

## Morphological techniques used in ichthyopathological diagnosis

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In this review, different diagnostic techniques for identifying etiology of fish disease are described. Among microscopic techniques, we find rapid diagnostic tests to observe parasites, gill diseases and bacteria; routine histopathological techniques are also used. In samples having bone tissue, decalcification methods are employed. In recent years, apart from using immunohistochemistry and electron microscopy, morphological techniques allow a deep study of disease pathogenesis. At present, molecular techniques for pathogen identification and atomic-absorption spectrophotometry to measure the concentration and distribution of toxic metals in tissues they also use. In conclusion, different techniques can contribute to the evaluation of disorders and provide tools for a better understanding in fish medicine.

**Keywords:** histopathology; fish; Polymerase Chain Reaction; atomic-absorption spectrophotometry

### 1. Introduction

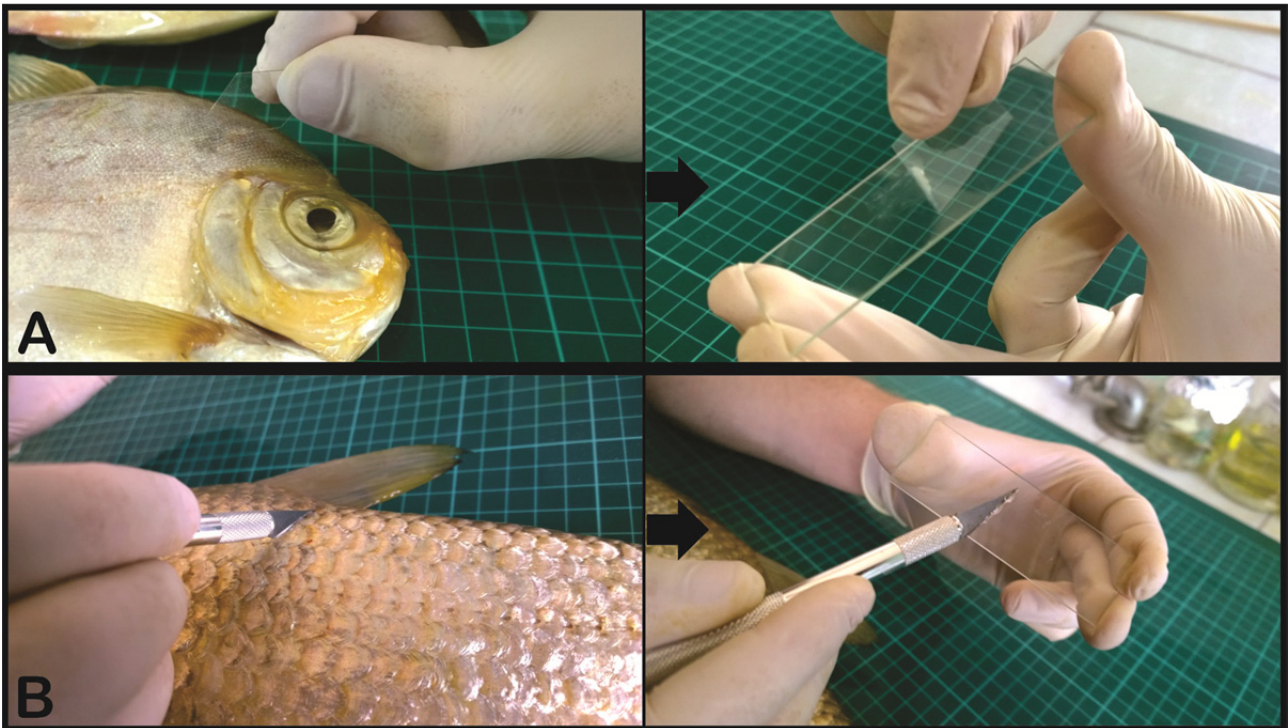
Fish farms cultivate and breed a large number of fish in an enclosed environment for consumption, ornamental purposes or recreational fishing. Although these fish move freely within these aquatic environments, they may be subjected to stress, due to intra- and interspecific competition or an unfavorable environment. This situation facilitates the appearance of traumatic, nutritional, genetic and infectious disorders, which generally result in macroscopic and microscopic lesions. Ethical considerations require the optimization of husbandry practices and advances in aquatic animal health [1].

Over recent decades, there has been an increase in the number of studies on fish medicine, a discipline that incorporates aspects of medical management, fish biology, pathological studies and disease diagnosis. Fish medicine focuses on clinical approaches, not only on the *post-mortem* appearance of diseases, but also on live fish, both as individuals and as populations [2]. Diagnosis of fish disease is established by means of data that combines specimen history, behavioral observation, macroscopic examination, skin and gill scraping, necropsy examination with squash preparations of internal organs and histopathology.

Below, we describe the main methods employed for ichthyopathological diagnosis, which include light and electron microscopy. We also mention some complementary studies currently used, such as molecular techniques for pathogen identification and atomic absorption spectrophotometry to measure the concentration and distribution of toxic metals in tissues.

