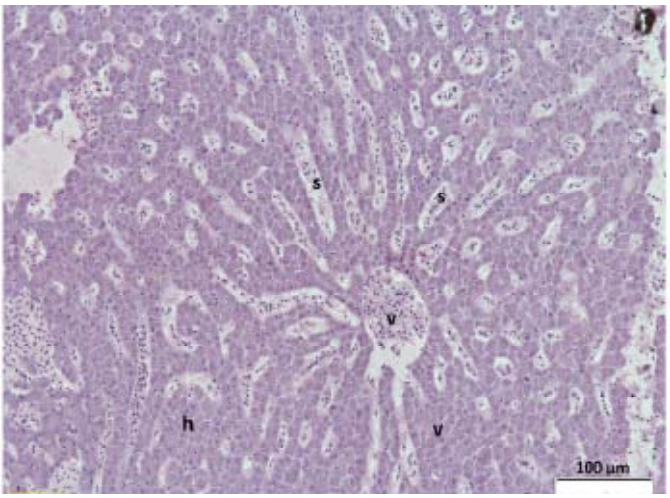

Figure 10. Section of normal hepatic tissue of a common sole showing a regular network of hepatocytes disposed in a bilayer and limited by sinusoids. h: hepatocyte, s: sinusoid, v: blood vessel (H&E).

b. Haemorrhage

Haemorrhages are blood leaking from blood vessels, especially when blood-swollen vessels and proliferation of sinusoids indicate some degree of inflammatory response. Erythrocytes from ruptured vessels are often observed to intrude into foci of necrotic tissue spreading from the periportal area (Figure 11). Haemorrhages have been frequently observed around blood vessels in the liver of soles exposed to contaminated sediments (Costa *et al.,* 2011).

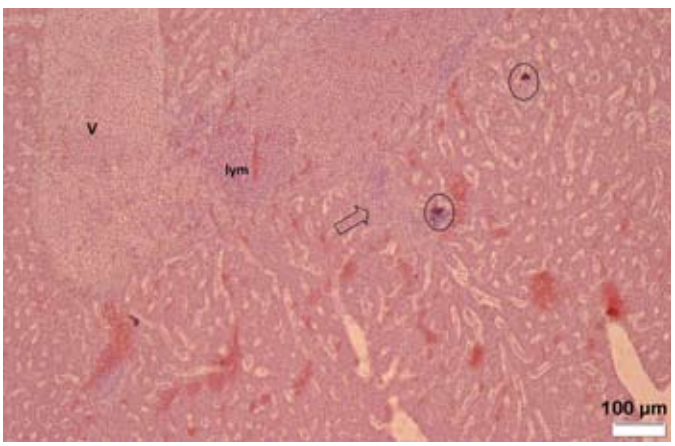

Figure 11. Hepatic tissue showing a slight haemorrhage (wide arrow) and hyperaemia in a blood vessel (v). lym: lymphocytes; circle: melanomacrophages (H&E).

c. Hyperaemia

Hyperaemia is the congestion of an organ caused by venous and arterial processes (Figure 11 and Figure 12). It is a result of an underlying pathologic process, usually inflammation. It is often associated with oedema. High prevalence of hyperaemia in fish liver have been related to the presence of organic compounds (Noreña-Barroso *et al.,* 2004; Agamy 2012).

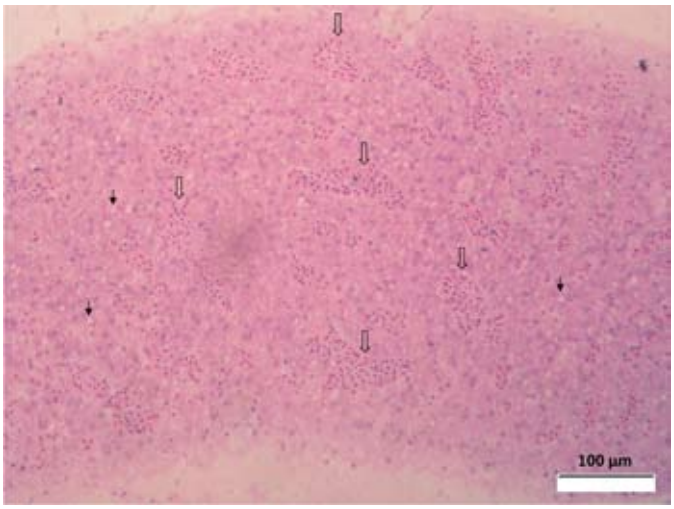

Figure 12. Hepatic tissue affected by hyperaemia in sinusoids (wide arrows) and slight fat vacuolation in hepatocytes (narrow arrows) (H&E).

