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Reviews

ADVANCES IN FIXATION TECHNIQUES FOR AQUATIC SPECIMENS: COMPARATIVE INSIGHTS FROM FISH AND CRUSTACEAN HISTOLOGY

M. N. AKMAL[1*], A. R. INTAN-SHAMEHA[1], S. ANNAS[2] and T. DIYANA[3]

[1]*Department of Veterinary Preclinical Sciences, Faculty of Veterinary Medicine, University Putra Malaysia, 43300, Serdang, Malaysia.*
[2]*Department of Veterinary Laboratory Diagnosis, Faculty of Veterinary Medicine, University Putra Malaysia, 43300, Serdang, Malaysia.*
[3]*Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, University Putra Malaysia, 43300, Serdang, Malaysia.*

SUMMARY

Histological assessment is essential in aquatic animal research. However, the fixation of fish and crustaceans poses challenges due to rapid postmortem autolysis, elevated endogenous enzymatic activity, and structural impediments like scales or calcified exoskeletons. This review highlights fundamental histological principles with contemporary empirical research on fixation optimisation in fish, specifically small fish (zebrafish), and crustaceans. Collectively, previous studies underscore common challenges while dealing with aquatic samples and illustrate how customised fixation techniques, including double-fixation protocols and species-specific decalcification procedures, can significantly improve tissue preservation, staining efficacy, and diagnostic accuracy. Double fixation with formalin, succeeded by Bouin's or Davidson's fluid, produces enhanced results in fish specimens, particularly in smaller species. At the same time, optimised methods for crustaceans entail abdominal excision, fixation in Davidson's fluid, and regulated decalcification. This review underscores the imperative of amalgamating chemical principles with morphological factors to formulate fixation methods that are consistent, reproducible, and tailored to the distinct physiological traits of aquatic species.

Keywords: autolysis, crustaceans, fixation technique, fish, histology

INTRODUCTION

Performing histology and histopathology provides a foundation for assessing tissue morphology, diagnosing disease processes, and facilitating research across various biological disciplines (Singh et al., 2019; Dagdeviren et al., 2024). Tissue fixation is a crucial aspect of this field. It halts autolysis and preserves the tissue structure for microscopic observation. The fixation technique must maintain cellular architecture, inhibit enzymatic decomposition, and enhance staining quality while minimising artefact incidence (Mokhtar and Abd-Elhafeez, 2013; Dagdeviren et al., 2024). A 10% formalin solution is widely employed as a universal fixative for all mammalian and fish tissue specimens (Layton et al., 2019). Additionally, compound fixatives that combine multiple chemical agents with fixative properties, such as Davidson's and Bouin's solutions, have been developed to enhance rapid tissue permeability and fixation (Dagdeviren et al., 2024; Wild et al., 2025).

Aquatic organisms pose unique challenges with fixation due to their rapid deterioration via the autolysis process, which is significantly influenced by their aquatic environment that completely differs from the terrestrial ambience. Removal of fishes from the water disrupts various physiological processes in their organs, accelerating autolysis (Mokhtar and Abd-Elhafeez, 2013). Furthermore, fish possess numerous catalytic enzymes, particularly in cold-water species, which further hasten degradation (Malinowska-Panczyk and Kołodziejska, 2018). Anatomical features, such as scales or calcified carapaces, also contribute to this degradation by hindering the penetration of the fixative into internal organs (Cervellione et al., 2017). Therefore, to prevent aquatic tissue samples from decomposing and producing artefacts, an appropriate fixation method must be employed. Hemolysis and cellular cavitation are the most frequently observed artefacts, typically occurring when epithelial tissue separates from the underlying connective tissue, especially in the gills, intestines, and kidneys (Miwa, 2017). Recent studies have addressed similar issues in both fish and crustaceans, leading to improved fixation methods. A double-fixation procedure for fish, particularly small species, has been introduced to minimise tissue disruption and artefact formation caused by fixatives (Miki et al., 2018). Additionally, there are supplementary steps in the fixation process specifically for preparing histology samples of crustaceans. These steps include decalcification, enzymatic digestion, and coelomic incision (Wild et al., 2025).

This review synthesizes the findings to highlight the challenges of fixing aquatic specimens, innovative fixation techniques, and practical guidance for achieving optimal histology results in fish and crustacean research.

