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Fish in focus: Navigating organ damage assessment through analytical avenues – A comprehensive review

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ABSTRACT

Aquatic ecosystems, critical for biodiversity and food production, confront escalating threats from anthropogenic activities like pollution and climate change, impacting fish health. This review outlines various assays used to study organ damage in fish, ranging from traditional histopathology to advanced molecular and biochemical methods. The aim is to guide researchers in selecting suitable assays for their specific questions, considering the advantages and limitations of each technique. Covered methods include histopathological assessment, biomarker analysis, genotoxicity assays, oxidative stress indicators, and non-invasive imaging. The review explores their application in monitoring environmental stressors' impacts on fish organs, emphasizing emerging trends like omics technologies and non-destructive imaging for comprehensive assessments. These innovations hold promise for early detection and understanding the implications on fish populations and ecosystem health.

1. Introduction

Aquatic ecosystems are of paramount importance, serving as critical reservoirs of biodiversity, sources of sustenance through food production, and centers for recreational activities. However, these invaluable ecosystems are under increasing threat from a myriad of anthropogenic activities, including pollution, the inexorable march of climate change, and habitat degradation. Among the inhabitants of aquatic environments, fish stand as pivotal components, yet they are particularly susceptible to the deleterious effects of these environmental stressors, which can manifest as organ damage and a decline in overall health.

Given the intricate relationship between fish and their habitat, these creatures have become indispensable subjects for scientific study, serving as model organisms in the exploration of how environmental contaminants, diseases, and other stressors impact aquatic ecosystems. A critical facet of such research is the evaluation of organ damage in fish, offering insights into the health and resilience of these populations and their associated ecosystems.

This review endeavors to provide a comprehensive examination of the diverse assays and techniques routinely employed in the assessment of organ damage in fish. By delineating the methods and their applications, our aim is to furnish researchers with a comprehensive guide to aid them in choosing the most suitable assays for their specific research inquiries. This knowledge is vital not only for monitoring the impacts of environmental stressors on fish but also for implementing measures to mitigate these effects.

The array of methods explored in this review ranges from conventional histopathological assessments to cutting-edge molecular and biochemical assays, each with its distinct advantages and limitations. We delve into the intricacies of histopathology, biomarker analysis, oxidative stress indicators, and the application of non-invasive imaging techniques. Through these approaches, we aim to unveil the complex landscape of organ damage in fish, including the effects of stressors on organs like the liver, gills, kidneys, and reproductive systems.

In addition, this review highlights new developments in the field, such as the incorporation of omics technologies for a comprehensive knowledge of organ damage, including transcriptomics, proteomics, genomes, and metabolomics. Additionally, the utilization of non-destructive imaging techniques, like ultrasound and magnetic resonance imaging (MRI) in live fish, offers novel and promising avenues for the early detection of organ damage and its implications on fish populations and the health of aquatic ecosystems Figs 1–6.

To sum up, this review offers a thorough and insightful summary of the techniques and uses for evaluating organ damage in fish, offering vital insights into the health and adaptability of aquatic ecosystems. A combination of these assays can provide a holistic understanding of

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organ damage in fish. Researchers should carefully select and integrate these methods to address their research objectives effectively. Advances in technology and interdisciplinary approaches continue to enhance our ability to study and mitigate organ damage in fish, contributing to the conservation and management of aquatic ecosystems.

1.1. Importance of assay

Fish can suffer from various injuries and deformities that can adversely affect their health and production performance. Injury is characterized as physical harm, whereas deformity results from either acquired irregularities in an organ or congenital, potentially resulting in organ damage and impaired function. Both injuries and malformations can manifest in wild and farmed fish at any point in their life cycle. Common injuries are often noted in the eyes, mouth, fins, and skin, given that these areas contain a multitude of external structural attributes prone to damage during aquaculture and animal care practices [2]. The disease in fish can originate either from internal factors within the fish's body or external sources. Internally, diseases in fish can result from genetic factors, internal secretions, weakened immune systems, as well as neurological or metabolic disorders. On the other hand, externally induced diseases and damage to the fish's organs can be attributed to pathogen exposure due to environmental factors, improper management of fish feed, and the use of antibiotics [3].

Microplastics and pesticides are newly recognized pollutants in marine life, and they result in numerous adverse impacts on aquatic organisms, with fish being particularly affected. In agricultural settings, agrochemicals are employed for pest management, and a significant portion, approximately 90 %, remains in the environment without breaking down [4]. Reactive oxygen species (ROS) are produced when fish are exposed to different types of nanoparticles, pesticides, and microplastics. These ROS can cause inflammatory responses, oxidative stress, genotoxicity, immunotoxicity, and DNA damage. It also causes changes in the gut microbiota's makeup, which eventually lowers fish quality and growth. Furthermore, changes in fish behavior, such as feeding patterns and locomotion (Swimming), have been witnessed when exposed to these contaminants. Furthermore, these toxins affect a range of signaling pathways, including JNK, Nrf-2, NF-κB, MAPK and ERK. The Nrf2-KEAP1 signaling system is involved in overseeing

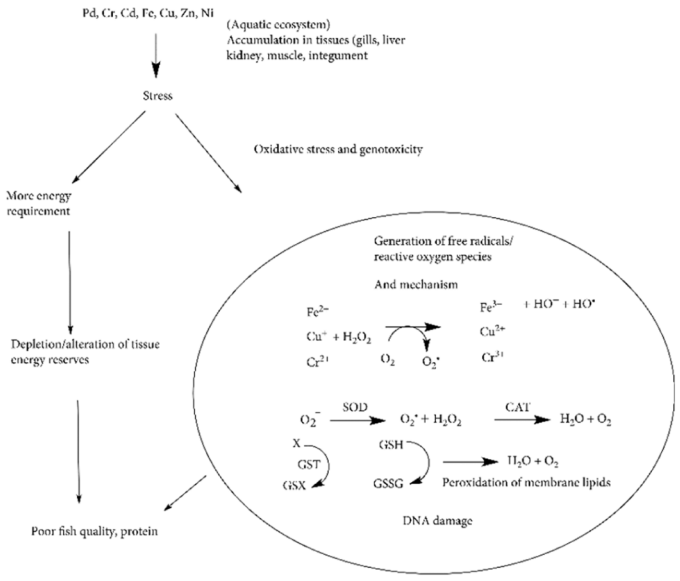

Fig. 2. A schematic representation illustrating the presence and movement of heavy metals within an aquatic system [8].

antioxidant enzymes in fish and upholding their redox equilibrium. Additionally, these pollutants have been observed to modify various antioxidant enzymes like catalase, superoxide dismutase and the glutathione system [5,6].

Pollution in the environment stands as a significant factor leading to organ damage in fish. It may arise from heavy metal pollution due to the harmful characteristics of heavy metals, including their toxicity, persistence, tendency to accumulate in organisms, and increase in concentration up the food chain. Fish primarily absorb heavy metals through their gills, body surfaces, and digestive tracts when they consume food containing accumulated metal. Arsenic, chromium, cadmium, copper, nickel, mercury, zinc, and lead are the prevalent heavy metal contaminants that can lead to intense toxicity in fish [7]. The primary molecular process underlying metal toxicity is the induction of

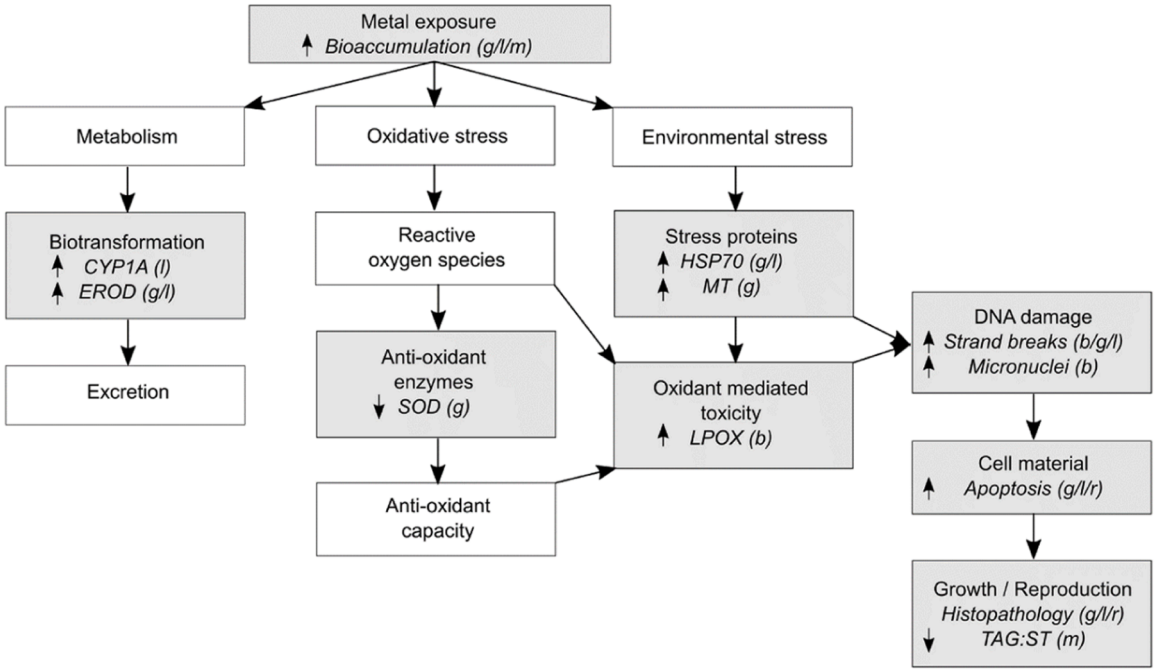

Fig. 1. Flowchart illustrating changes in chemical, biochemical, physiological, and other responses triggered by exposure to metals [1].

oxidative stress. This stress undermines the immune system, leads to harm in tissues and organs, stunts growth, and diminishes reproductive capabilities [8]. Heavy metals induce non-lethal impairment to the kidneys, liver, respiratory, neural and gonadal system in aquatic organisms. The buildup of these metals in different fish organs can result in structural abnormalities and disrupt their normal functioning[9].