d. Melanomacrophage centers (MMCs)

Clusters of pigmented cells, termed "macrophage aggregates" or "melanomacrophage centers" are present in liver to varying degrees, either singly or in large numbers throughout the liver parenchyma (Figure 13). They also vary considerably in size. Melanomacrophages are sometimes surrounded by a layer of connective tissue and lymphocytes. According to the variety of pigments and the cellular content, melanomacrophages in H&E stained sections vary in appearance from yellow to golden brown and black. Proliferation of MMCs has been related with several factors such as age, reproductive stage, infectious diseases and PAH contamination (Couillard *et al.*, 1999; Agius and Roberts, 2003; Rabitto *et al.*, 2005). Hence, MMC prevalence and intensity has been proposed as a potentially useful biomarker for environmental pollution and degradation (Murchelano and Wolke, 1991; Manera *et al.,* 2000).

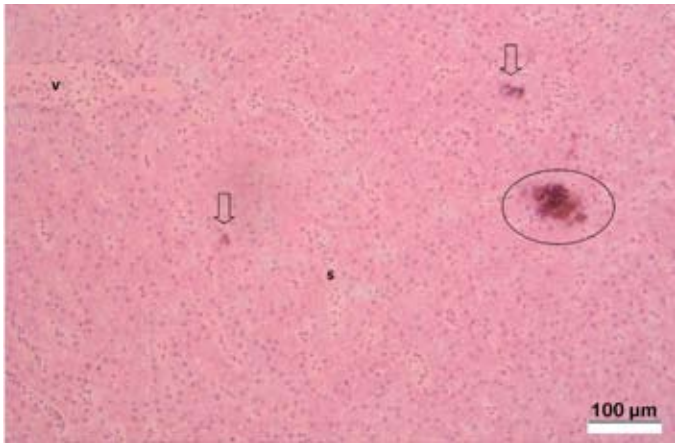

Figure 13. Hepatic parenchyma with several melanomacrophage centers (circle and arrows). v: vessels; s: sinusoids (H&E).

e. Lymphocytic infiltration

Another lesion associated with the cellular immune response is the lymphocytic infiltration. Infiltrates of lymphocytes (Figure 14) occur very frequently in close proximity to blood vessels or granulomas and connective tissue. Lymphocytes are characterized by a large nucleus, occupying most of the cell volume, and by a granular cytoplasm. In some cases, infiltrations affect large areas of the liver. Lymphocytic infiltration has been induced in fish exposed to 3% WAF for fifteen days (Agamy, 2012).

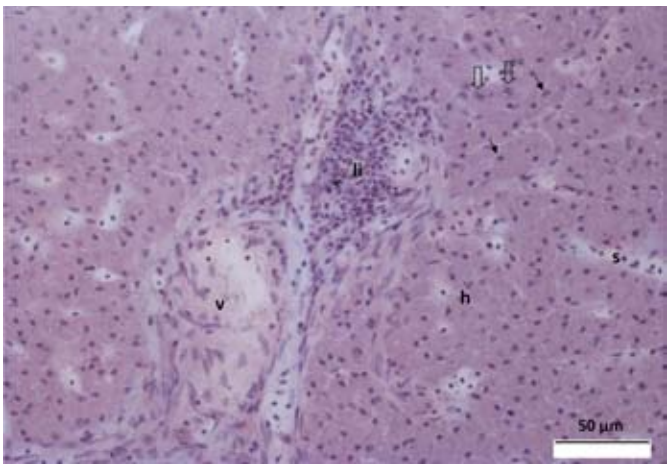

Figure 14. Hepatic tissue showing lymphocytic infiltration close to a blood vessel. Note that nuclear pleomorphism (wide arrows) is shown in surrounding hepatocytes. li: lymphocytic infiltration; v: blood vessel; h: hepatocytes; s: sinusoids; narrow arrows: normal nucleus (H&E).

f. Hepatocellular and nuclear pleomorphism (HNP)

Hepatocellular and nuclear pleomorphism leading to a marked variation in morphology, is regularly recorded in the liver (Figure 15). Pleomorphism is characterized by a remarkable change in size either of the hepatocytes or the nuclei, with the latter being more common. Affected cells display an enlarged and occasionally

misshaped nucleus, exceeding twice the size of nuclei observed in non-affected neighbouring hepatocytes. Despite this increased nuclear size, affected hepatocytes often do not display an increase in diameter. HNP is usually accompanied by marked disorganization of the liver parenchyma. Nuclear and cellular pleomorphisms are associated with the exposure of carcinogenic compounds (Becker *et al.,* 1987; Myers *et al.*, 1987; Köhler, 1990).