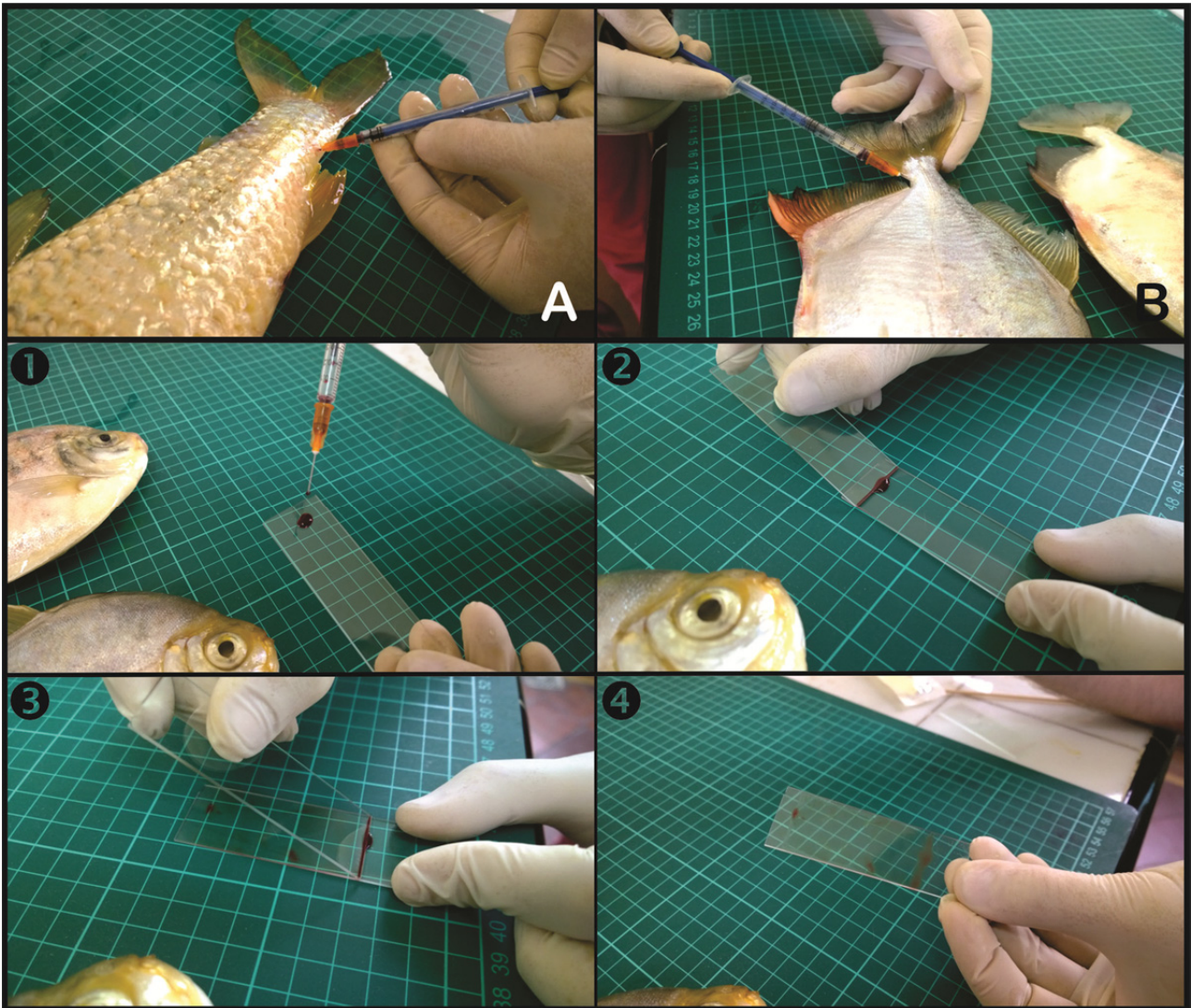
### 2. Tissue and fish samples

In order to make an ichthyopathological diagnosis, we may collect samples in two ways, *in vivo* or *post mortem*. The former includes samples of mucus or epithelial tissues that are scraped off with a small scalpel or a coverslip from different parts of the fish body and mounted on a glass slide (Fig. 1) [3]. Moreover, punctures may be performed to obtain blood or fluid samples; they are subsequently placed directly on a glass slide (Fig. 2). Using sterile cotton swabs (Fig. 3), samples of the affected areas may be collected for microbiological analysis [2].



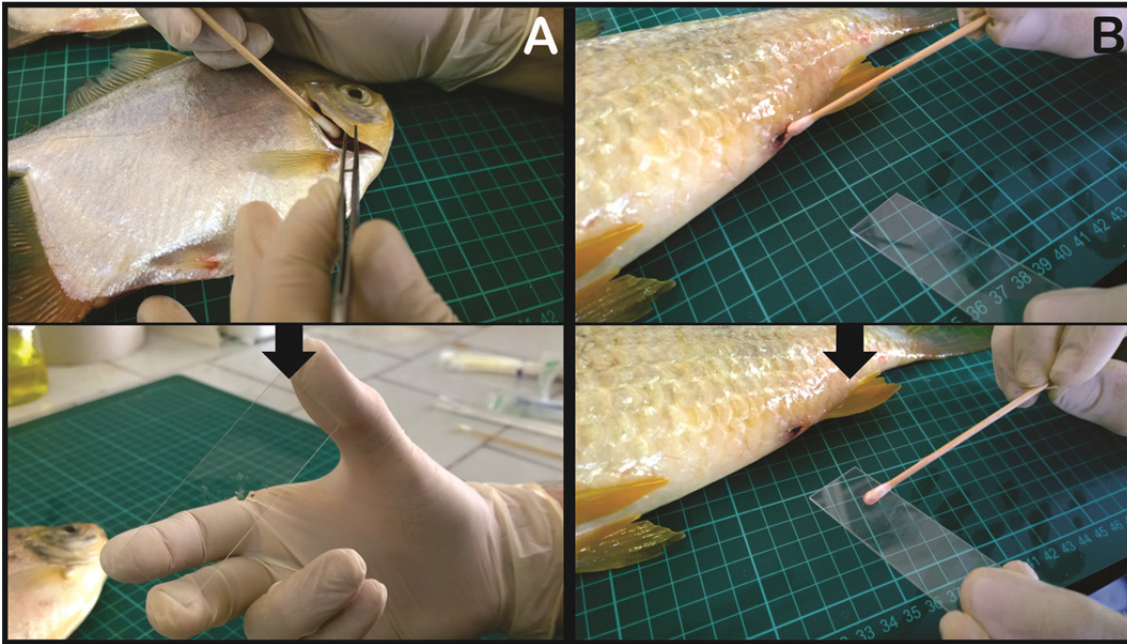
**Figure 1.** In the affected areas, small samples of mucus and epithelial tissues may be scraped off with a glass cover (A) or a small scalpel (B).