*Corresponding author: Dr. Mohd Akmal Mohd Noor (M. N. Akmal).
Email: akmal.noor@upm.edu.my

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Principles and Challenges of Fixation in Aquatic Species

Chemical and Biological Basis of Fixation

In general, the core fixation process relies on chemical fixatives that maintain tissue integrity via several primary mechanisms (Figure 1), including cross-linking (protein cross-linking via the formation of methylene bridges), coagulation (coagulating the proteins that are prone to being degraded and commonly used for preservation of the fibrous connective tissue), and dehydration (removing water molecules that precipitate in the hydrogen bond of a hydrophilic protein's side and partially reversing the hydrophobic group outside, which could potentially disrupt the tertiary structure of the proteins) (Layton et al., 2019; Singh et al., 2019; Dagdeviren et al., 2024). Formaldehyde, often used as 10% neutral buffered formalin (NBF), is a fixative that crosslinks proteins in animal tissues, keeping their molecular structure as close to normal as possible. When formaldehyde is present in an aqueous solution, it forms methylene glycol, which reacts with the protein's side chains, making them reactive. To stabilise this reactive side chain, the protein will be covalently linked to another protein via a methylene bridge (Layton et al., 2019). It remains the most widely used fixative due to its broad applicability, cost efficiency, and consistent compatibility with standard hematoxylin and eosin (H&E) staining protocols (Wild et al., 2025).

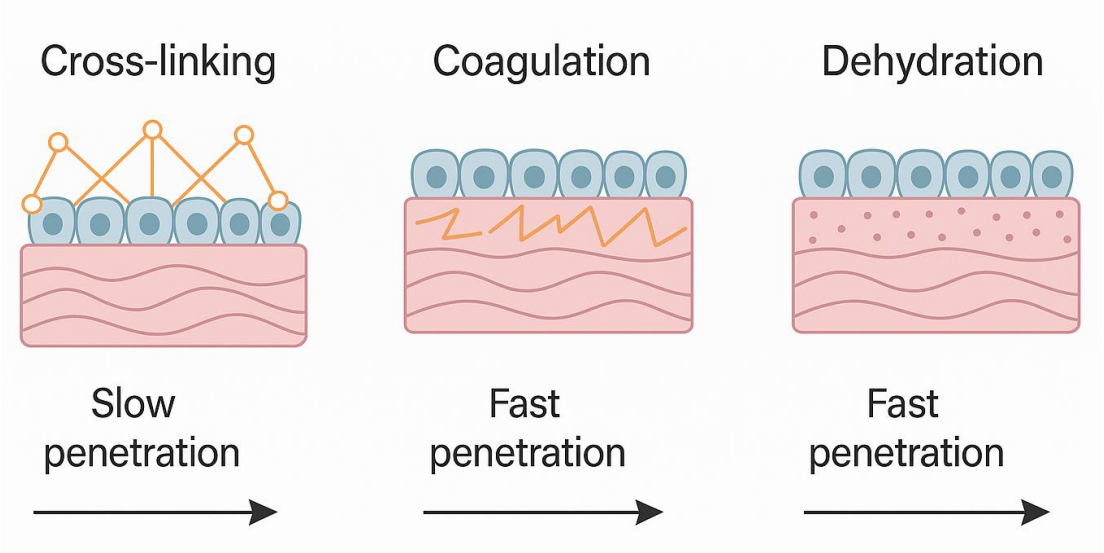

Figure 1: Conceptual diagram of fixative mechanisms in aquatic tissue preservation

Although it is widely utilised, there is no universal or perfect fixative, as each alternative has its own advantages and disadvantages. These trade-offs encompass essential considerations such as maintaining tissue morphology, preserving antigenicity and molecular integrity, the extent of tissue reduction or swelling caused by fixation, and the stability of the material during prolonged storage (Miki et al., 2018; Feng et al., 2021; Wild et al., 2025).

Aquatic organisms, particularly susceptible to rapid postmortem autolysis, further intensify these limitations (Mokhtar and Abd-Elhafeez, 2013). In fish and crustaceans, structural deterioration may commence within minutes following death, caused by elevated levels of digestive enzymes, ongoing metabolic processes, and external anatomical barriers, such as fish scales and crustacean exoskeletons, that substantially hinder the penetration of fixatives (Mokhtar and Abd-Elhafeez, 2013; Cervellione et al., 2017; Malinowska-Panczyk and Kołodziejska, 2018). Consequently, achieving sufficient and consistent fixation in aquatic species poses distinct technical challenges that require meticulous attention in histological procedures (Miki et al., 2018).