2. Histological studies

Histology serves as a valuable tool for comprehending and averting diseases, enhancing production outcomes, and using histological changes as potential markers of environmental pollution. The method essentially involves acquiring extremely thin sections of animal organs, allowing for the detection of cellular and tissue abnormalities once they are stained. The degree of these anomalies, which are frequently seen in organs like the intestines, liver, spleen, heart, and gills, could be directly related to the conditions under which the animals were kept in captivity or to their exposure to the outside world [10].

This involves the microscopic examination of afflicted tissue, representing a crucial investigative method within the medical domain. This practice is rooted in the scrutiny of histology, which pertains to the microscopic anatomy of humans or animals. The procedure entails the inspection of slender tissue slices under light microscopes. Histotechnique encompasses a series of methods that facilitate the visualization of microscopic characteristics of tissues and cells, aiding in the identification of distinct structural alterations associated with various diseases [11].

Histological examination appears to be a highly sensitive method, playing a critical role in detecting cellular alterations that can occur in vital organs like the gills, muscles, liver, and kidneys. Consequently, histological examination has the potential to serve as a cost-effective method for evaluating the health of organisms, offering insights into the overall well-being of an aquatic ecosystem. The histological examination of the liver of fish can serve as a useful model when examining the impact of stressors, such as contaminants, infectious agents, parasites, biological contaminants, and physical-chemical variables. Exposure to these stressors can induce pathological alterations in fish, including kidney tubular damage, abnormalities in gill lamellae, and liver necrosis. Consequently, it is crucial to conduct histopathological studies to characterize and evaluate potential abnormalities in aquatic animals exposed to diverse infestation and noxious materials in aquaculture environments [12].

2.1. Tissue preparation

Tissue samples must undergo a number of preparation procedures, such as fixation, processing, embedding, sectioning, and occasionally antigen retrieval, before specialized staining is applied. These days, most of these processes are automated in histology labs [13].

2.2. Fixation

Tissue fixation serves several important functions, including chemical stability, increased tissue hardness for sectioning, as well as ultimately, stopping autolysis and degradation. Chemical fixatives preserve tissues by inducing protein denaturation through mechanisms like cross-linking (e.g., formaldehyde), coagulation (e.g., acetone, methyl Carnoy's), or a combination of these processes (as observed in mercuric

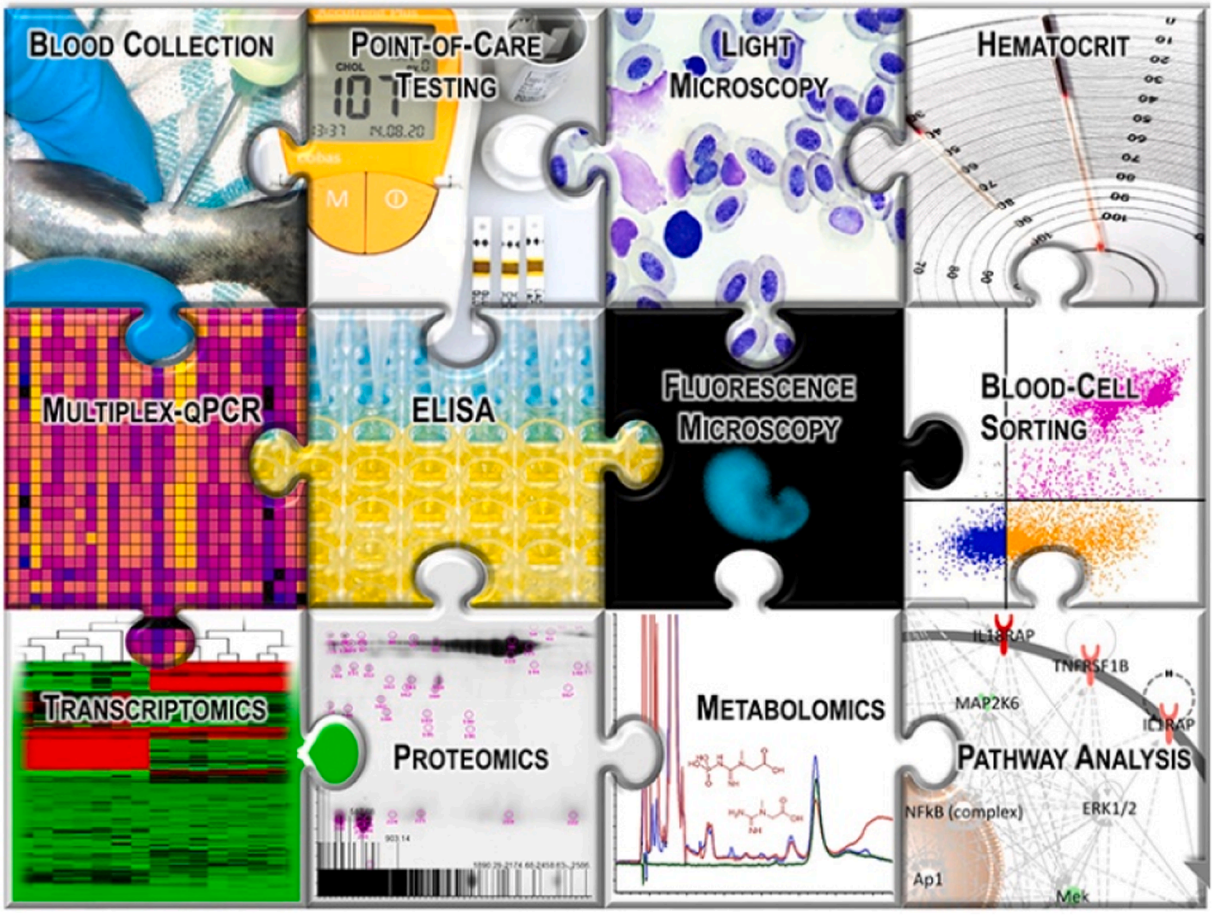

Fig. 3. Visual Summary of Chosen Analytical Methods Utilized with Fish Blood [22].

formalin). Alterations in the molecular structure typically establish a subtle equilibrium between maintaining tissue integrity and preserving its structure. Furthermore, fixation can affect the penetration of tissues and antigen being exposed, which can be beneficial or detrimental. Although various fixatives cater to specific applications, the routinely used fixatives in histology commonly involve solutions based on formaldehyde, such as 4 % paraformaldehyde and neutral buffered formalin (NBF) [14]. Bouin's fixative is particularly useful for sensitive and fragile tissues, like the brain and embryos, along with small tissue fragments. It preserves glycogen and nuclei well, though it penetrates tissues more slowly and may distort kidney and mitochondrial tissue. Mercuric formalin fixation is a popular method for achieving enhanced nuclear clarity in tissues such as lymph nodes and bone marrow because it yields remarkable nuclear staining [15].

To ensure effective fixation, it is crucial to initiate the process promptly upon the removal of selected organs. Tissues or organs should be sliced into 3–5 mm thick sections for rapid immersion in the chosen fixatives. Fill the vials to about 2/3 of their capacity with the chosen fixative. It's important to maintain a specific ratio of 1 part of organs to 20 parts of fixative volume. Subsequently, place the organs into separate vials filled with the appropriate fixative solution. These vials should be stored to allow for the necessary fixation time, which can vary depending on the type of fixative employed: approximately 10–12 hours for formalin, 7–10 hours for alcohol formalin, and 4–6 hours for Bouin's fixative. This meticulous process ensures the proper preservation and preparation of tissue specimens for further analysis and research [16].

2.3. Paraffin infiltration

In the preparation of tissue samples for histological processing, paraffin wax is typically melted in a hot air oven, with the temperature set in the range of 50–60 degrees Celsius, depending on the specific melting point of the wax. It is of utmost importance to exercise caution and avoid surpassing this temperature by more than 5 degrees Celsius above the wax's melting point, as exceeding this limit can lead to tissue hardening, shrinkage, and potential crystallization of the wax, rendering it unusable. Afterward, the tissue is submerged in the molten wax, with the volume of wax being approximately 25–30 times greater than that of the tissue. During the impregnation process, it is crucial to subject the tissue to 2–3 changes in the molten wax to ensure the complete elimination of any residual clearing agent. This detailed procedure is essential for the successful embedding of tissue samples in paraffin, facilitating subsequent histological analysis [17].

2.4. Dehydration and embedding

The introduction of ethanol serves to dehydrate the sample, eliminating water content and additionally enhancing tissue firmness for eventual examination under light microscopy. Once ethanol has been utilized for tissue dehydration, the subsequent step involves the use of xylene to eliminate the ethanol.

3. Microscopic analysis

The typical method for detecting structural alterations in organs is through the microscopic evaluation of tissue sections, a practice known as histopathology. This procedure encompasses the inspection of slender tissue slices under a microscope to detect any modifications in cells, tissues, or organs. The technique comprises various stages, including the collection of tissue samples, their preparation, slicing, staining, and scrutiny [11].

