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Methods for the Collection of Fish Mucus: A Systematic Review

Marta Fernandez Cunha[a], Ezequiel R. Coscueta[a], María Emilia Brassesco[a], Frederico Almada[b], David Gonçalves[c] and Manuela Pintado[a]

[a]Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Porto, Portugal; [b]MARE—Marine and Environmental Sciences Centre, ISPA Instituto Universitário de Ciências Psicológicas, Sociais e da Vida, Lisbon, Portugal; [c]Institute of Science and Environment, University of Saint Joseph, Macau, S.A.R., China

ABSTRACT

The aquatic environment holds a vast source of organisms that provide numerous opportunities to bioprospect new molecules. Notably, fish are producers of an epidermal mucus that offers protection against pathogens, making it a promising source of bioactive molecules. This source of molecules, however, has yet to be thoroughly explored, and particularly, optimization of methods for collection and study is needed. This review concentrates on the methods of mucus collection employed to secure high-quality samples, enabling the extraction and characterization of molecules with bioactive potential. A comprehensive search was conducted, and publications were selected based on the following criteria: (i) the mucus has been collected from the external body of the fish, not involving dissection or damage; (ii) mucus crude extracts have undergone a chemical or genetic characterization; (iii) mucus was used in bioactivity assays (e.g., antimicrobial or immune-related). Scraping, bagging, and absorption are the primary methods for collecting fish mucus. They were assessed based on fish handling, sample volume, and processing, including anesthesia and starvation. Scraping with a soft tool, such as cotton balls or sponges, proved most effective and minimized contamination, dilution, and injury risk. This review aids future studies of mucus composition and properties.

KEYWORDS

Bioprospection; bioactivity; skin mucus; fish immunity; aquatic organisms

Introduction

Over the last decades, there has been an increasing interest in exploring the aquatic ecosystem to discover new products (Lindequist 2016; Tiralongo et al. 2020). This trend has been fostered by increasing demand for innovative products for the health sector due to the selective pressures of infectious diseases and multiresistant pathogens (Rugină 2018). Within the diversity of aquatic animals, marine sponges, with more than 9000 described species (WORMS 2023), have been the most extensively explored group, representing 50% of current research for new products (Snelgrove 2016). In contrast, other organisms, such as vertebrates (e.g., fish), have been poorly explored, representing just 5% of the studies involving marine organisms until recently (Snelgrove 2016). There are more than 32,000 fish species (Nelson et al. 2016), representing a largely untapped resource for bioprospection. Fish is well known for its high nutritional value and has been promoted as a health enhancer with high-quality biofunctional proteins already explored for nutraceutical and pharmacological usage (Abdelhedi and Nasri 2019; Khan et al. 2020). This is related to the fact that a highly competitive and harmful environment surrounds fish (Cipolari et al. 2020; Yin et al. 2020), possessing unique characteristics for survival. Studies have shown remarkable physiological characteristics of fish epidermal mucus, with multiple ecological roles, including protection against mechanical impact, a chemical barrier against toxins and pathogens, intraspecific communication, and parental feeding (Reverter et al. 2018).

The fish body mucus is mainly composed of 95% water and mucins. Mucins are glycosylated proteins of high molecular weight which contribute to the viscosity of mucus. Depending on the degree of hydration, they can alter the rheological, viscoelastic, and adhesive properties (Roberts and Powell 2005; Guardiola et al. 2017; Fernández-Montero et al. 2021). Additionally, mucins contain several molecules with

CONTACT Ezequiel R. Coscueta ecoscueta@ucp.pt Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Rua Diogo Botelho 1327, 4169-005 Porto, Portugal.