Diagnostic procedures most often require the sacrifice and sampling of individual fish. Fish exhibiting lesions (ulcers, erosions, hemorrhages) or abnormal behavior should be selected and euthanized by an overdose of anesthetic [2, 3]. In that case, the same types of samples previously mentioned may be taken; besides, squash preparations of gill filaments may be examined under the microscope. Fish necropsy is performed after completing the external examination, in which a gross view of the internal organs may help identify different pathologies. The target organs generally used for histopathology are the gills, kidney, liver and spleen; however, all organs may be sampled, depending on the presumptive diagnosis. If there is an injury, samples should contain the damage and some healthy tissue surrounding it (Fig. 4).

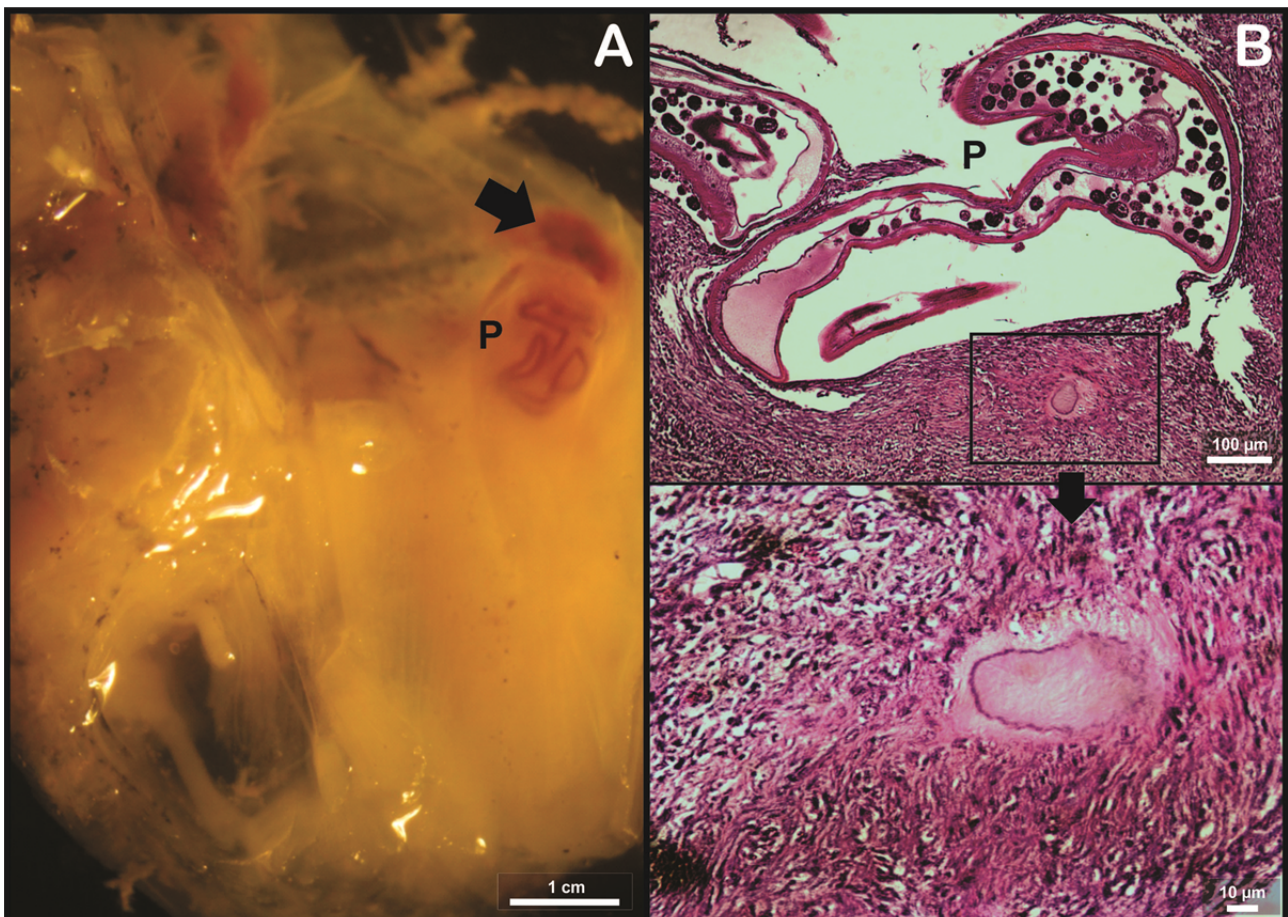


**Figure 2.** (A) and (B) Photographs show blood collection from a fish using the tail vein. (1)–(4) Steps to follow to obtain a blood smear. (1) On one end of a glass slide, place a small drop of blood; (2) place a second glass slide (spreader) at a 45° angle and wait until the blood spreads along to the edges of the glass; then, while holding the glass slide at the same angle, allow the drop to spread over its surface sideways in a thin line; (3) push the blood forward along the length of the lower slide; and (4) allow it to dry completely before staining.





**Figure 3.** In the affected areas, samples of gill mucus (A) and perianal mucus (B) may also be collected with a sterile cotton swab.



**Figure 4.** Organ infected with a nematode parasite. (A) Gross view showing a subcutaneous parasite (P) and an ulcer (arrow) caused by it. (B) Section of the organ musculature showing the nematode's body (P) and an ulcerative area (inset); around it, the tissue reaction of the host can be observed.