Anatomical and Physiological Barriers in Fishes and Crustaceans

Based on the previous study, it documented significant autolysis in the liver and gut of fish sample, particularly in the small fish when standard formalin was used as an exclusive fixative (Miki et al., 2018). The degeneration was attributed to insufficient penetration of formalin and the slow inactivation of endogenous enzymes, which allowed for significant tissue disintegration before fixation was completed (George et al., 2016). Wild et al., (2025) similarly revealed that crustaceans face rapid autolysis, especially in metabolically active organs like the hepatopancreas, unless immediate and effective fixation is conducted.

Numerous anatomical and practical characteristics further hinder fixing in aquatic species (Figure 2). Fish scales significantly impede the infiltration of aqueous fixatives, thereby increasing the risk of autolysis (Grunow et al., 2015). The chitinous exoskeleton of crustaceans obstructs the diffusion of fixatives to internal tissues and may also damage microtome blades during sectioning (Costa et al., 2021). The diminutive size of numerous fish and crustacean species constrains the possibilities for organ-level dissection, frequently requiring whole-body fixation, which

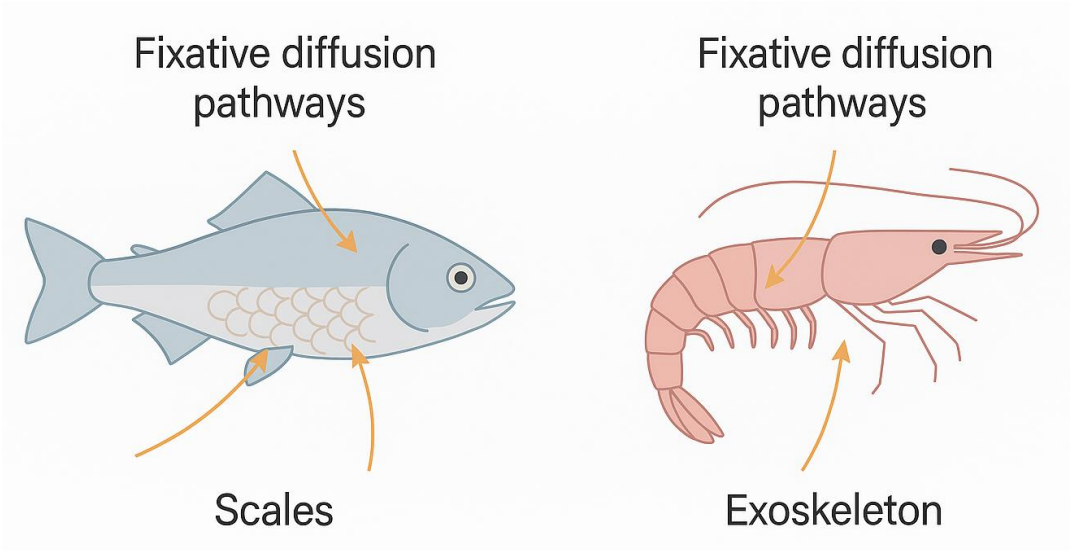

Figure 2: Diagram shows the anatomical barriers preventing fixative diffusion (arrows) in fish and crustaceans

subsequently extends the duration needed for fixative infiltration (Miki et al., 2018; Wild et al., 2025).

These limits highlight the necessity for specialized fixation techniques that surpass standard formalin immersion to ensure the proper preservation of tissue architecture in aquatic species (Miki et al., 2018).

Fixation Approaches in Fish: Evidence for Double Fixation

Limitations of Single-Fixative Methods

The constraints of conventional fixation methods are apparent in comparative evaluations of fixatives. Miki et al. (2018) assessed 20% formalin, Bouin's fluid, and Davidson's solution, noting the incidence of artefacts in various tissues. These included epithelial detachment in the gills, the creation of an artificial cavity in the colon, hemolysis, and autolysis in the gallbladder region of the liver. While Bouin's and Davidson's solutions facilitated accelerated tissue penetration, their application resulted in coagulation-related artefacts (Dagdeviren et al., 2024), including disrupted epithelial layers, shrinkage around renal tubules, and detachment of mucosal tissues. These results indicate that enhanced penetration speed does not inherently ensure good tissue preservation, and rapid-acting fixatives may jeopardise fine structural integrity (Miki et al., 2018).