bioactive functions, such as glycoproteins, proteolytic enzymes, lectins, galectins, lysozymes, immunoglobulins, C-reactive proteins, and antimicrobial peptides (Soltanian and Gholamhosseini 2019; Uyan et al. 2020). To study these molecules, researchers must collect the mucus from the fish, which involves handling live animals. Whenever possible, nondestructive and noninvasive techniques to collect mucus should be applied (Fæste et al. 2020). Therefore, it is crucial to have a collection method that avoids or reduces the contamination of samples with damaged epithelial cells or body secretions other than mucus. For example, in fish immunological studies, it is essential to differentiate between cellular skin and mucus matrix proteins (Tartor et al. 2020). This approach is also critical to study mucus properties such as antimicrobial, antioxidant, antihypertensive, or anti-inflammatory. Furthermore, proper sample processing is vital for accurate chemical analysis through chromatography techniques (e.g., high-performance liquid chromatography, size exclusion chromatography, liquid chromatography-tandem mass spectrometry) to identify molecules of interest or determine selected biological activities. The selected pre-processing aims to eliminate potential contaminants in the sample, increase concentration, and modify the sample to be suitable for detection and separation (Smith 2003).

Previous assessments of fish mucus properties (e.g., Reverter et al. 2018; Lee et al. 2020; Tiralongo et al. 2020) did not compare fish mucus collection techniques in terms of their effectiveness (e.g., total volume collected, sample concentration, sample integrity, and preservation method). This information, however, based on method standardization, is crucial for future comparative studies on mucus bioactivity.

A comprehensive literature review was conducted concerning mucus collection to tackle practical obstacles such as small mucus volumes, which impede bioactivity assessments. Thus, this review aims to present a comprehensive overview of fish mucus collection techniques and suggest a standardization of the pre-processing of mucus samples and storage conditions.

Within the last six years, there has been an increasing number of publications about fish mucus. A critical aspect of the research development is that most literature has been focused on fish immunology. That is probably related to the development of the aquaculture industry, a response to the increasing world population and demand for cheap animal protein (Adel et al. 2021). Aquaculture fish circulate in closed systems or nearby and are often subjected to stressful conditions and infectious diseases (Adel et al. 2021; Djordjevic et al. 2021). Bacterial and viral infection can lead to the death of all fish in the system, and the innate immune defenses of mucus play an essential role in fish survival (Akbari et al. 2021; Sridhar et al. 2021). Consequently, many studies tested different diets with plant extracts, bioactive compounds, and probiotics to enhance the immune system in fish. Several studies aimed to evaluate the enhancement of fish immunity by analyzing lysozyme, proteases, esterases, and antioxidant activities (Sanahuja et al. 2018; Reyes-Becerril et al. 2019; Hernández-Contreras et al. 2021; Mehrinakhi et al. 2021; Rashidian, Boldaji, et al. 2021).

Other studies focus on the mucus from the gastrointestinal tract and gills as a first line of innate immune defense triggered by mucosa-associated lymphoid tissues (MALTs) (Chieng et al. 2020; Mansour et al. 2020). Studies on the diet effects involving mucus from the gastrointestinal tract, however, require the sacrifice of the fish, which is not of interest to this article (Hamed et al. 2019). Furthermore, publications on histopathology and histology were excluded because these studies required skin removal instead of only mucus.

Classification of collection methods

The collection methods described in the literature fit into three main categories: scraping, bagging, and absorption (Figure 1). The scraping method consists of exerting a slight pressure on the surface of the fish skin using a tool (e.g., cell scraper) to scrape off the mucus (Hernández-Contreras et al. 2021). The bagging method consisted of placing the fish inside a polyethylene bag with a saline solution and handling the bag, massaging the fish for a few minutes to increase mucus production (Khoei 2021). The mucus collected by these two previous methods is usually centrifuged to remove possible contaminants. The absorption method involves covering the fish with medical wipes for a short time until thoroughly soaked. Then, the medical wipes are gently removed and placed in tubes for centrifugation to collect the mucus (Fæste et al. 2020; Tartor et al. 2020). The most common collection technique was the scraping method, which accounts for 58% of the papers analyzed in this review (Figure 1A).