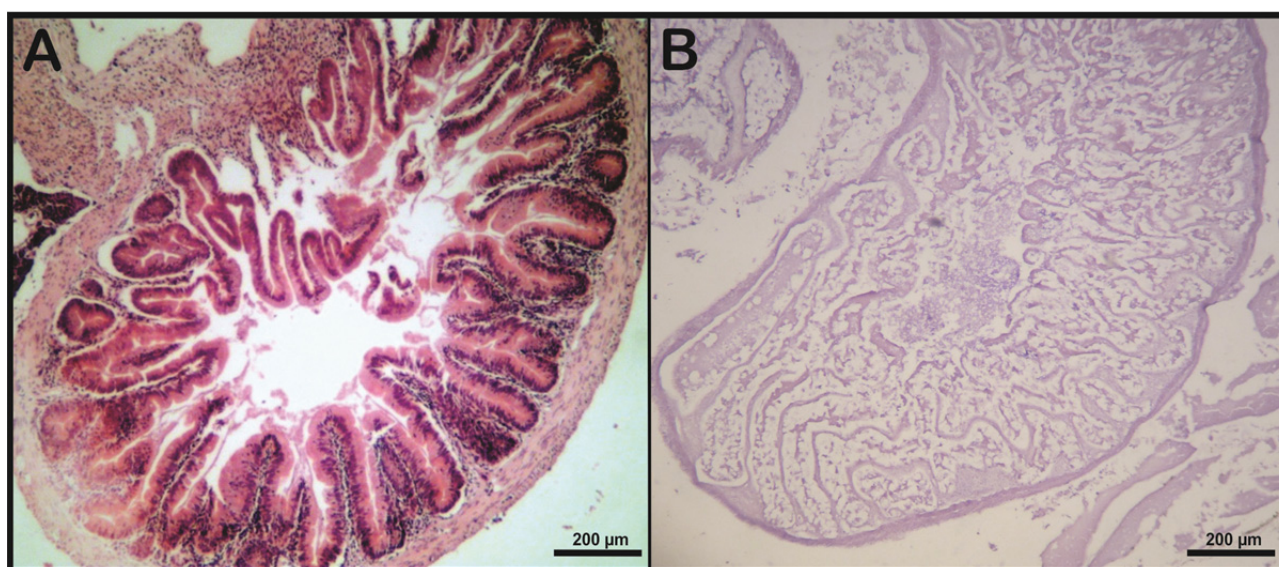


## 2.1 Fixation

Samples from the necropsy must be processed for subsequent cutting and coloring. The material should be treated with fixative solutions appropriate to each one of the study methods. This is the most important step in tissue preparation, because it stops the processes of autolysis and putrefaction. In the case of hollow organs, such as the digestive system, care should be taken to make fixative solutions enter them, as the mucosa of these organs is very sensitive to *post-mortem* changes (Fig. 5). There are two types of fixing processes:

Physical fixation methods: they may be accomplished by high temperature or cryopreservation; these procedures rapidly inhibit cellular activity [4]. In blood smears, after spreading the blood over the surface of the slide, samples are allowed to dry at room temperature or flame-dried, and then colored. To carry out atomic absorption spectrophotometry, small pieces of tissue are placed in cryoprotectant and frozen in liquid nitrogen [5].

Chemical fixation methods: they may be performed by perfusion or by immersion. In the perfusion method, the fixative solution is injected into the heart and the circulatory system, and used to circulate the perfusants uniformly through the brain and body; this method is not used in ichthyology. Instead, the immersion method is the most common approach used in biological specimen preservation [4]; samples are submerged in fixative solution to allow diffusion through the tissue. The size of the sample is important and should not exceed 1 cm<sup>3</sup>. Neutral-buffered formalin and seawater Davidson's fixative are formaldehyde-based fixatives commonly used in fish histopathology; generally, for electron microscopy, 3% glutaraldehyde in cacodylate buffer is used. When the possibility of immunohistochemistry studies is considered, it is desirable that the samples should not be kept more than 24–48 h in formalin.



**Figure 5.** Cross-section of catfish gut. In both microphotographs, a normal intestinal wall fixed by formaldehyde and stained with hematoxylin-eosin can be observed. (A) A good histological sample showing the different tissues. (B) A bad histological sample due to a poor fixation process, showing autolysis and putrefaction of the intestinal mucosa.

## 3. Light microscopy

### 3.1 Rapid diagnostic tests

These tests include scraping off, puncturing and squash preparation. These methods are suitable for the observation and identification of parasites [6], gill diseases and the presence of bacteria. In the case of parasites, these may be present in the mucus scrapings and quickly die after sampling [7]. Samples of mucus, epithelial tissues, squash preparations and punctures are mounted on a glass slide, and then may be colored or not with quick and simple techniques. By contrast, in blood samples, colorations such as May Grünwald/Giemsa or Wright's stain should be made.