The anatomical constraints, physiological traits, and observed fixation artefacts collectively suggest that standard immersion in formalin or other typical fixatives is inadequate for effectively preserving the microanatomy of aquatic specimens, particularly small aquatic fishes (George et al., 2016; Miki et al., 2018; Costa et al., 2021). Thus, there is an apparent need for specialised fixation approaches, including modified formulations, staged fixation techniques, or double-fixation protocols, specifically designed to address the constraints imposed by rapid postmortem autolysis and limited fixative diffusion. Optimising techniques is crucial for generating high-quality histological preparations and guaranteeing precise analyses of tissue structure and disease in fish and crustacean studies (Miwa, 2017; Miki et al., 2018).

Double-Fixation Method: Mechanism and Outcomes

Miki et al. (2018) introduced an improved fixation technique, a two-step sequential process aimed at mitigating rapid autolysis observed in fishes, particularly small aquatic species. The initial phase, or primary fixation, involved submerging specimens in 20% unbuffered formalin at 4°C for one hour. This technique aimed to rapidly inhibit enzymatic activity and preserve nuclear components before significant postmortem changes (Layton et al., 2019). After the swift initial stabilisation, a secondary fixation phase was implemented, during which tissues were immersed in either Bouin's fluid or Davidson's fluid, maintained at 4°C, for an additional five to six hours. This subsequent phase aimed to augment fixative penetration and enhance overall tissue morphology while reducing artefacts often associated with single-fixative methods (Dagdeviren et al., 2024).

The sequential technique produced numerous significant improvements in tissue preservation, which aligned with a previous study reporting a similar finding (Chaudhari et al., 2025). The gills exhibited consistently well-preserved lamellae, with much less epithelial lifting relative to specimens treated with traditional single-step techniques. The liver displayed negligible autolysis with only sporadic, minor localised degradation, indicating a significant enhancement compared to the marked hepatic deterioration commonly observed in fast-autolysing fish species. The intestinal tissues exhibited no signs of artificial space creation between the mucosa and submucosa, signifying enhanced structural cohesiveness and diminished mechanical artefact. Likewise, the kidney and epidermis exhibited less shrinkage and fewer artefact separations,

respectively, indicating that the sequential method offered more consistent stabilisation across various tissue types (Miki et al., 2018).

Of the combinations evaluated, the regimen employing 20% formalin, followed by Bouin's fluid, yielded the most consistently advantageous outcomes, resulting in enhanced preservation across nearly all assessed organs (Miki et al., 2018). This discovery indicates that the preliminary cross-linking attained with concentrated formalin, subsequently enhanced by the rapid penetrative and coagulative characteristics of Bouin's solution, offers a harmonious and synergistic method for regulating both autolysis and morphological deformation (Singh et al., 2019; Layton et al., 2019). All the findings for the fish samples are summarised in Table 1.

Table 1: Summary of single and double-fixation outcomes in the fish samples

Fish Samples			
Tissue	**Fixative Agent**	**Advantage**	**Limitation**
Gills	20% FS	Gill filaments and lamellae well-preserved	N/A
	20% FS & DS	Gill filaments and lamellae good-preserved	N/A
	20% FS & BS	Prevented epithelial lifting on the gill filaments and lamellae	Poorly separated gill filaments and lamellae
Liver	20% FS	N/A	Mild to moderate liver parenchyma autolytic changes vicinity to the gall bladder
	20% FS & DS	Liver parenchyma is well-preserved	Mild autolytic changes detected at the liver parenchyma periphery to the gall bladder
	20% FS & BS	Liver tissue is well-preserved	Mild autolysis of the liver parenchyma surrounding to the gall bladder
Intestine	20% FS	N/A	Mild to moderate autolysis detected in the intestinal tissue
	20% FS & DS	Intestinal tunics are well-preserved	N/A
	20% FS & BS	All the intestinal tunics are well-preserved	N/A
Kidney	20% FS	Renal parenchyma is well-preserved	N/A
	20% FS & DS	Renal parenchyma is preserved	Sporadic tubular shrinkage
	20% FS & BS	Renal parenchyma is preserved	Mild tubular shrinkage
Skin	20% FS	Skin tissue is well-preserved	N/A
	20% FS & DS	Good in preserving the fish skin tissue	Slight artifact cavitation at the epidermal-dermal junction
	20% FS & BS	Good in preserving the fish dermal tissue	Mild artifact spacing in between of epidermis and dermis layer