Scraping

Scraping fish skin mucus includes tools such as spoons, scalpels, spatulas, microscope slides, cell scrapers, and cotton swabs. According to the current

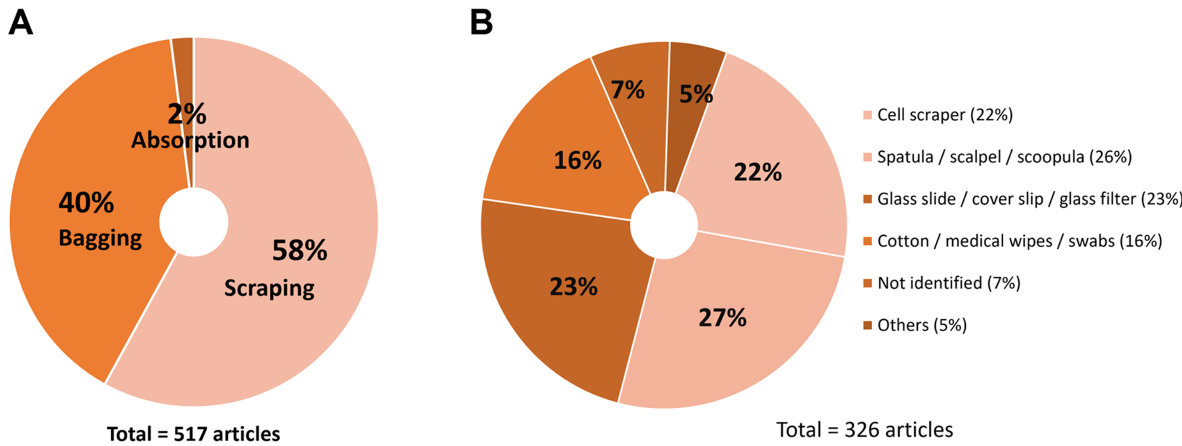

Figure 1. Pie chart depiction of: A) Literature-derived proportional representation of the three primary methods employed for mucus collection: scraping, bagging, and absorption. B) The proportion of articles within the 'scraping' category, segmented according to the tools employed: cell scraper, spatula/scalpel/scoopula, glass slide/microscope slide/cover slip/glass filter, cotton/medical wipes/swabs, unidentified tools, and others.

analysis, cell scrapers, spatulas, and glass slides were the most frequently used (Figure 1B). The skin is scraped from the dorsal-lateral surface of the fish, avoiding contamination of the sample by blood, urine-genital, or intestinal excretions (Díaz et al. 2019; Guluarte et al. 2019; Pelusio et al. 2022). Using tools such as medical wipes and sponges may be a better option for scraping mucus because it has proven to be gentler and less stressful than a spatula (Carbajal et al. 2019; Tartor et al. 2020). It has been suggested that the process should take no longer than two minutes to prevent the degradation of mucus metabolites (Firmino et al. 2021). Scraping the fish for mucus collection also stimulates the fish to secrete extra mucus (Salimi Khorshidi et al. 2021). The category of others includes electrical stimulation to induce mucus secretion before scraping. Liu et al. (2019) scraped the mucus from *Boleophthalmus pectinirostris* using an electric tool based on the method described by Tyler et al. (1992). The authors reported that the stimulus strength varies with animal size, skin thickness, and conductivity.

Bagging

The bagging technique involves putting the fish in a plastic bag with a saline solution and massaging it to release the mucus. Factors such as the solution and the time the fish remains in the bag may vary between studies. Rufchaei et al. (2021) used a 50 mM NaCl solution for freshwater fish and 100 mM NaCl for seawater fish. Fish are typically kept in the bag for one to two minutes during the massage (Van Doan et al. 2020b; Ghafarifarsani et al. 2021), although some authors have reported more extended periods of 10–20 min (Syed Salman et al. 2020).

Absorption

In the absorption method, the fish is placed on its ventral side, cotton wipes are placed on the body surface, from the caudal peduncle to the head, and removed only when saturated (Ouyang et al. 2020; Tartor et al. 2020; Wen et al. 2020). Also, soaking paper has been used as an alternative for collecting mucus (Saha et al. 2017).

Homogenization solution and centrifugation

After collection, mucus samples are usually homogenized with a range of stabilizing solutions: saline solutions, buffered saline solutions, or water. Homogenization is generally followed by centrifugation to discard scales and debris, using the supernatant for analysis (Hajirezaee et al. 2020). Table 1 outlines the homogenization conditions and centrifugation settings for processing samples using the scraping and bagging method.