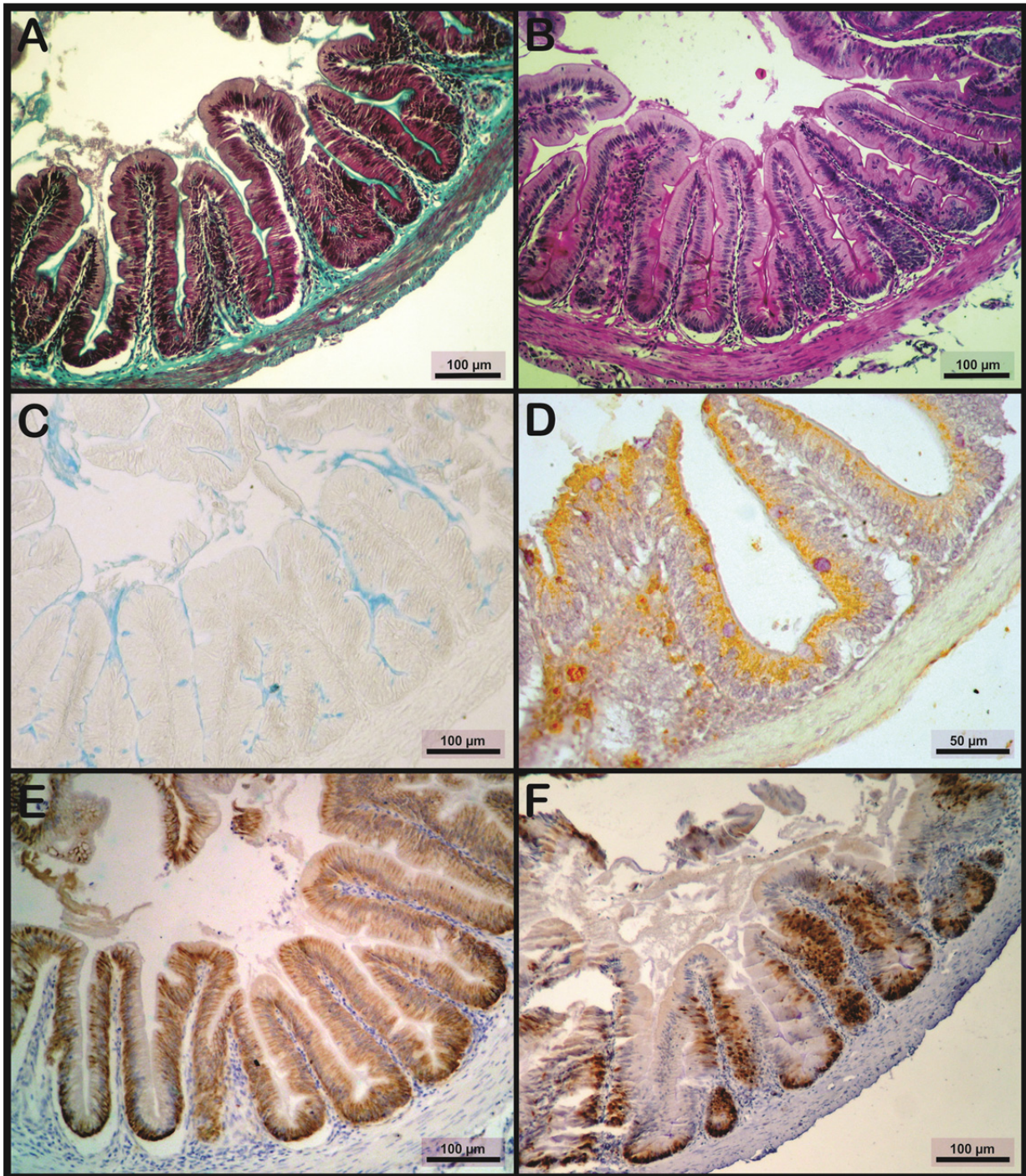
### 3.2 Routine techniques

Routine tests may be performed using culture media or histological methods. As mentioned above, with cotton swabs, samples of the affected areas are obtained and placed in a suitable culture medium for cultivation and subsequent diagnosis.

Histological techniques are used to visualize and identify microscopic structures enhanced by means of stains. Thus, the evaluation of histological sections of diseased tissue by light microscopy is considered an essential tool to arrive at a diagnosis. The main staining technique used in routine examination of histopathological damage caused by injuries is



coloration with hematoxylin and eosin (Fig. 5A); other stains, such as trichrome techniques (Fig. 6A), are also commonly used [8]. However, there are many other staining techniques that may be used to selectively stain cells and cellular components. The choice of technique will depend on the specific components that are being looked for in tissues.



**Figure 6.** Microphotographs of the same intestinal wall of a catfish obtained with different techniques can be observed. (A) Trichrome stain to highlight the collagen fibers; (B) PAS/hematoxylin for neutral glycoconjugate observation; (C) Alcian blue, allowing glycoconjugate and acid glycosaminoglycan identification; (D) lectin histochemistry to identify residues of sugars present in glycoconjugates; and immunohistochemical characterization of the intestinal wall with anti- $\text{Na}^+/\text{K}^+$ -ATPase-labeled enterocytes (E), and also with anti-PCNA (F), showing labeling in the enterocyte nuclei.

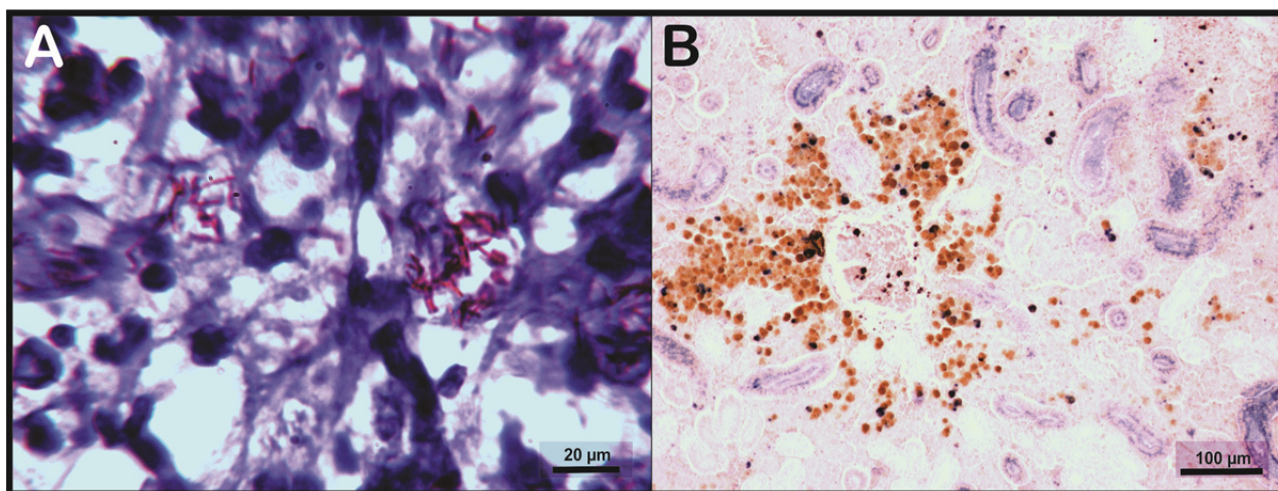
### 3.3 Histochemical staining

The importance of these techniques lies in their ability to locate, identify and differentiate variations in the cellular carbohydrate sequence with great sensitivity and specificity, as they can discriminate different cell populations [9]. A