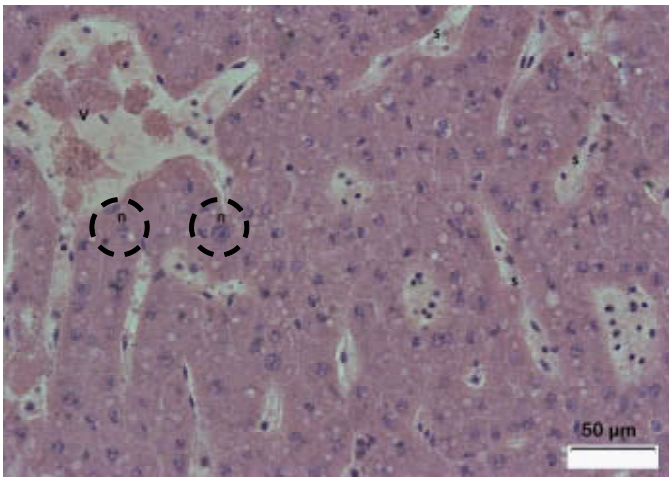

Figure 15. Hepatic tissue showing hepatocyte nuclear pleomorphism (n), v: vessel; s: sinusoids (H&E).

g. Necrosis

Necrosis is a degenerating alteration characterized by the presence of necrotic hepatocytes usually showing pyknosis or hypertrophy. In necrotic tissue the cellular structure is not maintained, and eosinophilic cytoplasm elements and free pyknotic nuclei are visible within the liver section (Figure 16). Necrosis in fish usually implies regional vascular impairment (Metcalfe, 1998) and necrosis of epithelial cells is believed to be a direct effect of irritants (Hinton *et al.,* 2008).

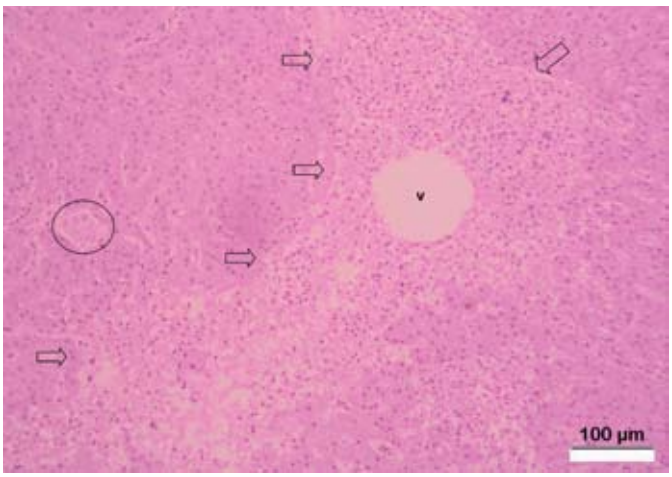

Figure 16. Necrotic hepatic tissue (arrows) around a blood vessel (v) and coagulative necrosis in a focal area surrounded by normal hepatocytes (circle). Note the eosinophilic and pale staining and nuclear condensation of necrotic cells (H&E).

h. Fat vacuolation

The degree of hepatocyte fat vacuolation is highly dependent on the stage in the reproductive cycle and the availability of an adequate food supply (Davies and Vethaak, 2012). However, fat vacuolation is characterized by the presence of highly vacuolated hepatocytes, enlarged due to an increase in volume of fat (Figure 17). The nuclei of these cells are compressed to the cell periphery by the large fat vacuoles. Excessive fat accumulation of hepatocytes is generally regarded as a pre-neoplastic toxicopathic change in mammalian studies, but its significance in fish is less certain. Fat vacuolation in hepatocytes is a common response to chemical exposure in fish (Metcalfe, 1998; Marty *et al.,* 2003).