Specific solutions and centrifugation conditions are commonly applied for each tool feasible in the scraping method. For example, cell scrappers are usually used with tris-buffered saline (TBS, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl), with homogenization and centrifugation settings as 2000 g, 10 min, 4 °C (Guardiola et al. 2017; de Mattos et al. 2019; Ceballos-Francisco et al. 2020). Other procedures in mucus processing include filtration with 0.45 or 0.22 µm filters (Escribano et al. 2020; Zhao et al.

Table 1. Scraping and Bagging method procedure.

Tool	Homogenization Solution	Centrifugation	Filtration	Lyophilization	References
Cell Scraper (Scraping)	Tris-buffered saline (TBS, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl)	2000 g, 10 min, 4 °C			(de Mattos et al. 2019)
		3000 rpm, 10 min, 4 °C			(Franco-Martinez et al. 2019)
		500 g, 10 min, 4 °C			(Guardiola et al. 2014)
				X	(Guardiola et al. 2017; Reyes-Becerril et al. 2017b)
		500 g, 4 °C			(Hernández-Contreras et al. 2021)
		1400 g, 10 min, 4 °C			(Reyes-Becerril et al. 2019)
		1500 rpm, 10 min, 4 °C			(Brandts et al. 2018; Cerezuela et al. 2016; Espinosa et al. 2017)
				X	(Espinosa-Ruiz et al. 2021; Guardiola et al. 2014; Oliveira et al. 2018; Reyes-Becerril et al. 2017a)
	2.5 volume of 0.01 M phosphate buffered saline (PBS)	15000 rpm, 20 min, 4 °C		X	(Alijani Ardeshir et al. 2020)
	500 µL sterile water	2000g, 10 min, 4 °C			(Jakiul Islam et al. 2021)
	PBS	800 g, 10 min	0.22 µm		(Lange et al. 2018)
		12,000 g, 10 min, 4 °C			(Hamed et al. 2019)
			X		(Lange et al. 2020)
		X			(Sequeiros et al. 2022)
			1.5, 0.45 and 0.22 µm		(Farmer et al. 2021)
	5.0 mL of 10 mM Tris-Cl buffer (pH 7.0)	X			(Lange and Webster 2017)
	0.67% NaCl			X	(Nigam et al. 2017)
	150 µL of phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4 \cdot 12H_2O$, 1.47 mM KH_2PO_4; pH 7.5)	1500 g, 10 min, 4 °C			(De Mercado et al. 2018)
	50 mM NaCl	1500 g, 10 min, 4 °C			(Taheri Mirghaed et al. 2020; M. Yousefi et al. 2021)
	Sterile sea water	2000 rpm, 10 min, 4 °C			(Piazzese et al. 2019)
		2000 g, 10 min, 4 °C			(Campos-Sánchez et al. 2021)
	No Solution	12,000 g, 10 min, 4 °C			(Espinosa and Esteban 2020; Espinosa-Ruíz and Esteban 2021)
		400 g, 10 min, 4 °C			(García Beltrán et al. 2020)
		300 g, 10 min, 4 °C			(Espinosa et al. 2020)
		1400 g, 10 min, 4 °C			(Chen et al. 2020; Chen et al. 2020; Tapia-Paniagua et al. 2018)
		1500 g, 10 mim, 4 °C			(Hu et al. 2021)
				X	(Almaida-Pagán et al. 2018)
		2000 g, 10 min, 4 °C			(Cámara-Ruiz et al. 2021; Carbajal et al. 2019; Ceballos-Francisco et al. 2020; Conforto et al. 2021; Dawood et al. 2020; García Beltrán et al. 2019; Guardiola et al. 2017; Valero et al. 2019)
			0.2 µm		(Escribano et al. 2020; Guardiola et al. 2019)
		13,200 g, 20 min, 4 °C			(Xiong et al. 2020)

(*Continued*)

Table 1. Continued.