Examining tissue sections through microscopy is a widespread approach for detecting structural alterations in the organs of fish. This method entails the creation of slender tissue slices, subsequent staining,

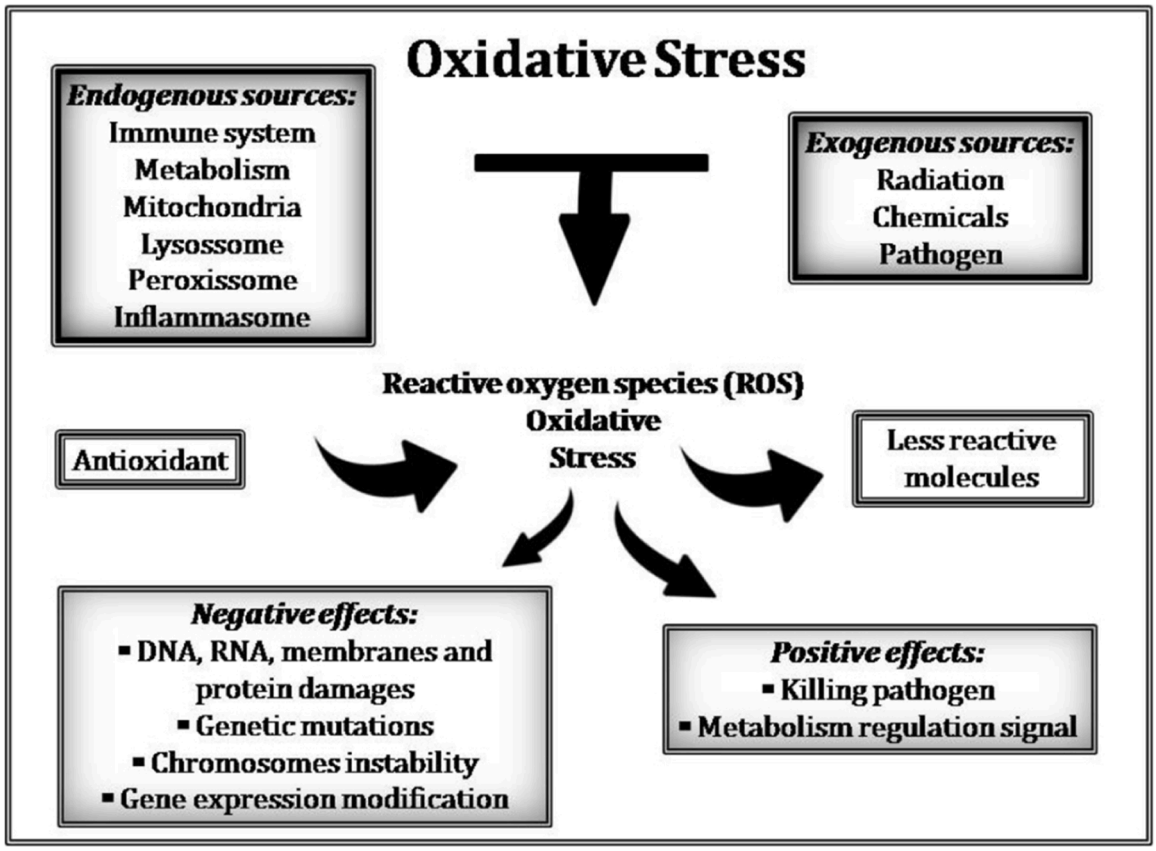

Fig. 4. The impacts of oxidative stress [32].

and observation using a microscope. Staining is crucial for accentuating distinct tissue structures, aiding in the detection of any potential modifications [13]. Numerous varieties of dyes are available for this particular application, one of the most frequently employed dyes is Hematoxylin, which imparts a blue hue to proteins, and Eosin, which imparts a pink hue to proteins. These two dyes are commonly utilized in combination to delineate intracellular organelles and proteins. Some other dyes are Masson's trichrome stain, and periodic acid-Schiff (PAS). Given the diverse array of proteins in existence, certain dyes have been developed to emphasize particular proteins. The advantage of using a specialized dye is its exceptional ability to highlight a specific protein. However, due to its selectivity, it may not reveal other structures. Consequently, it is common to generate multiple slides from a given sample, enabling the application of various dyes to obtain a comprehensive range of necessary information [14].

3.1. Gray's method

Processing of tissue, formation of block and staining procedures adhered to the methodology outlined by Gray. Tissue alterations were examined using histological analysis. To fix tissue samples, they were first gathered and submerged in a 10 percent formalin solution. Following fixation, the tissue was rinsed multiple times in water that was distilled to remove any remaining fixative. Next, it was subjected to a series of alcohol baths at progressively higher concentrations to achieve dehydration. Subsequently, the dehydrated samples were cleared using xylene and then embedded in molten wax, ultimately forming wax blocks. These resulting wax blocks were prepared in pre-glycerine-coated cavity blocks using warm water. The tissue samples were cut into small sections, approximately 5 microns thick, using a rotating microtome, namely the Leica RM 2125 RTS. After that, these sections were attached to spotless glass slides that had already been covered with Mayor's albumin. They were then allowed to air dry for 12–16 hours at room temperature. Eventually, the sections underwent deparaffinization and Harris hematoxylin-eosin (HE) staining in order to facilitate histological analyses. The data were subjected to statistical analysis using the analysis of variance, also known as ANOVA, and the Duncan Multiple Range Test (DMRT) [4].

4. Biochemical assays

Biochemical assays are a common method for gauging the level of organ damage in fish, especially when assessing harm to the liver and muscle due to chemical contaminants or illnesses. In fish, a diverse array of serum proteins found in complex combinations plays a pivotal role in a broad spectrum of physiological functions, relevant to both their well-being and when they are afflicted by diseases. These proteins hold significant value in comprehending various physiological aspects of fish [18]. The serum biochemical profile serves as a valuable tool for gaining insights into the internal health of fish, often detecting issues before visible disease symptoms arise due to their close association with aquatic environments. Research in hematology has indicated that exposure to pesticides can result in diminished red and white blood cell counts in fish [19]. This profile examines a range of parameters, including creatinine, urea, glucose, albumin, and enzymes like alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and aspartate aminotransferase (AST), providing essential information about their overall health status.

5. Hematological analysis

The hematological analysis assesses overall health and potential organ damage resulting from stress. The electrolyte and water balance in fish blood can also have an impact on the hematocrit and hemoglobin values in relation to erythrocyte count. Stress causes a drop in plasma sodium levels in freshwater fish, which activates ion channels on the membranes of erythrocytes that carry ions in opposing directions. Water enters as a result of the elevated ion concentration, expanding the erythrocytes and improving their capacity to bind oxygen. To meet the increased oxygen requirements in response to these changes, the spleen releases more erythrocytes [20]. Large, fast-moving fish usually have an increased oxygen requirement in their muscles. This need may cause the head kidney's erythropoiesis to be stimulated. As a result, fish that move quickly in the ocean and fish that live in benthic habitats have different natural hematocrit levels [21].

Gene expression studies are typically based on RNA samples obtained from the organs of fish that have been previously euthanized. In contrast, collecting blood from fish can be done in a non-lethal manner, making it an alternative and more ethically preferable matrix. While blood sampling is relatively non-invasive, it can be stressful for fish. However, the advantage of repeated blood sampling from the same fish is that it allows for tracking various processes over time after a treatment or assessing the well-being of fish during their development [23].

Flow cytometry provides an alternative approach for analyzing the composition of blood cells and offers the benefit of processing a large number of samples. In a flow cytometer, individual blood cells are sequentially exposed to a laser beam, and the light they scatter carries distinctive characteristics associated with a particular blood cell type, enabling their separation. The inclusion of specific antibodies aids in achieving a more accurate assessment of the proportions of distinct immune cell subgroups in the blood [24]. Size and granularity of the cells have been the main factors used for cell sorting in the context of fish. Because only a few model fish species have access to fish-specific antibodies, this dependence on cell characteristics results from the scarcity of fish-specific antibodies [25].

It is commonly known that high temperatures can activate certain heat-shock protein (HSP) genes, including HSP90 (HSP90AA1) and HSP70 (HSP1A1). Additionally, a great deal of research has shown that heat stress modifies the expression of immune-related genes in the blood cells of various fish species. Interestingly, transcripts that code for cytokines seem to reflect the immune system's reaction to stress. In this context, cytokines such as transforming growth factor (TGF), interleukins (IL), interferon (IFN), and tumor necrosis factor α (TNF) are especially important. The liver, spleen, and kidney are the typical tissue choices when measuring transcripts linked to the immune system in stressed or stimulated fish. On the other hand, the skin, gills, and blood

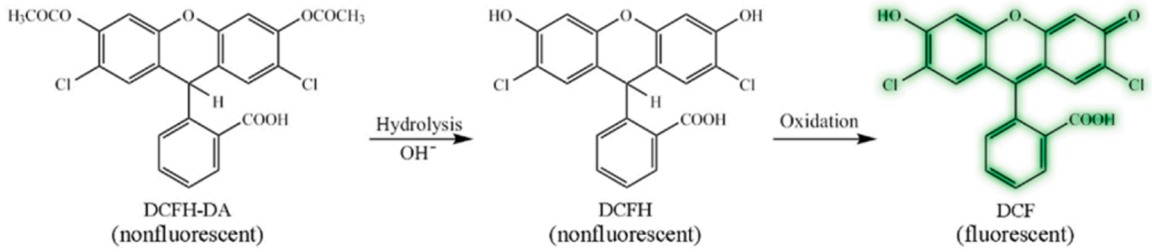

Fig. 5. The molecular compositions of DCFH-DA, DCFH, and DCF, as well as the fluorescence signal emitted by DCFH-DA [54].

are used to detect homeostasis abnormalities [26].