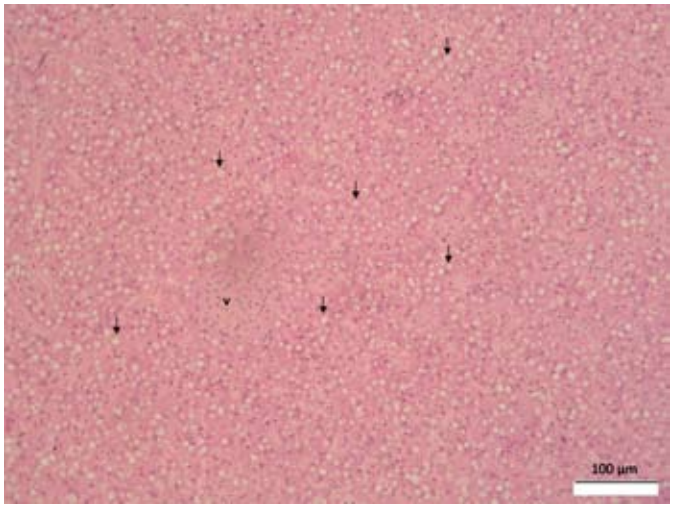

Figure 17. Hepatic tissue showing a medium degree of fat vaculation (narrow arrows), v: blood vessel, (H&E).

i. Hydropic vacuolation of epithelial cells of bile duct

Hydropic vacuolation of epithelial cells of bile duct is clearly identified by large lipid droplets. It usually appears associated with advanced concentric periductal fibrosis (Figure 18). Hydropic vacuolation of epithelial cells of bile duct in certain flatfish species has been associated with exposure to hydrocarbons (Vethaak and Wester, 1996; Simpson *et al.,* 2000).

j. Concentric periductal fibrosis

Concentric periductal fibrosis also known as "onion skin" tends to be non-specific alteration, which is characterized by inflammation and ductal proliferation. In general, early concentric periductal fibrosis is marked by inflammation and later, as the disease progresses, fibrosis predominates (Figure 18). Eventually the bile duct can be replaced by a solid fibrous cord. Concentric periductal fibrosis has been related to parasite presence but the causal link is not clear (Fricke *et al.,* 2012).

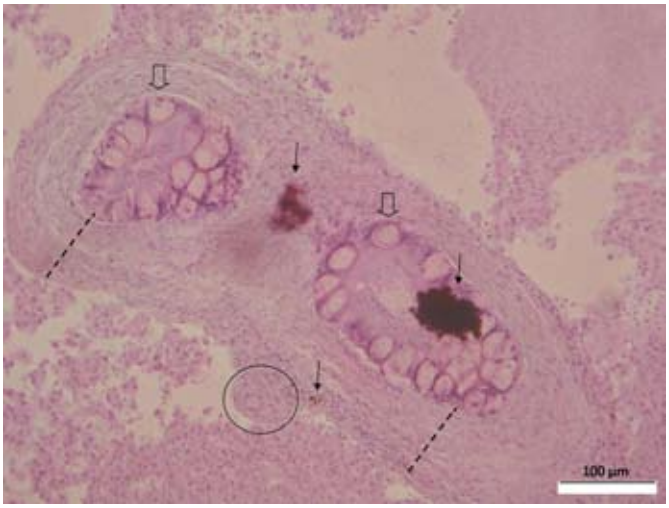

Figure 18. Hydropic vacuolation of epithelial cells of bile duct (wide arrows) surrounded by a concentric periductal fibrosis (broken line) characterized by the presence of multi-layer fibrillar connective tissue (H&E). narrow arrows: melanomacrophage centers; circle:normal bile duct.

k. Spongiosis hepatis

Spongiosis hepatis is a focal lesion that consists of spaces filled with pale eosinophilic material (Figure 19). Spongiosis hepatis also called "cystic degeneration" occurs as clusters of small cyst-like structures. The typical multilocular appearance of the lesion is produced by a network of interconnected perisinusoidal cells (ito cells). Cysts occur either singly or as multiple large, clear cavities in the hepatic parenchyma. Spongiosis hepatis has been reported in fish exposed to chemicals in the laboratory and in fish from contaminated regions (Myers *et al*., 1987; Lauren *et al.,* 1990; Couch, 1991; Agamy 2012).