Tool	Homogenization Solution	Centrifugation	Filtration	Lyophilization	References
		3000 g, 10 min, 4 °C			(Cai et al. 2020; Guardiola et al. 2018)
					(Djordjevic et al. 2021; Parma et al. 2020; Ramos-Pinto et al. 2021; Suzuki et al. 2017; F.-E. Sylvain and Derome 2017)
Glass slide/microscope slide/slide (scraping)	0.85% Normal Saline	10,000 rpm, 20 min			(Gong et al. 2021)
	10 mM sodium phosphate buffer, pH 6.5, with 0.1 mM phenylmethylsulfonyl fluoride (PMSF)				(Benktander et al. 2019, 2021)
	RNase-free PBS		Sterile membrane 0.45 µm		(N. Zhao et al. 2020, 2021)
	Phosphate buffer, pH 7	1500 g, 5 min			(Rashidian, Boldaji, et al. 2021)
	1:1 filtered and autoclaved salt water				(Fernández-Montero et al. 2019)
	0.5 mL sterile PBS	4500 rpm, 20 min, 4 °C			(Sun et al. 2020)
		4000 rpm, 10 min, 4 °C			(Tang et al. 2017)
	1:1 sterile phosphate-buffered saline (PBS – 0.01 M, pH 7.2)	7000 g, 10 min			(Sutili et al. 2020)
	TRI reagent				(Parida et al. 2018)
	PBS (0.01 mM, pH 7.4) with EDTA and PFSM				(Fei et al. 2018)
	1:3 50 mM NaCl	18,000 g, 40 min, 4 °C			(Serradell et al. 2020)
Scalpel/ Spatula/ Scoopula (Scraping)	1× phosphate-buffered saline and sample buffer (7 M Urea, 2 M Thiourea, 2 mM PMSF)	15,000 g, 15 min			(Nurhikmah et al. 2022)
	Tris-buffered saline (TBS, 50 mM Tris–HCl, pH 8.0, 150 mM NaCl)	4000 rpm, 30 min, 4 ° C		X	(Palaksha et al. 2008)
		500 g, 10 min, 4 °C		X	(Rahimnejad et al. 2018)
		30,000 g, 15 min, 4 °C	Whatman No. 1 filter paper		(Adel et al. 2021; Adel et al. 2020; Adel et al. 2021; Akbari et al. 2021; Gholamhosseini, Adel et al. 2020; Saeidi Asl et al. 2017)
		3000 g, 15 min, 4 °C			(Gholamhosseini, Hosseinzadeh, et al. 2020)
	Ultrapure water at 4 °C	14,000 g, 30 min, 4 °C			(Borges et al. 2018)
	Ultrapure water	14,000 g, 15 min, 4 °C			(Chieng et al. 2020)
		850 g, 5 min, 4 °C	0.22 µm		(Levipan and Avendaño-Herrera 2021)
		2200 g, 10 min			(Americus et al. 2020)
		20,000 g, 20 min, 4 °C	Advantec No. 1 filter paper	X	(Ueki et al. 2019)
	0.05 M Tris–HCl buffer (pH 7.4)	18,800 g, 10 min, 4 °C			(Nagashima et al. 2021)
	Phosphate-Saline Solution at pH 7.4	16,000 g, 16 min, 4 °C			(Pimentel et al. 2017)
	Tris-buffered saline	500 g, 30 min, 4 °C	Whatman no.1 filter paper		(Mehrinakhi et al. 2021)
	Sterile PBS	2×(20,000 g, 30 min, 4 °C)	0.22 µm sterile filter		(J. Li et al. 2021)
		12,500 g, 10 min, 4 °C			(Rodríguez et al. 2021)
		8000 g, 10 min, 4 °C			(Chirapongsatonkul et al. 2019)
	PBS	12,000 rpm, 10 min, 4 °C	0.45 µm Millipore filter		(Giri et al. 2012)

(*Continued*)

Table 1. Continued.