Note: FS: formalin solution; DS: Davison's solution; BS: Bouin's solution; N/A: Not applicable

The work underscores the essential role of successive fixation in the histological preparation of fishes, especially the small aquatic animals. This two-step technique efficiently addresses the shortcomings of individual fixatives by combining the initial quick inactivation of degradative enzymes with a subsequent phase of morphological stabilisation (Chaudhari et al., 2025). This method illustrates how the deliberate integration of complementary fixation methods can significantly improve tissue integrity, providing a practical and reliable alternative for researchers dealing with specimens susceptible to rapid postmortem deterioration (Miki et al., 2018; Layton et al., 2019).

Fixation Optimization in Crustacean: Multistep Fixation Approaches

Wild et al. (2025) performed a comparative investigation of three fixatives: 10% neutral buffered formalin (NBF), Bouin's fluid, and Davidson's solutions, to assess their efficacy in preserving crustacean tissues without alterations to the protocol. Their findings exhibited distinct variations in fixation performance. NBF yielded the most unfavourable results, characterised by ripping, shrinkage, and significant autolysis, signifying insufficient preservation for fragile aquatic specimens. Bouin's fluid reduced shrinkage but caused a considerable loss of nuclear detail, hence reducing its diagnostic value. Conversely, Davidson's fluid produced the most advantageous outcomes, distinguished by excellent chromatin visibility, negligible autolysis, and enhanced muscle integrity, rendering it the most efficacious of the three fixatives evaluated. This aligned with a recent study that reported a similar finding (Longakit et al., 2025).

The research furthermore investigated the impact of additional enzymatic digestion, particularly using trypsin, to alleviate the rigidity of exoskeletal components that hinder

microtomy in crustaceans (Nakamura et al., 2019). Despite its supposed advantages for sectioning, trypsin digestion repeatedly yielded adverse results. It expedited autolysis, particularly in metabolically active organs, including the hepatopancreas and pancreas, and led to widespread degradation of tissue integrity. The treatment did not produce noticeable enhancements in section quality, indicating that enzymatic digestion poses considerable risks without providing substantial technical benefits. Consequently, the scientists determined that enzymatic softening methods are inadvisable for delicate aquatic tissues (Ford et al., 2023).

The additional decalcification step was assessed as a potential strategy to enhance section smoothness and reduce microtome tearing in specimens characterised by significant mineralisation or chitinous features (Wild et al., 2025). Decalcification improved mechanical sectioning, resulting in smoother cuts and less tearing (Grunow et al., 2015). Nonetheless, this advantage led to increased autolysis, diminished staining specificity, and increased tissue fragility. These results demonstrate the intricate equilibrium between enabling fixative infiltration and maintaining tissue structure, highlighting that decalcification, although beneficial, must be executed judiciously and refined to prevent the degradation of histological integrity (Wild et al., 2025). All these findings are well summarised in Table 2.

The recommended approach for crustaceans involved targeted tissue exposure, appropriate fixation chemistry, and regulated decalcification (Costa et al., 2021; Wild et al., 2025). The optimal final tissue-quality scores were achieved using a procedure that included abdominal excision to reveal internal organs, fixation in Davidson's fluid, and subsequent decalcification. This combination improved fixative diffusion, reduced autolytic degradation, enhanced hematoxylin and eosin (H&E) staining quality, and enabled superior sectioning. These results underscore the need to customise fixation methods to the anatomical and metabolic characteristics of aquatic samples, indicating that deliberate alterations in specimen handling and fixation procedures can significantly enhance histological outcomes (Srinivasan et al., 2002; Wild et al., 2025).

Integrating Theoretical and Experimental Findings

Fixative Chemistry as Explanation for Observed Trends

A previous study elucidates that aldehyde fixatives (formalin and glutaraldehyde) function by generating hydroxymethyl adducts that stabilise proteins, albeit with delayed penetration (Layton et al., 2019). Coagulant fixatives (such as picric acid and acetic acid in Bouin's fluid) exhibit rapid penetration but may induce tissue atrophy and the extraction of macromolecules (Dagdeviren et al., 2024). These mechanistic insights align with experimental findings and can be summarised in Table 3.