5.1. Staining methods

Staining is a crucial technique in histology that enhances the visibility of tissue and cellular structures under a microscope. By using specific dyes, it allows for detailed examination of organs, aiding in the diagnosis of diseases and assessment of tissue damage. This method plays an essential role in understanding cellular function and pathology.

5.1.1. Hematoxylin and Eosin (H&E) staining

Hematoxylin and eosin (H&E) staining is one of the cornerstone techniques in histology, valued for its ability to reveal intricate cellular and tissue architecture. Hematoxylin, a basic dye, binds to nucleic acids within cell nuclei, staining them a distinct blue-purple color, which makes nuclear details highly visible under a light microscope. Eosin, an acidic dye, counterstains the cytoplasm, connective tissue, and other extracellular components, rendering them in various shades of pink and red. This contrast between the basophilic (nucleus) and eosinophilic (cytoplasm) elements enables clear differentiation of cellular structures, allowing pathologists to assess the overall tissue organization and identify pathological changes. H&E staining is indispensable in evaluating organ damage, particularly in cases of fibrosis, necrosis, and inflammation, which are common indicators of tissue injury. For example, in liver cirrhosis, H&E staining can highlight fibrotic bands, which replace normal liver parenchyma, while in cases of ischemia or toxin-induced injury, the staining reveals areas of necrosis, where tissue architecture is disrupted or lost. The method also allows for the identification of inflammatory infiltrates, which are often present in tissues undergoing damage due to infection, autoimmune conditions, or chronic injury. H&E staining serves as a foundational diagnostic tool in pathology, essential for detecting and understanding the extent of organ damage across various conditions [27].

5.1.2. Masson's trichrome staining

Masson's trichrome staining is a specialized histological technique that differentiates tissue types, offering valuable insights into tissue architecture and pathology. By using a blend of dyes, it distinctly stains key components: collagen fibers in blue, muscle fibers in red, and cell nuclei in black. This color contrast is especially useful for identifying and assessing fibrosis, a condition marked by excessive connective tissue formation in response to chronic injury or inflammation. Fibrosis commonly affects organs such as the liver, lungs, and kidneys, where ongoing damage leads to the replacement of functional tissue with scar tissue. In liver cirrhosis, for instance, Masson's trichrome highlights the deposition of collagen fibers, making it easier for pathologists to detect and measure the extent of fibrotic damage. This technique is vital in determining the severity of fibrosis, aiding clinicians in tracking disease progression and evaluating treatment efficacy. In conditions like chronic kidney disease and pulmonary fibrosis, the staining similarly reveals the fibrotic changes, offering a clear view of tissue remodeling. Thus, Masson's trichrome is a critical tool in diagnosing and monitoring fibrotic diseases, helping to uncover the structural transformations that occur within affected tissues [28].

5.1.3. Periodic Acid-Schiff (PAS) staining

Periodic Acid-Schiff (PAS) staining is a specialized histochemical technique designed to detect polysaccharides and mucosubstances, such as glycogen, glycoproteins, and mucins, which are stained in a distinctive magenta color. This method is particularly effective for visualizing carbohydrate-rich structures, especially basement membranes, which are critical for maintaining the structural integrity and function of various organs. PAS staining is commonly used in pathology to identify abnormalities in these membranes, which are often implicated in disease processes. One of its key clinical applications is in diagnosing diabetic nephropathy, where it detects the thickening of the glomerular basement membrane, an early indicator of kidney damage due to prolonged high blood sugar levels. This capability makes PAS staining essential for early detection and ongoing monitoring of kidney conditions. Additionally, it is useful for identifying glycogen storage disorders in the liver, as it highlights abnormal glycogen deposits, and for detecting mucin-secreting tumors, such as adenocarcinomas. By revealing excess

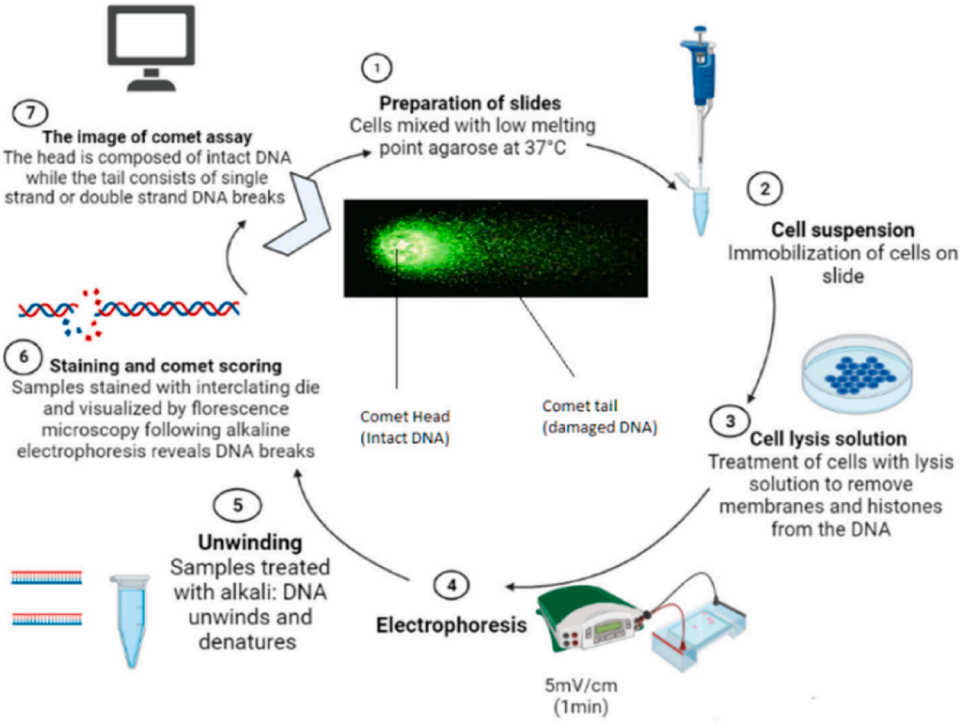

Fig. 6. A visual representation illustrating the procedural steps involved in conducting a comet assay [62].

mucin production, PAS aids in the classification and diagnosis of various cancers. Overall, PAS staining provides precise visualization of carbohydrate-containing structures, making it a crucial tool for diagnosing a broad range of diseases, from metabolic disorders to malignancies [29].

5.1.4. Immunohistochemistry

Immunohistochemistry (IHC) is an advanced staining technique that uses antibodies to specifically bind to and identify proteins, antigens, or other cellular components within tissue samples. This method offers valuable insights into molecular changes linked to organ damage by detecting and localizing key biomarkers. IHC is particularly effective in visualizing proteins involved in critical cellular processes such as apoptosis, fibrosis, and inflammation, making it indispensable for understanding the underlying mechanisms of tissue injury. For example, the detection of caspase-3, an enzyme central to apoptosis, allows pathologists to assess the extent of cell death in affected tissues. Similarly, IHC can identify alpha-smooth muscle actin (α-SMA), a biomarker for fibrosis, which aids in evaluating fibrotic conditions in organs like the liver, kidneys, and heart. This makes IHC crucial for investigating tissue responses to damage, such as liver fibrosis in chronic liver disease, kidney damage in chronic kidney disease, or myocardial fibrosis following cardiac injury. By revealing the expression of such biomarkers, IHC provides critical information for disease diagnosis, monitoring, and treatment planning. Its ability to offer precise molecular analysis makes it a foundational tool in modern pathology, enhancing our understanding of organ damage and the progression of various diseases [30].

5.2. Metabonomics and metabolomics assays

Metabonomics and metabolomics assays are advanced analytical techniques that provide a comprehensive profile of biochemical changes within biological systems, particularly concerning organ damage and disease progression. Metabonomics, a subset of metabolomics, emphasizes the quantitative analysis of metabolites in biological samples, typically utilizing techniques like nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) to identify and quantify small molecules in complex biological matrices. This methodology enables researchers to uncover metabolic pathways and variations in metabolite concentrations associated with different pathological conditions. For instance, changes in metabolite profiles can signal early biochemical alterations in organs experiencing injury, offering valuable insights into the mechanisms underlying diseases [37]. Metabolomics assays are essential for characterizing how tissues and organs respond to stressors, toxins, or disease processes, allowing for the identification of potential biomarkers that facilitate early diagnosis and prognosis. In studies related to liver health, metabolomics has been employed to examine metabolic changes in conditions such as non-alcoholic fatty liver disease (NAFLD) and alcoholic liver disease, shedding light on lipid metabolism and the roles of specific metabolites in disease progression [38,39]. In models of kidney injury, metabolomics can detect alterations in metabolic profiles that correlate with functional impairment, potentially serving as biomarkers for the early identification of kidney damage [40]. Furthermore, metabolomics assays have become increasingly valuable in cancer research, aiding in the understanding of metabolic reprogramming within tumor cells and their microenvironments, which can lead to better therapeutic targets [41]. The integration of metabolomics with other omics approaches, such as genomics and proteomics, provides a holistic perspective on the biological processes related to organ damage, facilitating the discovery of novel therapeutic strategies and personalized medicine approaches. Overall, the development of metabonomics and metabolomics assays marks a significant advancement in biomedical research, enhancing our understanding of disease mechanisms and improving clinical outcomes through the identification of metabolic biomarkers that support early diagnosis and targeted therapies [31].