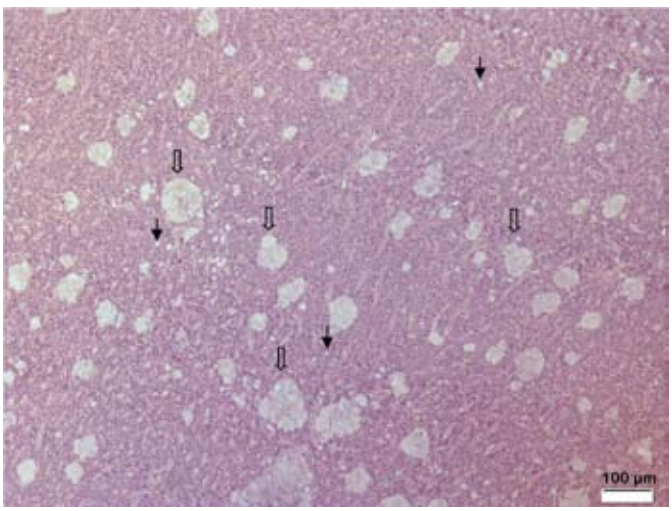

Figure 19. Hepatic tissue showing severe spongiosis hepatis (wide arrows) and a slight fat vacuolation of hepatocytes (narrow arrows) (H&E).

l. Hepatocellular adenoma

In the livers of common soles examined in the Basque coast neither pre-neoplastic focus of cellular alterations nor malignant neoplasms were observed. In other fish species such as in Baltic eelpout (*Zoarces viviparous*) this type of lesions are very rare too (Fricke *et al.,* 2012). Only one benign neoplasm was found in common soles of the Basque coast (Figure 20). Hepatocellular adenoma can be recognized by a set of morphological criteria. Usually there is a clear separation of tumour cells from the surrounding parenchyma (Figure 20). Additionally, parenchyma cells and vessels appear compressed and hepatocellular trabecules usually present a mild architectural disorganization and increased cellular density. Adenomas have been observed in fish exposed to PAHs (Reynolds *et al.,* 2003) but their presence and prevalence in the liver increase with the age of the fish (Vethaak *et al.,* 1996).

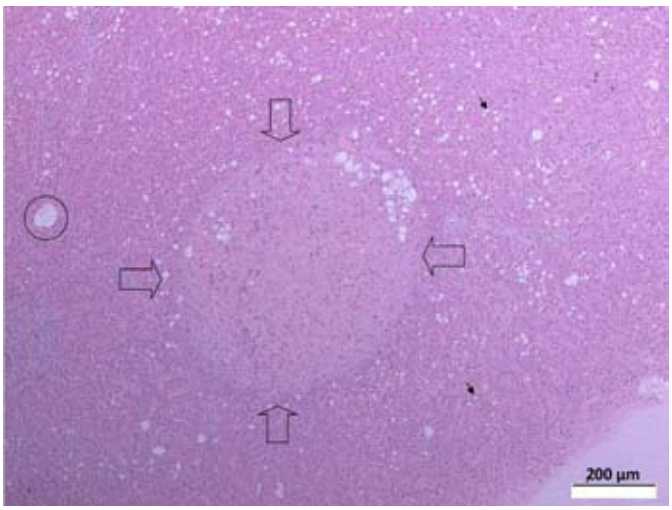

Figure 20. Hepatic tissue showing a hepatocellular adenoma (bening neoplasm, wide arrows) and slight fat vacuolation (narrow arrows) and spongiosis hepatis (circle). Note that a clear compression of adjacent hepatocytes is evident and that a clear separation of tumour cells from the surrounding parenchyma is observed (H&E).

n. Artefacts

Artefacts are features in the surveyed tissue that are not originally present in the living tissue. These artefacts interfere with normal histological diagnosis by changing the tissue appearance and hiding structures. Understanding artefacts is important for both histologists and pathologists as they have the potential to compromise accurate diagnosis. For more information and examples about histologic artefacts in fish sections the following websites could be visited:

http://training.fws.gov/resources/course-resources/fish-histology/Fish_Histology_Manual_v4.pdF

http://www.leicabiosystems.com/pathologyleaders/artifacts-in-histological-and-cytological-preparations/.