wide variety of histochemical stains may provide important information about many physiological and pathological processes in tissues; each of these can help identify particular structures. In fish pathology, specific histochemical techniques, such as PAS/H (Fig. 6B), Alcian blue (Fig. 6C) and lectin histochemistry (Fig. 6D), are used. This last technique is based on the use of lectins, proteins that make it possible to recognize the saccharides that form part of glycoproteins and glycolipids [9]. The bonding between saccharides and lectins is detected by systems that rely on the activation of chromogens, such as diaminobenzidine [10], similar to the ones used in immunohistochemistry (see above). In fish, the use of this technique is common in research [11, 12], but references to its use as a diagnostic technique cannot be found. In mammals, some lectins are useful for the recognition of endothelial cells for clinical diagnosis and research [13, 14]; however, there are no similar studies in fish.

Nevertheless, many other staining techniques are used to selectively stain cells and cellular components. The choice of technique will depend on the specific components that are being sought in tissues, e.g., a special bacteriological stain, such as Ziehl-Neelsen (Fig. 7A) or Perls' staining to identify pigments (Fig. 7B).



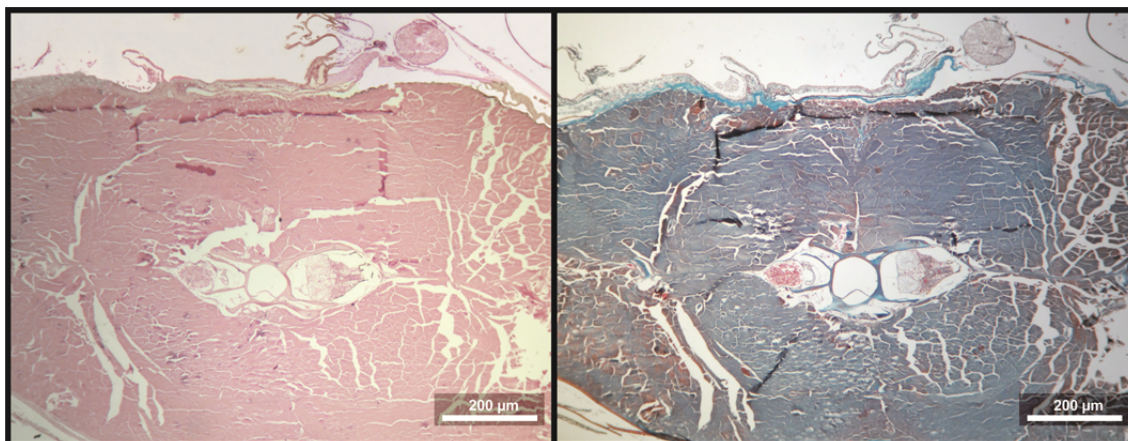
**Figure 7.** Special stain techniques, such as (A) Ziehl-Neelsen to identify bacteria (section of the intestinal wall), and (B) Perls' staining to identify pigments (section through the kidney).

### 3.4 Decalcification methods

Decalcification is a common technique used in processing ichthyological samples. Through this process, the inorganic constituents (calcium salts) are separated from the mineralized collagen matrix. It is used in whole animal samples if they are small in size (Fig. 8), or in samples containing bones, skin plates, fin rays or scales. Decalcification allows samples to be subjected to some of the above-mentioned techniques for light microscopy.

Descaling may be performed by the immersion of the samples in acidic solutions, solutions with chelating agents or a mixture of both. For the selection of the descaling agent, the degree of mineralization of the sample must be taken into account. Hydrochloric and nitric acids are strong acids commonly used in 5–10% concentrations in aqueous solutions; these acids have the disadvantage of requiring extensive monitoring during the descaling and may affect subsequent staining. The advantage is that they allow a quick decalcification in a few hours, even in large specimens like Characiformes or Perciformes, or in highly mineralized skin plates, such as the ones that can be observed in Siluriformes. Formic, acetic and picric acids are weak acids commonly used in the laboratory. The first one is the only acid used in pure form for descaling. Acetic and picric acids are components of Carnoy's, Bouin's and Zenker's solutions [15], usually used in small, fragile or poorly mineralized samples with a dual purpose, for fixing and descaling.

Chelating agents are organic chemical compounds that have the ability to bind on metal ions, such as  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ . These include ethylenediaminetetraacetic acid (EDTA), which is the agent usually used in laboratories because it does not affect soft tissues or the staining. The only disadvantage is that the descaling process can take a few weeks, depending on the size or hardness of the sample.



**Figure 8.** Cross-section of a small fish; a decalcification process was carried out to subsequently stain it with hematoxylin-eosin (left) or trichrome technique (right).

### 3.5 Immunohistochemistry

Immunohistochemistry (IHC) techniques are a group of procedures that use the antigen-antibody reaction to recognize a molecule in a tissue. The observation may be undertaken with a light microscope or with a fluorescence microscope.

IHC has some advantages; for example, it allows the examination of fish morphology and it can be done in a histology laboratory with relatively simple equipment. However, it has some major complications, since many antigens are lost with fixing; so, short fixation times are required and, in many cases, the use of techniques to recover these antigens by procedures either chemical (use of enzymes) or physical (high temperature, ultrasound, etc.) [16, 17]. Also, when working with fish tissues, it creates new complications. Many of the specific epitopes have a high variation between taxa. Even those produced for use in a mammal species (e.g., human, mouse) cannot be used for other mammals; it is even more unlikely for them to work in fish. It may even be the case that while one epitope may work in a species of fish, it may not be used in another. On the other hand, some antigens are highly conserved phylogenetically: for example, the proliferation cell nuclear antigen (PCNA) may be used in a great diversity of organisms, including mammals, birds, fish and invertebrates. This is a very useful marker in fish cell proliferation (Fig. 6E). In other cases, specific antibodies have been generated for fish; for example, certain cell types involved in ion transport can be recognized by using specific antibodies (e.g., anti-Na-K-ATPase for teleosts) (Fig. 6F) [18].