6. Oxidative stress assays

An imbalance between the production of reactive oxygen species (ROS) and the existence of antioxidants that provide protection to an organism is what leads to oxidative stress (OS). The organism produces a variety of reactive oxygen species (ROS) during normal metabolic processes, but it also has an antioxidant defense system to keep these oxidative chemicals under control. Under certain circumstances, when there is an excessive production of oxidants, cells may experience severe damage and, in some cases, perish [32]. Reactive oxygen species (ROS) are mostly produced in the cytoplasm, plasma membrane, peroxisomes, and mitochondria. Oxygen is a biradical, meaning that it has two radicals in its orbitals, making it less reactive. However, reactions involving electron transfer or absorption of energy can contribute to the generation of reactive oxygen species (ROS) from molecular oxygen. These ROS include oxygen free radicals such as the alkoxyl (RO•), superoxide anion (O2•-), peroxyl (ROO•), nitric oxide (NO), and hydroxyl radical (HO•). They also include non-radical substances like oxygen, hypochlorous acid (HClO), hydrogen peroxide (H2O2), and transition metals like copper (Cu) and iron (Fe) [33].

6.1. Enzymatic assay

In fish research, enzymatic assays serve as vital method that offer important insights into a range of physiological processes. By assessing the activity of particular enzymes in fish tissues, these assays provide data on environmental conditions, stress, nutrition, and overall health. By measuring the activity of antioxidant enzymes that shield cells from damage brought on by reactive oxygen species (ROS), enzymatic assays are essential for assessing oxidative stress [34]. To determine the degree of damage brought on by environmental contaminants or other stressors, these enzymes are frequently measured in a variety of organs, including the liver, kidney, brain, gills, and muscle. Determining the equilibrium between oxidative stress and antioxidant defence mechanisms depends heavily on these enzymatic tests. They provide information about how well tissues or cells react to oxidative stress, which is crucial for research on aging, cancer, heart disease, and other disorders, by measuring the activity levels of particular enzymes that signify tissue damage or stress.

An antioxidant enzyme called superoxide dismutase (SOD) shields cells from oxidative stress by converting superoxide radicals into hydrogen peroxide. Fish's antioxidant defense systems, particularly in reaction to pollutants, toxins, and other stressors in aquatic environments, are frequently evaluated by measuring their SOD activity. A higher SOD activity may be a sign of increased oxidative stress and the body's reaction to counteract reactive oxygen species (ROS). As an illustration, SOD activity was assessed in the fish *Oreochromis niloticus* (Nile tilapia) following exposure to heavy metals, showing elevated enzyme activity in response to oxidative stress brought on by the toxicants [35]. Another crucial antioxidant enzyme that prevents oxidative damage to cells is glutathione peroxidase (GPx), which lowers lipid and hydrogen peroxides. As a component of the antioxidant defense system, GPx activity in fish is measured, especially in research examining the effects of oxidative stress, environmental pollution, and dietary supplements. Sparus aurata (gilthead sea bream) exposed to various dietary treatments containing polyunsaturated fatty acids had their GPx activity examined. According to the study, GPx was essential in helping the fish fight off oxidative stress brought on by lipid peroxidation [36].

Lactate dehydrogenase (LDH) is an example of metabolic enzymes that shed light on energy production, oxygen consumption, and stress reactions. An essential enzyme in anaerobic metabolism, lactate dehydrogenase (LDH) catalyzes the transformation of lactate into pyruvate. LDH activity is frequently measured in fish to evaluate muscle function and metabolic stress. As fish transition to anaerobic glycolysis for energy

production, elevated LDH levels are seen during intense exercise or in hypoxic environments [42]. When cells are damaged, lactate is released into the bloodstream by LDH (lactate dehydrogenase), which is involved in anaerobic respiration. Using spectrophotometric techniques, which track the conversion of lactate to pyruvate by measuring the change in absorbance at 340 nm, elevated levels in fish tissues signify organ damage [43,44]. Fish metabolic adaptation to pollution, temperature changes, and stress from exercise is studied using LDH assays. For instance, to evaluate the effects of environmental hypoxia, LDH activity was measured in the muscle tissues of *Cyprinus carpio* (common carp), which showed increased anaerobic metabolism in oxygen-limited conditions [45].

Enzymatic assays are used to measure key indicators of muscle function and metabolism, such as creatine kinase (CK) and myosin ATPase. These enzymes are crucial for energy production and muscle contraction, making them valuable biomarkers for studying muscle physiology, especially in fish. CK, a dimeric molecule with M and B subunits, forms isoenzymes, with four primary isoenzymes: CK-1, CK-2, CK-3, and CK-Mt. Damage to these tissues, such as hypoxia, can increase CK activity in the blood [46]. CK catalyzes the reversible transformation of creatine and adenosine triphosphate (ATP) into phosphocreatine and adenosine diphosphate (ADP), which is essential for muscle metabolism and the quick regeneration of ATP, the primary energy source for muscle contraction. Increased CK levels in fish muscle tissues may indicate disease, excessive strain, stress, muscle damage, or disorders [47].

Myosin ATPase is an essential enzyme for muscle contraction, responsible for hydrolyzing ATP to provide energy for muscle fibers' sliding filament mechanism. It is crucial for determining muscle fiber types and functional states in fish and has a direct impact on muscle contractility. Abnormalities in myosin ATPase activity can lead to muscle atrophy, exhaustion, and other pathological conditions. Monitoring absorbance at specific wavelengths can track the hydrolysis of ATP to ADP and Pi. Spectrophotometric techniques can quantify the amount of ATP hydrolyzed by myosin ATPase, including the release of inorganic phosphate (Pi). A colorimetric assay can identify Pi release during ATP hydrolysis, with a popular technique being the use of reagents like malachite green, which forms a complex with Pi and can be measured spectrophotometrically at 620 nm.

6.2. Non-enzymatic assay

For evaluating oxidative damage and fish antioxidant defence systems, non-enzymatic oxidative stress assays are essential. Without using enzymatic reactions, they quantify the amount of antioxidants and oxidative stress markers present. These tests shed light on how fish react to pollutants, environmental stressors, and physiological alterations. Antioxidants that are not enzymatic are essential for scavenging reactive oxygen species (ROS) and halting oxidative damage. These assays are therefore crucial for comprehending how fish react to environmental stressors.

6.2.1. Lipid peroxidation (MDA assay)

Malondialdehyde (MDA) measurement is a commonly used assay to evaluate oxidative stress in fish, and lipid peroxidation is an essential marker of oxidative damage to cell membranes. The most popular technique for figuring out MDA levels is the thiobarbituric acid reactive substances (TBARS) assay. ELISA or HPLC can be used to measure 4-Hydroxynonenal (4-HNE), another indicator of lipid peroxidation. When MDA and TBA react, a colored complex is created that can be measured spectrophotometrically, which is how the TBARS assay quantifies MDA. Increased oxidative damage and lipid peroxidation are indicated by elevated MDA levels [48,49]. In research on oxidative stress in heavy metal-exposed Oncorhynchus mykiss (rainbow trout), increased MDA levels have been employed as a biomarker of cellular membrane damage and lipid peroxidation [50].

6.2.2. Non-enzymatic antioxidant assays

Ascorbic acid (vitamin C) and glutathione (GSH), two non-enzymatic antioxidants that aid in scavenging reactive oxygen species (ROS) and shielding cells from oxidative damage, are measured by these assays. One of the most significant non-enzymatic antioxidants in cells is glutathione (GSH). There are two forms of it: reduced (GSH) and oxidized (GSSG). The redox state and oxidative stress in cells are indicated by the GSH to GSSG ratio. Assay for GSH/GSSG: Both reduced and oxidized glutathione in fish tissues is measured by this assay. Strong antioxidant defense is indicated by high GSH levels, whereas oxidative stress is suggested by a decrease in the GSH/GSSG ratio. The antioxidant capacity of fish can be evaluated by measuring the concentration of ascorbic acid in tissues or plasma, which is usually measured using colorimetric or HPLC methods. Ascorbic acid is a potent non-enzymatic antioxidant that scavenges free radicals and shields cells from oxidative damage. A decrease in ascorbic acid levels in fish tissues is frequently an indication of oxidative stress.

6.2.3. Protein carbonyl content (PCC assay)

Protein carbonylation is a significant marker of oxidative stress in fish, arising from the oxidative modification of proteins due to reactive oxygen species (ROS). The accumulation of protein carbonyls signals protein oxidation, which can compromise cellular function and is often linked to aging, disease, and responses to environmental stress. The protein carbonyl content (PCC) assay is widely employed to evaluate protein damage caused by oxidative stress. In this assay, carbonyl groups on oxidized proteins react with 2,4-dinitrophenylhydrazine (DNPH) to form a stable dinitrophenyl (DNP) derivative, which is then measured spectrophotometrically [51]. Elevated protein carbonyl levels indicate increased oxidative stress and structural protein damage. This type of protein oxidation has been observed in fish exposed to environmental contaminants, such as heavy metals and pesticides, making the PCC assay highly relevant for environmental toxicology studies [52]. Studies, for instance, have documented elevated protein carbonyl content in the liver and muscle tissues of fish subjected to various pollutants, demonstrating how environmental stressors can compromise protein integrity [53]. The PCC assay, when used alongside other methods like lipid peroxidation and non-enzymatic antioxidant assays, provides a thorough assessment of oxidative damage and antioxidant defenses in fish under environmental stress.

6.3. DCFDA assay

DCFDA stands for **2',7′-dichlorodihydrofluorescein diacetate**, Exposing DCFH-DA to light under mild alkaline conditions had a pronounced effect on enhancing the fluorescence signal. According to Kim et al. [113], this enhancement posed a challenge to conventional ROS detection using DCFH-DA. The alkaline conditions caused DCFH-DA to transform into DCFH through hydrolysis. This conversion initiated a series of cascading reactions, leading to the continuous conversion of DCFH into DCF, ultimately resulting in an amplified fluorescence signal. The rise in fluorescence signal could be attributed to the potential conversion of DCFH into DCF following exposure to light. DCFH-DA has the capability to undergo hydrolysis in alkaline conditions, leading to the transformation of DCFH into DCF, resulting in the manifestation of fluorescence [54].