Table 2: Summary of different fixative agents with additional step for crustacean samples

Crustacean Samples	
Fixative Agents and Additional Steps	**Outcomes**
10% NBF	i. Ripping the epithelial tissue ii. Cell and nucleus shrinkage iii. Significant tissue autolysis iv. Demonstared insufficient preservation
BS	i. Reduced cellular and nuclear shrinkage ii. Moderate nucleoplasm loosing
DS	i. Exceptional chromatin visibility ii. Very mild, sporadic autolysis iii. Enhanced muscle integrity histology
Digestion	i. Soften the exoskeleton and ease sectioning ii. Accelerate autolysis in hepatopancreas
Decalcification	i. Improve the sectioning process ii. Hasten the autolysis process iii. Reduce staining affinity to the tissue iv. Amplify tissue disintegration

Note: NBF: neutral-buffered formalin; BS: Bouin's solution; DS: Davidson's solution

Table 3: Mechanistic insights and corresponding experimental findings in fixative performance.

Observations	Authors' Opinions
Formalin alone is insufficient for fish and crustaceans	Slow penetration and lead to the delay inactivation of enzymes (Layton et al., 2019).
Bouin's & Davidson's solution cause epithelial lifting or shrinkage	Coagulating acids alter protein structure and tissue tension (Dagdeviren et al., 2024).
Double fixation improves tissue morphology	Sequential action will allow rapid stabilization and enhance deeper tissue penetration (Miki et al., 2019).
Decalcification in crustacean specimens hasten the autolysis process	Acidic solutions in the decalcification process accelerate hydrolytic degradation (Einbu et al., 2007).

Cross-Species Similarities in Fixation Challenges

Notwithstanding significant taxonomic disparities, fish and crustaceans demonstrate very analogous histology challenges (Miwa, 2017). Both populations have digestive organs that are very susceptible to rapid autolysis, requiring prompt intervention post-mortem to maintain structural integrity (Wenzlow et al., 2021). Moreover, exterior penetration barriers, like fish scales and the chitinous cuticles of crustaceans, obstruct the diffusion of aqueous fixatives, consequently hindering adequate tissue stabilisation (Grunow et al., 2015; Costa et al., 2021). The identical limits highlight the urgent necessity for rapid and efficient fixing to avert postmortem deterioration. Thus, researchers agree to arrive at the conclusion that tailored, species-specific fixation techniques are crucial for achieving accurate and reproducible histological outcomes in aquatic samples (Miki et al., 2018; Wild et al., 2025).

CONCLUSION

Fixation is an essential component in histology quality, and the unique biological traits of aquatic creatures require specialized methods for effective tissue preservation. The rapid postmortem autolysis, together with structural impediments like scales and chitinous exoskeletons, hinders the penetration and effectiveness of standard fixatives. This review synthesises fundamental histology concepts with contemporary experimental findings from the previous studies on fish and crustacean fixation to offer a thorough comprehension of fixation procedures appropriate for these taxa.

This review provides several significant conclusions. First, in accordance with histochemical principles, no single fixative is universally superior across all tissue types or species, highlighting the need for customised methodologies. Secondly, experimental studies indicate that double-fixation techniques, which generally consist of an initial immersion in formalin followed by Bouin's or Davidson's solution, significantly enhance tissue preservation in fish samples, particularly in small species, by minimising artefacts and improving morphological clarity. Third, research on crustaceans indicates that Davidson's solution is the most practical fixative, especially when abdomen excision precedes its application to expose internal organs and is succeeded by decalcification to enable microtomy (crucial for whole-body fixation). Fourth, enzymatic digestion methods, while designed to soften stiff tissues, are detrimental to fragile aquatic specimens as they hasten autolysis and compromise tissue architecture. The most effective fixation techniques exhibit common characteristics, such as fast enzyme inactivation, enhanced fixative penetration, and regulated chemical modification of tissue constituents.

Collectively, these results underscore the need for fixation techniques to be tailored to the organism's biological attributes, including body form, tissue composition, and autolysis vulnerability. Customized and standardized advanced techniques will markedly enhance the reliability and repeatability of histological investigations in fish and crustaceans. These developments will facilitate vital research domains like toxicology, aquaculture, developmental biology, and comparative physiology, where precise tissue preservation is crucial for significant interpretation and scientific progression.

CONFLICT OF INTEREST

The authors have agreed there is no conflict of interest in writing this review article.

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