Quantification of liver histopathology

a. Calculation of prevalence

The prevalence of each alteration is calculated as the number of cases of fish with a specific alteration with respect to the total fish analyzed and multiplied by 100.

b. Method for the categorization of lesion types: histopathological index

The estimation of the hepatic histopathological condition (*I*h) indices is based on the concepts of: (1) each lesion or alteration's relative biological importance (weight) and (2) the score value, a numerical attribute that reflects the degree of dissemination of the alteration within the studied organ. The semi-quantitative approach to calculate the liver histopathologic index proposed herein is based on the weighted histopathological condition indices developed by Bernet *et al.*, (1999) and Costa *et al.*, (2009) (Table 1). The indices are obtained for each individual and the histopathological alterations are divided by four reactions patterns, as defined by Bernet and co-workers: (1) circulatory disturbances; (2) inflammatory response; (3) regressive alterations (implying functional loss) and (4) progressive alterations (involving altered function).

According to Costa *et al.,* (2009) the global histopathological condition indices (Ih) for each fish and tissue is computed as:

$$Ih = \sum_{1}^{j} w_j \, a_{jh}$$

where, w_j is the weight of the j histopathological trait and a_j the score for the j alteration of the h individual. The weights may range from 1 to 3 (from a slight degree as a hyperaemia to a high severity as a necrosis) while the score can attain the values of 0 (unaltered condition or unobserved lesion), 2 (infrequent occurrence, < 1/3 of tissue affected); 4 (moderate occurrence, < 2/3 of tissue affected) or 6 (severely disseminated/diffuse, > 2/3 of tissue affected).

Table 1 summarizes the histopathological lesions that are assessed in the liver of common soles collected in the Basque coast and their respective weight.

Data treatment

There are a number of statistical procedures commonly applied in the analysis of fish disease data. The Chi square test is used to evaluate whether the prevalence (%) of liver histopathologic alterations or the prevalence (%) of externally visible diseases differs significantly among different experimental groups. In cases where the sample number is smaller than five, Fisher's Exact test may be used to analyze differences among prevalence. Data on histopathologic indices is tested using the non-parametric Kruskal-Wallis test followed by Mann-Whitney U test in order to determine statistical differences between different experimental groups. The significance level should be set at $\alpha = 0.05$.

Remarks

The protocol provided here to assess fish disease in common soles, in terms of externally visible diseases and liver histopathology is a useful tool to assess biological effects of contaminants in south-western Europe. Furthermore, the common sole seems to be a promising sentinel species to be used in the implementation of the MSFD in the Basque coast. However, there is a continuing need for the standardization of diagnostic criteria, as well as for establishing confounding factors and background levels, before implementing fish disease assessment in environmental monitorings.

Table 1. Summary of the histopathological traits (biomarkers) assessed in the livers of common soles of the Basque coast and their respective weight (Bernet *et al.,* 1999; Costa *et al*., 2009).

Reaction pattern	Histological alteration	Weight
Circulatory responses	Haemorrhage	1
	Hyperaemia	1
Inflammatory responses	Melanomacrophage centers	1
	Lymphocytic infiltration	2
Regressive changes	Hepatocellular and nuclear pleomorphism	2
	Necrosis	3
Progressive changes	Fat vacuolation	1
	Hydropic vacuolation of epithelial cells of bile duct	2
	Spongiosis hepatis	2
	Concentric periductal fibrosis	2

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ANNEX 1

Otoliths extraction for age determination

In order to determine the age of common soles, firstly gills should be removed in order to access to the brain cavity (Figure 21a). Secondly, a careful incision should be made in the brain cavity to dissect out both sagittal otoliths with the aid of forceps (Figure 21b). Then, otoliths should be washed with water to discard any adhering tissue and store for posterior examination.

In the case of common soles, otoliths reading does not require laboratory processing. The age is directly determined under a stereomicroscope to estimate the annual growth of otoliths (ICES, 2007) which supposes a great advantage for the use of common sole as sentinel organism (Figure 21c). For a better and easier age determination, a drop of glycerine should be added over the otoliths (personal communication). Two main bands should be distinguised in otoliths: (a) translucent bands which represent a period of fast growth and (b) opaque bands which represent a period of slower growth. For age determination, translucent bands should be counted as one year old (Figure 21d).