Fluorescent probes like 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) and dihydroethidium (DHE) are useful in various techniques such as microscopy, plate spectroscopy, and flow cytometry for detecting reactive oxygen intermediates (ROS). After undergoing deacetylation by intracellular esterases, H2DCF is transformed by hydroxyl, nitrogen, carbonate, or thiyl radicals into the green fluorescent DCF. According to Ng et al. [114], DCF derivative probes are valuable for their sensitivity in revealing drug-induced ROS production and intracellular, they are also susceptible to spurious signals, including self-oxidation from H2O2 produced during oxidation and

photo-oxidation due to the excitation source.

To assess stress biomarkers, gill and liver tissues from specific fish specimens were extracted, cleansed with phosphate buffer, and homogenized to create a 10 % homogenate using 0.1 M phosphate buffer at a pH of 7.4 in a Teflon homogenizer. This homogenate underwent lysis and was then subjected to centrifugation at 10,000 rpm for 20 minutes at a temperature of 4 ℃. Following centrifugation, both the pellet and the supernatant were collected and preserved at −20 ℃ until further analysis. The ultimate fluorescence signal's density exhibited a direct relationship with both the intensity of the optical power density and the duration of irradiation. The application of this assay involves the assessment of organ damage caused by environmental factors related to oxidative stress [54].

7. Molecular biomarkers

Molecular biomarkers are used to investigate the influence of contaminants or stressors on gene expression within specific organs. Environmental conditions can lead to a decrease in the oxygen supply, resulting in the occurrence of hypoxia. Aquatic hypoxia is a frequent and recurring phenomenon that puts fish under hypoxic strain as they attempt to thrive in environments with low or fluctuating oxygen concentrations. Fish raised in high densities are especially susceptible to abrupt drops in water oxygen levels, which are common in intensive fish farming. As a result, considerable attention has been directed toward studying oxygen levels, given that diminished ambient oxygen concentrations are recognized to influence food consumption, fish growth and their overall physical health [55]. Integral membrane proteins called glucose transporters actively aid in the movement of hexoses through cell membranes, including glucose, fructose, and galactose. Because these transporters, also referred to as GLUTs, are sensitive to hypoxia, a transcription factor known as hypoxia-inducible factor (HIF-1alpha) controls how these transporters are expressed in mammals. Certain hypoxia-responsive elements present in the GLUT genes are bound by HIF-1alpha. Mammalian tissues that are exposed to hypoxia exhibit increased GLUT gene expression, which results in a faster pace of glucose acceptance. According to [112], this upregulation shields cells from the damaging effects of oxygen shortage while supplying the energy needed for metabolism. Results from Real-Time RT-PCR analysis have shown a significant increase in GLUT2 transcription in response to both chronic and acute hypoxia. The GLUT2 isoform is particularly important in the context of hypoxia-induced enhancement of glucose transport, as it is essential for meeting the heightened ATP demand associated with anaerobic metabolism.

Northern blot analysis has been utilized to explore the decrease in the expression of biomarker genes following exposure to chemical substances [56]. Initially, using northern blot analysis, the transcriptional activation of the vitellogenin (VTG) gene in response to 17β-estradiol (E2), an endocrine disrupting chemical (EDC), was examined. Since then, a wide range of EDCs have been included in the Northern blot application, with the goal of analyzing the gene expression profiles of different biomarker genes. In the Northern blotting technique, RNA bands, separated through electrophoresis, are exposed to DNA fragments labeled with radioactive nucleotides. These DNA fragments are selected for their complementarity to the gene(s) of interest. This procedure is utilized to identify differentially expressed genes [57].

Acetylcholinesterase (AChE) is an enzyme that breaks down acetylcholine in the synaptic cleft and is involved in neurotransmission. AChE activity is a common biomarker for neurotoxicity in fish, particularly in response to pesticide exposure, such as organophosphates and carbamates, which inhibit AChE activity. AChE activity was inhibited after exposure to organophosphate pesticides in the fish *Dicentrarchus labrax* (European sea bass), indicating neurotoxic effects. This inhibition is frequently used to evaluate the effects of waterborne pollutants on fish nervous systems [58].

Fish detoxification from toxins and pollutants involves cytochrome P450 enzymes, especially CYP1A. A common biomarker for exposure to environmental pollutants like polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) is CYP1A activity. Fish's induction of CYP1A may be an indication of how the body is reacting to the breakdown and detoxification of these toxic substances. For instance, exposure to benzo[*a*]pyrene, a known PAH, significantly increased CYP1A activity in *Oncorhynchus mykiss* (rainbow trout), demonstrating the liver's detoxification process [59].

The most suitable biomarkers encompass indicators of bioaccumulation, markers of exposure (such as EROD, CYP1A, LPOX, SOD, MT, HSP, micronuclei, DNA strand breaks, apoptosis), and markers of the resultant effects (histopathological assessments, TAG:ST) (1). Among these assays, the Comet assay, also referred to as single-cell gel electrophoresis, is a notable example. This technique is utilized to measure DNA damage in various fish tissues like gills, liver, kidneys, and blood, both in vivo, in vitro, and in situ, subsequent to exposure to diverse pollutants found in aquatic environments [60].

7.1. COMET assay

The Comet assay, also referred to as alkaline single-cell gel electrophoresis (SCGE), is a straightforward, rapid, and highly sensitive method for assessing genotoxicity. It measures the extent of DNA damage at the individual cell level and serves as a crucial tool in environmental surveillance, contributing to the evaluation of the health of aquatic organisms. This technique detects DNA damage in diverse aquatic species, including fish, shellfish, mussels, and clams. Specifically designed to identify genetic damage in the form of DNA strand breaks, the assay provides an even more sensitive indicator. In aquatic environments, it is widely applied for evaluating and monitoring the genetic well-being of both chordates and non-chordates [61].

Cells were mixed at a concentration of 1 × 105 cells per ml with liquid low-melting agarose (referred to as LM Agarose) at a temperature of 37 °C at a ratio of 1 part cells to 10 parts LM Agarose by volume. Subsequently, 50 μL of this mixture were dispensed onto a comet slide, which was positioned on a level surface at a temperature of 4 °C in complete darkness for a duration of 10 mins.

The prepared slides, coated with LM Agarose and the cell suspension, were submerged in a lysis buffer solution (consisting of 2.5 M NaCl, 100 mM EDTA, and 10 mM Trizma base) at a temperature of 4 °C for a period ranging from 30 to 60 minutes. Afterward, the comet slides were immersed in a freshly prepared alkaline unwinding solution (comprising 300 mM NaOH and 1 mM EDTA) with a pH exceeding 13 for 20 minutes at room temperature.

Next, the slides were positioned in an electrophoresis slide tray and covered with a tray overlay. The power supply was configured to 21 volts, and this voltage was applied for 30 minutes. Following electrophoresis, the samples were dried at a temperature of 37 °C for approximately 10–15 minutes.

To preserve the samples, they were stored at ambient temperature along with a drying agent, with silica gel being a commonly used desiccant. For staining, a volume of 100 μL of diluted SYBR Green dye was added to each area of dried agarose and allowed to stain for a duration of 30 minutes at room temperature in the absence of light. Finally, the slides were thoroughly dried at 37 °C, then examined and observed using a fluorescence microscope, such as the one from Carl Zeiss in Germany, and photographs were taken [60].

7.2. Enzyme assays

Enzyme assays (e.g., alanine aminotransferase, aspartate aminotransferase) can indicate organ damage by measuring enzyme leakage from damaged cells. Alanine aminotransferase (ALT) is an enzyme located within the hepatocytes of fish and various other animals. Its role involves facilitating the liver's process of breaking down proteins to render them more readily absorbable by the body. Fish ALT levels can

serve as a precise indicator for assessing the efficiency of dietary protein utilization [63].

8. Instrumentation techniques

Sensitive detection methods improve a variety of enzymatic assays in fish. These methods offer accurate and thorough measurements of enzyme activity, which aid in evaluating physiological alterations, metabolic processes, and aquatic organisms' reactions to environmental stressors.

8.1. Fluorometry

A commonly used approach for determining the amount of fluorescence released by a fluorescent product or substrate during an enzymatic reaction is fluorometry. Low levels of enzymatic activity in fish tissues can be found using this technique because it is very sensitive and selective. Enzymes involved in oxidative stress, metabolic pathways, and cell signaling processes are frequently studied using fluorometric assays. Enzymes like lactate dehydrogenase (LDH) and glutathione S-transferase (GST), which are essential for metabolism and detoxification processes, are frequently measured in fish using fluorometry. The activity of glutathione S-transferase (GST) in *Danio rerio* (zebrafish) larvae exposed to environmental pollutants has been measured using fluorometry. Changes in GST activity, a sign of detoxification and an aquatic organism's reaction to oxidative stress, can be detected using the fluorometric assay [64].

8.2. Chemiluminescence

Another effective method for determining enzyme activity is chemiluminescence, which measures the light released during a chemical reaction that the enzyme catalyzes. It is a very sensitive technique for identifying enzymatic reactions in fish because the intensity of the light emitted is directly proportional to the activity of the enzyme. According to Elia et al. (2003), this approach offered comprehensive insights into the ways that oxidative stress impacts fish health in contaminated aquatic environments. Chemiluminescence is especially helpful when researching enzymes like myeloperoxidase (MPO) and superoxide dismutase (SOD) that are involved in oxidative stress and immune responses. These enzymes are essential for regulating immune responses and neutralizing reactive oxygen species (ROS). For instance, the activity of superoxide dismutase (SOD) in *Oreochromis niloticus* (Nile tilapia) exposed to environmental contaminants has been assessed using chemiluminescence assays. SOD is a crucial enzyme that catalyzes the transformation of superoxide radicals into hydrogen peroxide, thereby reducing oxidative damage [65].