The applications of IHC in fish disease diagnosis—as it occurs with other animal taxa—are manifold, as it identifies the recognition site of disease-producing agents. In the case of some diseases of fish of economic importance, Evensen and Rimstad [19] developed a protocol to recognize pancreatic necrosis virus infections in paraffin-embedded tissues of Atlantic salmon. Previously, by immunofluorescence technique, the presence of the virus was recognized, but without observing the injuries. IHC allowed them to identify the presence of the virus in damaged cells of the exocrine pancreas. Also, specific monoclonal antibodies were developed to detect important bacteria in salmon farming, such as *Renibacterium salmoninarum* (agent of kidney disease) and mycobacteria [20]. In aquarium fish, IHC has also been used to detect the presence of infectious agents. An interesting example is the work of Zerihun et al. [21], who determined the presence of mycobacteria in granulomas of different species of teleosts, including aquarium fishes. In recent years, IHC has been used for tumor diagnosis in fish from aquariums and farms, e.g., multicentric myxoma in European eels [22], olfactory neuroblastoma and schwannoma in goldfish [23, 24], and dysgerminoma in an orange-spot freshwater stingray [25]. In addition, different antibodies are being used for the diagnosis and prognosis of hyperplastic and tumor diseases in chondrichthyes [26] and catfish [27]. Finally, IHC has also been important for the study of other diseases, such as cardiomyopathies in salmonids [28] or liver disease in rainbow trout [29].

## 4. Electron microscopy

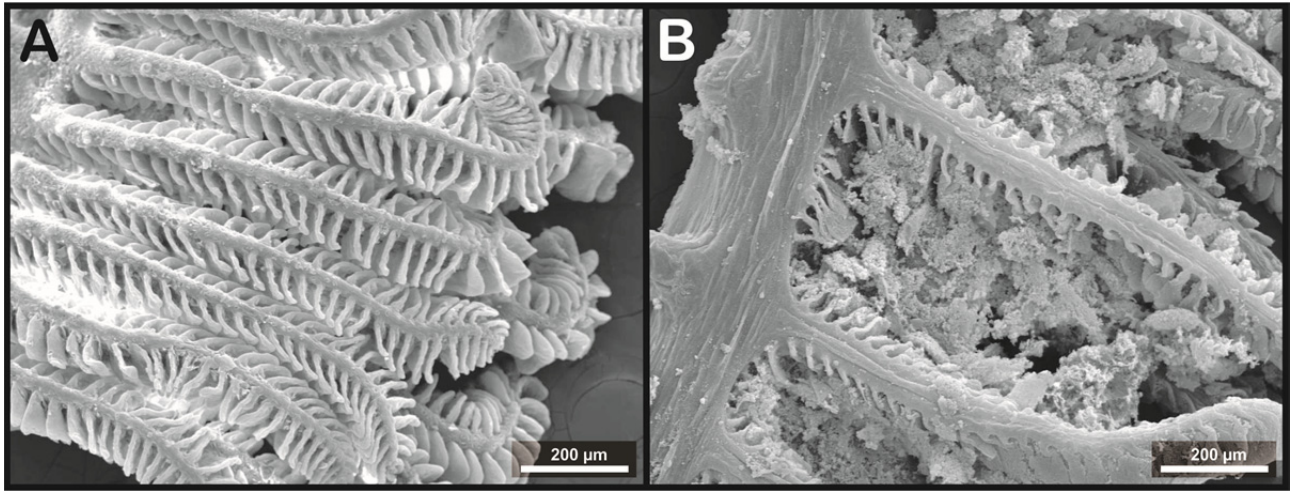
Electron microscopy (EM) is a valuable tool in basic research and teaching, and it remains the only technique that allows us to visualize cellular and tissue ultrastructure [18, 30]. At present, EM has an important role in some types of pathology, e.g., in the diagnosis of symptoms of cell intoxication [31]; both scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are useful in such evaluations [32]. Qualitative and quantitative changes in cellular organelles, fibrils, microvilli and intercellular junctions reflect hyperplasia, metaplasia or dysplasia of the cell, and may enable the identification of diseases [33].

However, despite the fact that it is a great tool for the identification and characterization of viruses, bacteria, protozoa and fungi, EM cannot replace the information obtained with conventional light microscopy [34]. Therefore, its use in diagnosis is limited, either for strategic reasons or budget constraints. Its main value, though, is as an ancillary tool to

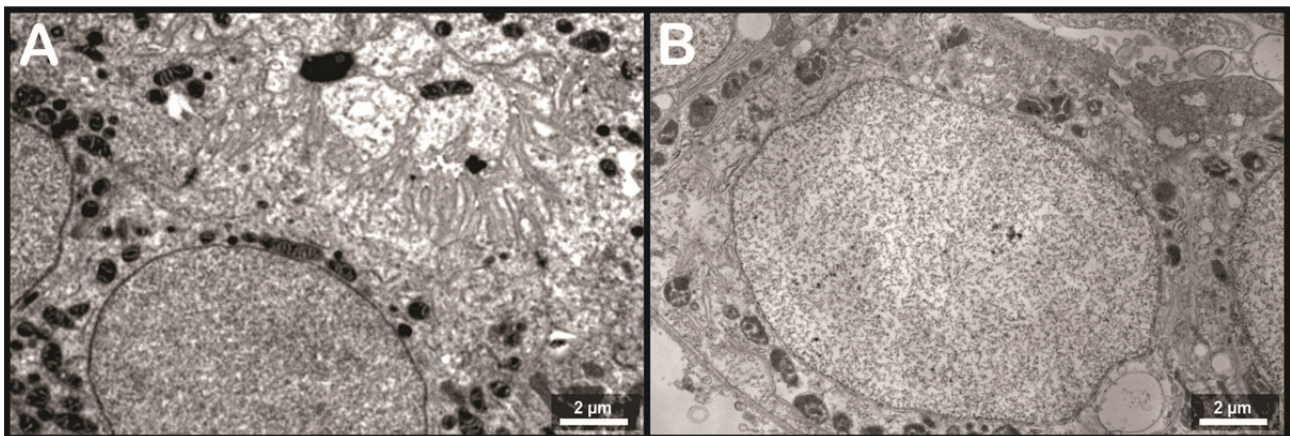


complement, support, or confirm data obtained with light microscopy, cytogenetics and molecular biology, among others, and make a correct diagnosis.