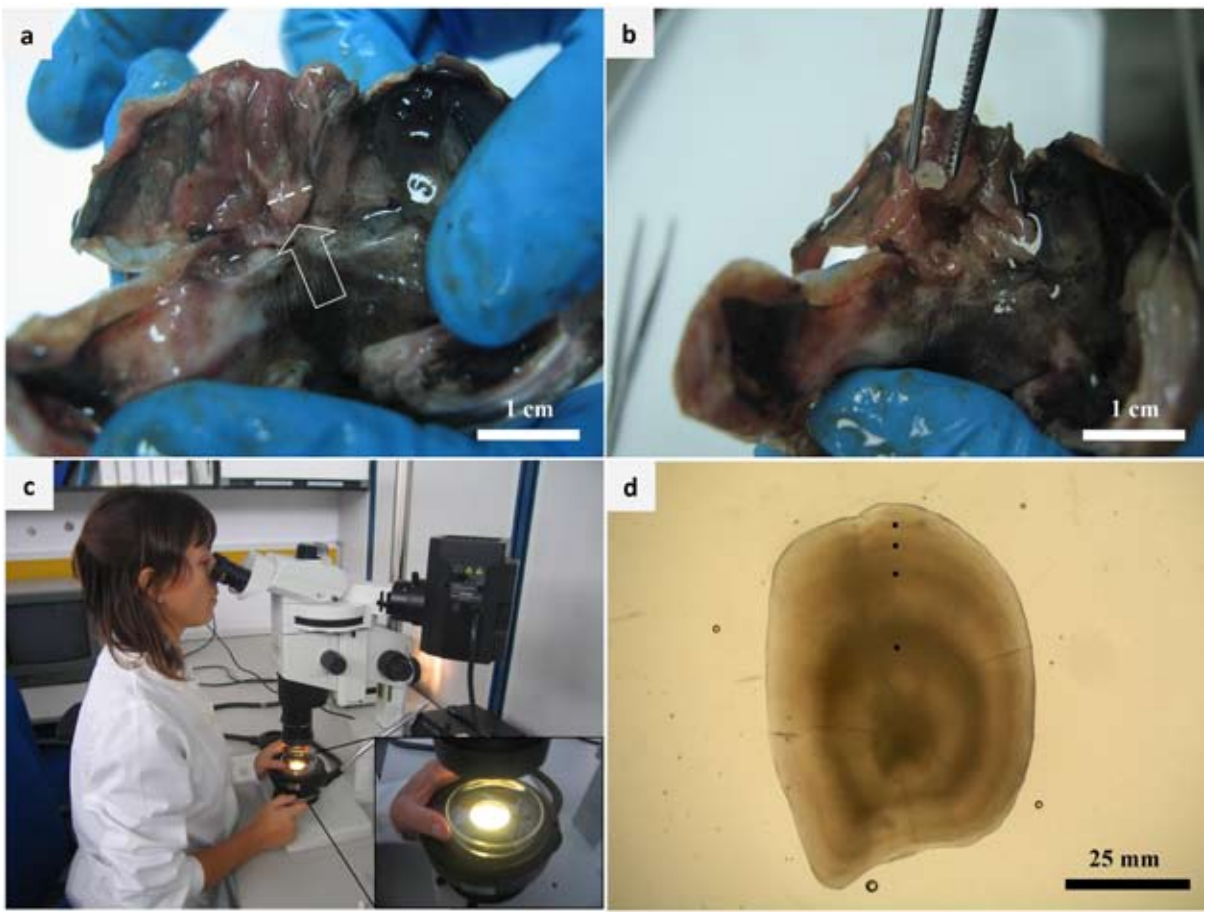

Figure 21. Otoliths extraction for age determination. (a) Brain cavity of a common sole where the incision (arrow and broken line) should be made; (b) Otoliths extraction from the brain cavity; (c) Age determination using a stereomicroscope; (d) Otoliths with four marked (spot) dark translucent bands, showing an individual of 4 years old.

ANNEX 2

Fixatives for use in fish liver histopathology

Requirements for 4% formalin (volume: 1000 ml):

Formaldehyde solution - 37% w/v	100 ml
Sodium di-hydrogen orthophosphate	9.4 g
Di-sodium hydrogen orthophosphate	4.7 g
Distilled water	900 ml

Preparation: Dissolve the buffer salts stirring in distilled water with gentle heat. Once the salts are dissolved add the formaldehyde solution (37%) and the remaining distilled water. Store in a bottle and label it.

Use: Dissect a piece of 5 mm thick of the pathological tissue. Place it into a cassette without squeezing and keep it in a jar containing the fixative. For a correct tissue fixation, the sample volume should not exceed $1/10^{th}$ of the volume of the fixative and occasional agitation is recommended. After 24 hours, samples should be washed with distilled water and stored in 70% alcohol until processing.