8.3. Electrochemical methods

The electrical current or potential produced by an enzymatic reaction usually a redox reaction in which electrons are transferred between an electrode and an enzyme is measured using electrochemical techniques. Enzymatic activity in metabolic pathways, such as those involving lactate dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase (G6PD), which are crucial for fish energy production and stress adaptation, can be detected with these methods. This technique has been used in aquaculture to quantify the amount of lactate dehydrogenase (LDH) present in the muscle and gill tissues of Atlantic salmon, or Salmo salar. Anaerobic metabolism depends heavily on LDH, and its activity is frequently used as a biomarker for stress response and muscle function. Fish LDH activity can be tracked in real time using electrochemical biosensors [66].

8.4. HPLC (high-performance liquid chromatography)

One method for separating and measuring particular enzymes or the byproducts of their reactions is HPLC. By identifying the substrate or product of enzymatic reactions, it is extremely accurate and frequently used to quantify enzymatic activity in complex biological samples. By separating substances according to how they interact with a stationary phase and a mobile phase, HPLC makes it possible to precisely quantify particular enzymes or the products of their reactions. Acetylcholinesterase (AChE) activity in fish exposed to organophosphate pesticides has been measured using HPLC. HPLC is used to separate and quantify the breakdown products of acetylcholine, which are catalyzed by AChE. This provides comprehensive information on the neurotoxic effects of pollutants. When measuring the amount of acetylcholine hydrolyzed in fish, samples are homogenized and the enzyme activity is determined. The concentration of the reaction products is then measured after they have been separated using HPLC [67,68].

8.5. Mass spectrometry (MS)

By measuring the molecular mass of reaction products, mass spectrometry can be used to investigate enzymatic reactions. This extremely sensitive method provides information on substrate specificity and enzyme kinetics by identifying and quantifying the products or substrates of enzymatic reactions.In lipidomics, MS is widely used to investigate the lipid makeup of fish tissues[69]. It determines changes in lipid metabolism under various circumstances, such as exposure to pollutants or dietary changes, by examining the lipid profiles. Identification of Products of Lipid Breakdown: Lipase activity produces glycerol and free fatty acids, which are byproducts of lipid metabolism that MS can identify and measure. This aids in comprehending the metabolic processes and how different factors affect the metabolism of fats. The impact of environmental stressors on fish metabolism has been investigated using MS. For instance, MS can identify molecular changes in lipid metabolism caused by pollution, temperature fluctuations, and other stressors [70].

8.6. NanoDrop

The NanoDrop spectrophotometer has become indispensable in genetic, physiological, and environmental studies, particularly for analyzing biomolecules like proteins, DNA, and RNA in fish tissue samples. In fish assay studies, where sample availability can often be limited, the NanoDrop method is especially beneficial due to its ability to measure both concentration and purity with minimal sample volumes, often as little as 1–2 microliters. By allowing for efficient, rapid, and precise quantification, NanoDrop technology enables researchers to reliably assess nucleic acid and protein concentrations without the need for extensive sample processing, which can be crucial when working with small or precious samples. This instrument uses absorbance measurements at 260 nm for nucleic acids and 280 nm for proteins, providing accurate readings on sample quality that help ensure reliable downstream applications such as PCR, sequencing, or proteomics [71]. Furthermore, the method's high sensitivity and ease of use make it ideal for integrating into a variety of analytical workflows, enhancing the capacity to monitor genetic and physiological responses in fish exposed to different environmental conditions.

9. Gene expression analysis

Over the past ten years, the adoption of next-generation sequencing (NGS) and microarray technologies has brought about a significant shift in the way gene analysis is conducted. This shift has moved from studying a specific group of genes to conducting comprehensive screenings of potentially all genes expressed in the genome. However, traditional microarray technology, often referred to as a "closed-end

transcriptomic technique," relies on pre-existing genetic information, making it impractical for particular species that lack comprehensive or any genetic data, especially non-model species [56].

Pyrosequencing technology was utilized to create a transcriptome-wide microarray for non-model species in order to overcome this constraint. On the other hand, in recent years, NGS technology has emerged as the preferred method for profiling transcriptomes due to its decreasing costs and high-throughput capabilities [72]. Various technologies are accessible for spanning from single-gene, quantifying gene expression, real-time PCR to high-throughput techniques like cDNA macro and microarrays, massively parallel signature sequencing (MPSS), and serial analysis of gene expression (SAGE). One of the most accurate techniques for assessing transcripts is Real-Time quantitative Reverse Transcription PCR (Real-Time qRT-PCR), which is based on two sequential steps: reverse transcription of mRNA and then using the resulting cDNA as a template for PCR. [55].

Due to the swift growth of transcriptome investigations in various fish species, a substantial volume of RNA-seq data has been made available, enabling a more methodical exploration of the overall characteristics and specifics of gene expression in fish. **FishGET** is committed to the aggregation and careful curation of RNA-seq data from fish, with the goal of identifying additional novel RNA types, encompassing both mRNA and long non-coding RNA (lncRNA). This endeavor aims to establish a more comprehensive reference transcriptome and offer more detailed and precise annotations of the transcriptome. **FishGET** features a visual expression comparison tool designed to emphasize variations in the expression levels of one or multiple genes or RNAs across different samples. Users can select the species, individual specimens, and input genes of interest to visualize their expression patterns on the expression page. The outcome typically takes the form of a dynamic and interactive heatmap [73].

Within the realm of aquaculture, the utility of real-time PCR has recently broadened, particularly in the detection of genetically modified organisms, parasites, and microbes. Nevertheless, the demand for rapid and accurate quantification of small quantities of nucleic acids in fish presents a opportune market for the application of real-time PCR. while technology advances, with more compact, more affordable and more user-friendly, it is likely that more real-time PCR-based applications will be realized in the field of aquaculture [55].

Quantitative PCR (qPCR) is a method employed to quantify the quantity of DNA or RNA within a specimen. It is frequently utilized for the identification of alterations in gene expression linked to organ damage. The analysis of gene expression entails the measurement of gene activity in a given sample, making it possible to identify variations in patterns of gene expression that might be associated with diseases or other circumstances. Various techniques are accessible for the analysis of gene expression, including qPCR, microarrays, and RNA sequencing. qPCR is a widely favored technique due to its exceptional sensitivity and specificity, enabling real-time quantification of gene expression levels [74].

qPCR involves a series of stages: (1) Extraction of RNA from the desired tissue or cells; (2) Conversion of mRNA into cDNA through reverse transcription; (3) Using custom PCR primers to amplify a specific cDNA section and monitoring the process in real-time; and (4) Using the exponential phase of the reaction to calculate the starting concentration of a chosen transcript in a particular tissue or cell type [75].

9.1. Microarray analysis

Microarray analysis is a high-throughput technique widely used to study gene expression levels across thousands of genes simultaneously, offering a comprehensive snapshot of genetic activity within a biological sample. The process involves hybridizing labeled RNA or cDNA samples to a microarray chip containing thousands of probes, each specific to particular genes. The fluorescence intensity measured at each probe reflects the gene expression levels, allowing researchers to quantify the activity of genes under different conditions. This technology has transformed genomics by enabling large-scale profiling of gene expression in various contexts, including development, disease, and responses to therapeutic interventions [76,77]. One of the most significant applications of microarray analysis is in cancer research, where it has been instrumental in identifying gene expression patterns that differentiate between tumor and normal tissue, leading to the discovery of potential biomarkers for cancer diagnosis and prognosis [78,79]. Beyond oncology, microarrays have been used to explore gene expression changes associated with various diseases such as cardiovascular disorders and autoimmune conditions, providing valuable insights into the underlying molecular mechanisms [80,81]. The technology also plays a pivotal role in drug discovery and development, where gene expression profiling after drug treatment helps in identifying molecular targets and assessing drug efficacy. Additionally, microarray analysis enables the exploration of complex cellular interactions and pathways, further advancing our understanding of disease biology and aiding the development of therapeutic strategies [82,83]. Despite its significant contributions to biomedical research, microarray analysis does have limitations, particularly concerning sensitivity and specificity when detecting low-abundance transcripts. Moreover, the technology requires specialized equipment and expertise, which may limit its accessibility in certain research settings [84]. Microarray analysis remains a cornerstone of genomic studies, continuing to provide critical insights into gene expression and regulation across a range of biological disciplines.

9.2. Nanostring technology

NanoString technology is an innovative high-throughput gene expression analysis method that utilizes unique molecular barcodes to detect and quantify RNA molecules with precision and sensitivity. Unlike traditional amplification-based methods like PCR, NanoString's digital readout system directly counts specific RNA molecules, offering accurate measurements of gene expression levels without the need for amplification [85]. A major strength of NanoString is its capacity for multiplexing, allowing researchers to analyze hundreds of genes simultaneously within a single sample, making it particularly advantageous in clinical and research settings where sample availability is limited [86]. This technology has found widespread applications across various fields, including oncology, immunology, and infectious diseases, where it provides critical insights into gene expression patterns associated with different conditions [87,88]. In cancer research, NanoString has been instrumental in profiling tumor samples, facilitating the identification of prognostic and predictive biomarkers that can help guide treatment decisions [89]. Additionally, in immunology, NanoString is used to assess immune responses by evaluating gene expression changes in immune cells during infections or in response to vaccines, offering a deeper understanding of immune dynamics and its implications for health and disease [90]. One of the key technical benefits of NanoString is its use of molecular barcodes, which minimizes cross-reactivity and reduces background noise, leading to highly reliable and reproducible results [85]. The nanostring technology does come with limitations, such as a narrower dynamic range compared to RNA-Seq [86].