Tool	Homogenization Solution	Centrifugation	Filtration	Lyophilization	References
		20,000 g, 30 min, 4 °C	0.45 and 0.2 µm pore size filters		(Lin et al. 2017)
		12,000 g, 30 min, 4 °C			(Xu et al. 2021)
	1 volume of sterile normal saline (9‰)	2000 g, 10 min, 4 °C			(Soltanian and Gholamhosseini 2019)
	Sterile saline solution	12,000 rpm, 5 min			(Fernández-Álvarez et al. 2019)
	Sterile sea water	2×(27,000 g, 15 min, 4 °C)			(Chabrillón et al. 2005)
		2000 g, 10 min, 4 °C			(Maldonado-Garcia et al. 2019)
	6% acetonitrile for 24 h at −80 °C	2h		X	(Charlie-Silva et al. 2019)
	10 µL of protease inhibitor cocktail	2500 g (3000 rpm), 15 min, 4 °C			(Ferreira et al. 2019)
	4 volumes of pre-heated distilled water with 1% acetic acid in a water bath for 5 min	20,000g, 30 min, 4 °C			(Go et al. 2019)
	20 mM phosphate buffer saline (PBS; pH 7.6)	20,000g, 10 min			(Tsutsui et al. 2018)
	0.9 mol/L sodium citrate, 0.1 mol/L Pipes (piperazine-N,N′-bis(2-ethanesulfonic acid)), stabilization buffer (pH 6.7)				(Schorno et al. 2018)
	Physiological saline (0.67% NaCl) at 4 °C for 10 min	10,000g, 10 min, 4 °C			(Srivastava et al. 2018)
	95% Ethanol				(Domingues et al. 2019)
	No solution	1000 g, 5 min, 4 °C			(Almeida et al. 2019)
		sequentially at 40 g, 400 g, and 10,000 g	0.45 µm		(Kelly et al. 2017)
		14,000 g, 15 min, 4 °C			(Fernández-Alacid et al. 2021; Firmino et al. 2021; Herrera et al. 2020; Sanahuja et al. 2019)
		1400g, 10 min, 4 °C			(Ceballos-Francisco et al. 2017)
		14,000 g			(Fernández-Alacid et al. 2019b; Ferreira et al. 2019)
		10,000 g, 6 min, 4 °C			(Palma et al. 2019)
		X	0.45 µm filter		(Burbank et al. 2017)
		X			(Zheng et al. 2019)
		14,000 g, 10 min, 4 °C	0.45 µm and 0.20 µm pore size filters		(Papadopoulou et al. 2017)
		10,000g, 10 min			(F. Yin et al. 2018)
		4500 rpm, 45 min			(Yildirim-Aksoy et al. 2018)
		12,000 rpm, 10 min, 4 °C			(Fazio et al. 2021)
		12,000 g, 10 min, 4 °C			(Abbas et al. 2020; A. A. N. Nurul et al. 2020; A. N. A. Nurul et al. 2019)
		1500 g, 15 min, 4 °C			(Vakili et al. 2021)
		1500 g, 10 min, 4 °C			(R. Safari, Hoseinifar, Imanpour, et al. 2020; Soltanian et al. 2018; Soltanian and Gholamhosseini 2018)
		1500 g, 15 min			(S. Yousefi et al. 2020)
		8000 rpm, 10 min			(M. Patel et al. 2020)
		5000 rpm, 10 min		X	(Hitit et al. 2020)
		5 min, 4 °C	Dissolved sterile milli-Q water (1:1) and filtered 0.22 µm		(Levipan et al. 2020)

(*Continued*)

Table 1. Continued.