SEM allows for the observation of alterations on the cell surface (Fig. 9) and TEM (Fig. 10) shows alterations in the number of organelles and loss of nuclear integrity. These findings in EM add to the understanding of the light microscopic images that are the mainstay in histopathology.



**Figure 9.** Scanning electron photomicrographs of catfish gill. (A) Normal organ in which rows of primary filaments showing secondary lamellae on each side can be observed. (B) Abnormal organ, with a marked absence of primary filaments.



**Figure 10.** Transmission electron photomicrographs of catfish kidney. (A) Cell of the first portion of the proximal tubule with the apical brush border and numerous mitochondria distributed all over the cytoplasm. (B) Cell of the first portion of the proximal tubule in which the apical brush border cannot be observed and the cytoplasm has scarce mitochondria.

## 5. Molecular techniques

The recent progress reached in molecular biology has given an impulse to the adaptation and development of new technologies applied to disease diagnosis in aquaculture. These techniques offer unquestionable advantages over other conventional methods. First, the speed, high sensitivity and specificity of the process, offering the possibility of identifying microorganisms of slow, difficult growth or that cannot grow *in vitro*, even detecting small amounts in samples or tissues of various origins. However, despite the fact that diagnostic methods based on molecular biology have been increasingly incorporated into all areas of pathology, in most cases they are used mainly as complementary tools to evaluate the risk of acute diseases or in their early diagnoses, and these cannot replace the conventional tests required in a final report [35].

Polymerase chain reaction (PCR) is an effective procedure for generating large quantities of a specific DNA sequence *in vitro*, being the most widely used in diagnosis. PCR reactions make use of the physicochemical properties of DNA in response to temperature changes.

Several kinds of PCR have been developed. A variant of PCR used to quantify mRNA is the reverse transcription polymerase chain reaction (RT PCR); in this method, an RNA template is, using a reverse transcriptase, converted into

a complementary DNA (cDNA) and later amplified. It is frequently used in expression pattern analysis for the identification of infections and diseases.

Another variant of PCR is random amplified polymorphic DNA (RAPD), which is based on the amplification of target genomic DNA with single arbitrary chosen primers; these can pair to a template with only two or more complementary sites. This technique does not require previous knowledge of the target sequence because DNA polymorphisms are detected based on the observation of the different amplified band patterns after electrophoresis. An alternative tool for genetic mapping is restriction fragment length polymorphism (RFLP) analysis. It is applied to specific nucleotide sequences in DNA that are recognized and cut by restriction enzymes whose band patterns are also visualized and analyzed by gel electrophoresis. These methods are valuable in epidemiological studies to identify individual strains and for the differentiation of closely related strains. Finally, other molecular tools used for diagnosis based on amplification of DNA are nucleic acid sequence-based amplification (NASBA) and loop-mediated isothermal amplification (LAMP) [36].

## 6. Atomic-absorption spectrophotometry

Heavy metals are found in low concentrations in the environment, some of them are even essential for organisms. However, due to natural or anthropogenic causes, the concentrations of these elements may increase, becoming toxic metals because of their negative effects [5, 37].

In aquatic environments, toxic metals are combined with organic compounds present in the sediments or in water ions, and enter the successive links in the food chain, where processes of bioconcentration, bioaccumulation and biomagnification occur [38]. Therefore, the periodic analysis of the pollution levels in tissues of vertebrates and invertebrates may identify risk areas [5, 39, 40].

In recent years, the atomic absorption spectrophotometry technique has been incorporated into the study of ichthyopathology samples. The aim of this technique is to determine the concentrations of toxic metals in target organs and tissues, such as liver, kidney and muscle, allowing the quantitative analysis. According to the methodology proposed by Woody and Van Vleet [41], the samples are fractionated into sub-samples, and processed using a spray flame, where ions and atoms are excited by heat, producing molecular, atomic and ionic emission spectra. For the detection of each element, a filter and a different source of radiation can be used; however, most equipment uses ultraviolet/visible monochromators that allow the detection of many elements [42].

The main advantages of using this method are its high sensitivity, speed of analysis and ease of operation. On the other hand, it has the disadvantages of using a destructive method of analysis, the need for benchmarks and that, as it is very specialized equipment, its conservation will depend on the quality of the surrounding environment and power supply.

## 7. Summary

In this paper, the main sample preparation techniques used in ichthyopathological diagnosis are described. As mentioned above, the choice of technique primarily depends upon the specific components that we are looking for in tissues and organs.

The importance of fish medicine, which is useful for both production and pet fish operations, determines that most of the modern techniques used in veterinary sciences may be applied to fish medicine diagnosis in order to identify causes of a disease. These techniques are very different and they include rapid diagnostic tests, routine and specific histomorphological techniques, molecular analysis and atomic absorption spectrophotometry. All of these can provide qualitative and quantitative information on disease properties, including the morphology, surface texture, pathogen identification, concentration and distribution of toxic metals.

**Acknowledgements** We would like to thank Rubén Mario for this technical work, Martín Montes for his contribution, and Magdalena Ponce for reading the MS. The support by National University of La Plata is gratefully acknowledged.

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