9.3. CRISPR/Cas9-mediated gene activation (CRISPRa)

CRISPR/Cas9-mediated gene activation (CRISPRa) is an advanced method for selectively enhancing gene expression without altering the underlying DNA sequence. Unlike the traditional CRISPR/Cas9 system, which induces DNA breaks to knock out genes, CRISPRa employs a catalytically inactive Cas9 protein (dCas9) fused with transcriptional activators. This dCas9 protein is designed to bind to specific DNA regions via a guide RNA (gRNA) but lacks the ability to cut the DNA. Instead, it recruits transcriptional activators such as VP64, p300, or the VP64-p65-Rta (VPR) complex, which facilitate the upregulation of gene expression by attracting the cell's transcriptional machinery to the

target gene's promoter region [91]. The modular nature of the system, where different gRNAs can be designed to target various genes, makes CRISPRa highly adaptable and useful for functional gene studies, particularly in organ damage research [92]. In studies investigating organ damage, CRISPRa holds significant promise for activating genes involved in repair, regeneration, and inflammatory responses. By upregulating key genes associated with tissue regeneration and anti-inflammatory processes, researchers can gain valuable insights into how these genes contribute to mitigating damage in organs such as the liver, kidneys, and heart [93]. The precision and versatility of CRISPRa also allow researchers to dissect complex gene regulatory networks in damaged tissues, facilitating the discovery of novel therapeutic targets. Moreover, CRISPRa can be combined with advanced molecular techniques such as RNA sequencing and chromatin immunoprecipitation (ChIP) to gain a deeper understanding of gene activation mechanisms in disease contexts. The system's inducible versions provide further flexibility, enabling the temporal control of gene expression, which is crucial for studying the dynamic changes in gene activity during the progression of organ damage and subsequent repair [94]. Such studies are essential for identifying time-sensitive gene functions and could lead to the development of more precise therapeutic strategies for conditions like acute kidney injury, myocardial infarction, or liver fibrosis [95].

10. Non-invasive methods

Recent advancements in technology, digital photography is emerging as an essential and versatile component of contemporary biology. Despite their relatively high cost, non-invasive digital imaging devices, high-throughput, such as CT and MRI scanners, have emerged as powerful tools for the three-dimensional evaluation and inspection of anatomical characteristics in the context of entire organisms. Because CT and MRI are not constrained by restrictions pertaining to signal penetration depth, they do not always necessitate particular specimen preparation before imaging. This has a number of important benefits, particularly in cases when handling rare or threatened species makes it impractical or undesirable to dissect specimens, as is often the case in museum collections. Furthermore, these methods provide an effective way to store large amounts of quantitative digital information permanently in a small amount of physical area. They make it possible to apply cutting-edge methods for analyzing and displaying data and to share such data online in common forms [96].

Three fundamental contrast mechanisms underpin most common types of anatomical MRI scans: Firstly, the proton density, or water content, of a voxel; The MR signal decay time, also known as transverse relaxation, or T2, comes in third. The second is the MR signal recovery time, also known as longitudinal relaxation, or T1. Each of these techniques is contingent upon the local tissue microstructure, which is composed of components such as water content, fat content, and protein content. Therefore, depending on the specific MR pulse sequence used, subtle structural variations between tissues may be visible [97]. On the other hand, the available MR signal-to-noise ratio (SNR) at a given voxel resolution determines the efficacy of MRI in addition to the physical characteristics of the scanned material and the software techniques employed to generate tissue contrast. The hardware's ability to recognize the generated MR signal inside the specimen's tissues and the scanner's magnetic field strength both have an impact on this SNR [98].

A non-invasive method for determining the concentration of particular metabolites generated by enzymatic reactions is NMR spectroscopy. A deeper comprehension of enzyme mechanisms is made possible by the structural information that NMR provides on substrates and products [99]. The activity of energy metabolism-related enzymes, like creatine kinase, in fish muscle tissues has been investigated using NMR. NMR can evaluate fish energy stores in a variety of environmental settings by identifying changes in chemical resonances [100]. Fish lipid metabolism, including fatty acid composition and lipid class analysis, has been studied using NMR spectroscopy. This aids in comprehending how environmental stressors or dietary supplements impact fish metabolism. By measuring indicators like the K value and trimethylamine nitrogen (TMA-N) content, it can quickly determine the freshness and quality of fish [101].

Ultrasonic imaging, a non-invasive method, employed to assess the reproductive status of various fish species. It proves to be a valuable instrument for scientists and resource managers as it allows them to obtain data regarding the reproductive condition of small-sized salmonids without causing any disturbance during the breeding season [102]. Research employing this method has typically concentrated on fish ranging from 200 mm to 1–2 m in total length (TL) and has explored inquiries concerning gender, gonad dimensions, and spawning status throughout the spawning season [103]. Ultrasound imaging in fish serves as a valuable tool for identifying organ harm resulting from different factors like pollution, illness, or physical injuries. This imaging technique can reveal alterations in the dimensions, configuration, and consistency of organs, providing insights into potential damage or diseases. However, it's critical to recognize that there is a chance that ultrasonic imaging will harm living tissue, mostly via a pair of processes: mechanical and thermal [104].

The development of useful ultrasonic imaging methods has the potential to significantly help fisheries managers implement this technology in difficult-to-reach locations, such as isolated aquaculture sites, lakes, ravines, and marine vessels. Using ultrasonography in this isolated and protected area presents many challenges, such as intense heat, sand abrasion, exposure to water, and turbulent white-water rapids that can harm electronic devices. In addition, screen visibility is negatively impacted by limited shade availability, and battery charging opportunities are limited. Biologists have a limited amount of time to catch fish and collect data because sampling trips can last up to eighteen days. These unfavorable circumstances restrict the use of ultrasound examinations and present difficulties for electronic devices. The effective application of ultrasonography for image capture in this harsh desert environment would demonstrate the technology's versatility in a range of field settings, providing managers, researchers, and fisheries biologists with a powerful tool to better understand fish reproductive cycles even in remote field settings [105].

10.1. Multiplex FISH (mFISH)

Multiplex Fluorescent In Situ Hybridization (mFISH) is an advanced cytogenetic tool that allows for the detailed visualization of complex chromosomal abnormalities by using fluorescently labeled DNA probes specific to each chromosome [106]. This method enables the simultaneous detection of chromosomal rearrangements such as translocations, deletions, and inversions, which is invaluable in cancer diagnostics and genetic research. By utilizing distinct combinations of fluorescent colors, mFISH creates a comprehensive chromosomal map, facilitating the identification of cryptic aberrations that conventional techniques may miss [107]. It is particularly significant in oncology for detecting chromosomal instability in tumors like leukemia and solid cancers. Additionally, mFISH is applied in prenatal testing to detect potential genetic disorders early on in development [108].

10.2. Spectral karyotyping

Spectral Karyotyping (SKY) is an advanced cytogenetic technique that utilizes fluorescently labeled probes to assign each chromosome a unique spectral signature [109]. Unlike mFISH, which distinguishes chromosomes through a combination of fluorescent colors, SKY employs spectral imaging to generate distinct color patterns for each chromosome [110]. This allows for precise identification of even complex chromosomal rearrangements. SKY is particularly valuable in cancer research, as it can uncover hidden chromosomal alterations that contribute to tumor development and malignancy. Spectral Karyotyping helps in the study of hematological malignancies, solid tumors, and

toxicology, where environmental factors may induce chromosomal damage [111]. The high-resolution imaging offered by SKY is essential for investigating chromosomal instability in cancer and other genetic disorders.

11. Conclusion

In conclusion, aquatic ecosystems are vital resources supporting biodiversity, food production, and recreation. However, these ecosystems face escalating threats from human activities, such as pollution, climate change, and habitat degradation. Fish, as integral components of these ecosystems, are particularly susceptible to these environmental stressors, which can result in organ damage and compromised overall health. Fish serve as valuable model organisms for investigating the impacts of environmental contaminants, diseases, and other stressors on aquatic ecosystems. An essential aspect of this research is the evaluation of organ damage in fish. Histology and its associated techniques are indispensable tools for understanding disease processes, optimizing biological productivity, and monitoring environmental impacts. Through detailed preparation and analysis of tissues, histological methods reveal structural abnormalities indicative of organ damage, offering vital insights into the health of organisms and ecosystems.

The integration of histology with biochemical, hematological, and molecular analyses enhances the ability to detect and understand organ damage. Enzymatic and non-enzymatic biomarkers, alongside advanced tools like the Comet assay and gene expression profiling, provide a comprehensive framework for assessing oxidative stress, metabolic dysfunction, and cellular responses to environmental stressors. Techniques such as H&E staining, metabolomics, and advanced imaging allow for detailed structural and functional evaluations, bridging gaps between cellular-level changes and broader physiological outcomes.

Emerging technologies, including real-time qRT-PCR, CRISPR-mediated gene activation, and non-invasive imaging, offer transformative potential in diagnosing and mitigating organ damage. These innovations enable precise detection, early intervention, and improved understanding of gene-environment interactions. As environmental stressors increasingly challenge aquatic ecosystems, these methodologies become ever more critical for sustainable management and conservation efforts.

Overall, combining traditional histological approaches with modern analytical and molecular techniques ensures robust evaluations of organ health, enabling targeted strategies for disease prevention and environmental monitoring. This multidisciplinary approach is essential for advancing biological research, improving aquaculture practices, and safeguarding biodiversity.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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