Tool	Homogenization Solution	Centrifugation	Filtration	Lyophilization	References
		2600g, 30 min, 4 °C			(Ritchie et al. 2017)
		1500 rpm, 10 min, 4 °C		X	(Al-Rasheed et al. 2018)
				X	(Abdel-Shafi et al. 2019; Kwan and Ismail 2018; Manoharan et al. 2017)
					(Ahmed et al. 2021; Almeida et al. 2019; Bahamonde et al. 2019; Cardona et al. 2022; Charoenwai et al. 2021; Chaudhary et al. 2018; Fæste et al. 2020; Fernández-Alacid et al. 2018; Fernández-Montero et al. 2021; Hodkovicova et al. 2019; Honda et al. 2018; Igarashi et al. 2017; Y. Jiang et al. 2019; Kroska et al. 2019; Kumari et al. 2019; K. Li et al. 2017; Liamnimitr et al. 2018; Machado et al. 2021; Magnadóttir et al. 2018; Magnadóttir et al. 2018; Micallef et al. 2017; Montelongo-Alfaro et al. 2019; Nolan and Britton 2018; Ordóñez-Grande et al. 2020; Padra et al. 2019; Pérez-Sánchez et al. 2017; Phusantisampan et al. 2020; Ponce et al. 2021; Rajan et al. 2017; Reyes-López et al. 2020; Richards et al. 2017; Rosli et al. 2019; Ruiz-Rodríguez et al. 2020; Saleh et al. 2018, 2021; Salimi Khorshidi et al. 2021; Torrecillas et al. 2019; Uren Webster et al. 2020; Uyan et al. 2020; Vaz Farias et al. 2020; Weththasinghe et al. 2021; Winter, Nolan, et al. 2019; Winter, Nyqvist, et al. 2019)
Cotton/ Medical Wipes/ Paper filter/ swabs (Scraping)	1 mL of PBS (pH 7.4)	2000 g, 10 min, 4 °C			(Dawood et al. 2016; Yan et al. 2017; Zaineldin et al. 2021)
		3000 g, 5 min, −4 °C			(Dawood et al. 2015; Dawood et al. 2017; El Basuini et al. 2021; Y. Zhao et al. 2017)
		3000 g, 5 min, −4 °C (filter tube)			(Dawood et al. 2019; Dawood et al. 2017; Zaineldin et al. 2018)
					(Dawood et al. 2017)
	PBS	12,000 rpm, 10 min, 4 °C			(Zhi et al. 2020)
		10,000 rpm, 10 min			(Cid García et al. 2020)
	PBS (pH 7.2)		dialysis membrane (12 kDa cut off) at 4 °C overnight		(Babu et al. 2017)
	200 µL of Xpedition Lysis/ Stabilization Solution				(Terova et al. 2021)
	500 µl of sterile water				(Murphy et al. 2020)
	3 mL of PBS with 0.02% (w/v) of sodium azide, overnight chilled at 4 °C	3000 g, 10 min			(Nor et al. 2020)
	70% ethanol				(Ek-Huchim et al. 2019)

(*Continued*)

Table 1. Continued.