Requirements for Bouin's fixative (volume: 1000 ml):

Distilled water	750 ml
Formaldehyde solution - 37% w/v	200 ml
Glacial acetic acid	50 ml
Picric acid	add to saturation

Preparation: Mix formaldehyde solution (37%), glacial acetic acid and distilled water. Afterwards, add picric acid to saturation (this takes usually a few hours). Store at 4ºC.

Use: As formalin.

Requirements for Davidson's fixative (volume 1000 ml):

Glycerol	100 ml
Glacial acetic acid	100 ml
Formaldehyde solution -37% w/v	200 ml
96% Alcohol	300 ml
Sodium chloride	7.5 g
Distilled water	300 ml

Preparation: Mix formaldehyde solution (37%), glacial acetic acid, glycerol, 96% alcohol and distilled water. Sodium chloride is dissolved in distilled water by stirring and then is added to the other components.

Use: As formalin and Bouin's fixative but fixation time, in this case, is 48 hours.

ANNEX 3

Histological processing schedules by resin (methacrylate)

After fixation samples should be washed with distillated water in order to remove the excess of fixative and then they should be transferred to 70% alcohol for transportation and long-term storage. (Note that samples should be maintained in 70% alcohol at least during 48 hours before dehydration and subsequent embedding procedures).

1. Dehydration:

- **1.1.** Immerse in 90% alcohol during 16 hours
- **1.2.** Immerse in 96% alcohol during 8 hours
- **1.3.** Immerse in a mixture of 100% alcohol and activated resin* (1:1) during 48 hours at 4ºC (* activated resin is prepared mixing 1 g of activator per 100 ml of resin)
- **1.4.** Immerse in activated resin* during 72 hours at least (store at 4ºC)

2. Embedding

- 2.1. Mix 15 ml of activated resin with 1.5 ml of hardener.
- **2.2.** Fill the embedding moulds with a slight layer of activated resin + hardener
- **2.3.** Leave it air drying during 45 minutes
- **2.4.** Put in the tissue blocks
- **2.5.** Set the blockholders
- **2.6.** Polymerize for 24-48 hours at room temperature

3. Sectioning

- **3.1.** Cut sections (3-5 µm) with a microtome
- **3.2.** Float out sections in a water bath full of distilled water (≈50ºC)
- **3.3.** Pick up sections with pre-labelled glass slides
- **3.4.** Dry slides in a hot plate

ANNEX 4

Staining schedule for haematoxylin and eosin (H&E)

1. Place slides in Papanicolaou's solution Harris' haematoxylin for 12 minutes
2. Wash slides in running tap water for 5 minutes
3. Place slides in Eosin yellowish hydroalcoholic solution 1% DC for 45 seconds
4. Wash slides for 5 minutes in running tap water
5. Dry slides in a hot plate
6. Mount with a coverslip in a synthetic mounting medium and leave to dry.

ANNEX 5

Material required for common sole liver histopathology

Item	Code	Company
Dissection tweezers	1000580	SELECTA
Scalpel handle	1001361	SELECTA
Scalpel blade	1001364	SELECTA
Dissection scissor	1000807	SELECTA
Tricaine methanesulphonate	MS-222	Sigma-Aldrich
Acetic acid	AC03421000	Scharlau
Glycerol	GL00271000	Scharlau
Formaldehyde 35-40% w/v stabilized with methanol QP	211328.1214	Panreac
Na_2PO_4 x $12H_2O$	141679.1211	Panreac
NaH_2PO_4 x H_2O	141677.1211	Panreac
NaCl	131659.1210	Panreac
100% Alcohol	251086.1214	Panreac
96% Alcohol	121058.1212	Panreac
Xylene	XI00512500 2.51	Scharlau
Isoparaffin H (substitute of xylene) DC	255069.2711	Panreac
Nitrile gloves	Labglon-00S-1	Naturflex
Megacassettes	44125	Deltalab
Glass slides	5161120	Menzel glaser
Coverslips	9161060	Menzel glaser
Methacrylate resin Technovit 7100	14653	Technovit
Papanicolaou's solution Harris' hematoxylin	1.09253.0500	MERCK
Eosin yellowish hydroalcoholic solution 1% DC	251301.1611	Panreac
Mounting medium for substitutes of xylene	2.558.110.008	Panreac
Immersion oil DC	251002.1207	Panreac

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