Tool		Homogenization Solution	Centrifugation	Filtration	Lyophilization	References
		50 mL of clean seawater	60 rpm, 40 min, 23 °C			(Charpentier et al. 2019; Forward and Rittschof 1999)
		0.2 mL of PBS and protease inhibitor cocktail	10,000g, 15 min, 4 °C			(C. Zhang et al. 2022)
		No solution	500 g,10 min, 4 °C (0.22 µm-filter in the Spin-X® tubes)			(Fæste et al. 2020; Ivanova et al. 2018)
			X	\		(Yokoyama et al. 2019)
					Dried at 60 °C for 48 h	(Maruyama et al. 2017; Shigeta et al. 2017)
						(Berger and Preisfeld 2018; Cano et al. 2020; Díaz et al. 2019; Gadoin et al. 2021; Hamilton et al. 2020; Jolodar 2017; Llewellyn et al. 2017; Minniti et al. 2017; Montenegro et al. 2020; Mosley et al. 2018; Müller et al. 2021; Riepe et al. 2021; Roux et al. 2019; F.-É. Sylvain et al. 2019; F.-E. Sylvain et al. 2020; Taslima et al. 2017; Tilley et al. 2020; Uren Webster et al. 2020)
Not identified		1:1 sterile saline solution (0.85%)	2655 g, 5 min	0.22 µm		(Matanza et al. 2021)
		0.9% saline solution	1190 g	0.22 µm		(Coelho Thomazi et al. 2020)
		4 volumes Tris-buffered saline (50 mM Tris HCl, pH 8.0, 150 mM NaCl)?	1792 g, 30 min, 4 °C	Whatman no.1 filter paper		(Mousavi et al. 2021)
			1500 g, 10 min, 4 °C			(Gobi et al. 2018)
		1 mM PMSF and protease inhibitor mixture, pH 7.2 (incubated slight shaking at 4 °C overnight)	10,000 g, 10 min, 4 °C	0.45 µm		(Fu et al. 2021)
		4% SDS and cocktail (ThermoFisher Scientific, USA)	20 min, 4 °C			(Huang et al. 2021)
		2 volumes of distilled water	13,000 rpm, 30 min		X	(Hilles et al. 2019; Hilles, Mahmood, Kaderi, et al. 2019)
		Potassium phosphate buffer (20mM, pH = 7.5)	1000 g, 10 min			(Goulart et al. 2018)
		Phosphate buffered saline (PBS)				(Monteiro dos Santos et al. 2019)
		Ultra-pure water	13,000 rpm, 30 min, 4 °C			(Hilles et al. 2022)
		1.5 mL RNAlater™				(Lorgen-Ritchie et al. 2022)
		No solution	10,000 g, 15 min, 4 °C			(Mansour et al. 2018, 2020)
			12,000 g, 10 min, 4 °C			(S. Liu et al. 2019)
			400 g, 10 min, 4 °C			(D.-X. Zhang et al. 2019)
					X	(Al-Rasheed et al. 2020)
						(Cordero et al. 2017; da Silva et al. 2021; Djordjevic et al. 2021; Karlsen et al. 2017; Van et al. 2017)
Others (e.g., hand, inoculating loop and forceps) (Scraping)	Spoon		12,000 rpm, 4 °C	0.22 µm filter		(Zhou et al. 2021)
		200 µL of sterile PBS	5000 rpm, 20 min, 4 °C			(H. Jiang et al. 2017)

(*Continued*)

Table 1. Continued.

Tool		Homogenization Solution	Centrifugation	Filtration	Lyophilization	References
	Hand					(Difford et al. 2022; Ikert et al. 2021; Spychalski et al. 2020)
		Phosphate buffered saline (PBS) solution				(Sadoul and Geffroy 2019)
	Forceps					(Bulloch et al. 2020; Heimroth et al. 2018)
	Plastic tab/ Plastic scoop					(Bulfon et al. 2020; L. Yin et al. 2020)
	Electrode			dialysis (MWCO: 1 kDa)	X	(H.-H. Liu et al. 2019)
	Sponge		15,000 rpm, 15 min		X	(Kumar et al. 2019)
	Inoculating loop	DNA extraction buffer (200 µL TE: 10 mM Tris-HCl pH 8, 1 mM EDTA)				(Galbraith et al. 2018)
		Hank balanced salt solution (HBSS) viral transport media				(Leis et al. 2018)
	Microcentrifuge tube					(Caballero et al. 2020)
Plastic bag (Bagging)		5 mL of 50 mM NaCl	3500 rpm, 10 min, 4 °C			(Hoseinifar et al. 2018)
			1500 g, 10 min, 4 °C			(Ahmadniaye Motlagh et al. 2020; Arani et al. 2021; Hoseinifar, Safari, et al. 2017)
		10 mL of 50 mM NaCl	1500 g, 10 min, 4 °C			(Bisht et al. 2020; Doan et al. 2019; Doan et al. 2020; Doan et al. 2020; Ghafarifarsani et al. 2021; Heydari et al. 2020; Hoseinifar et al. 2021; Hoseinifar, Hosseini, et al. 2019; Hoseinifar, Khodadadian Zou, et al. 2019; Hoseinifar, Sohrabi, et al. 2019; Hosseini et al. 2020; Hosseini Shekarabi et al. 2021; Khodadadian Zou et al. 2016; Khoei 2021; Kurian et al. 2020; Paknejad et al. 2020; Rashmeei et al. 2020; R. Safari et al. 2017a; Sarhadi et al. 2020; Shiry et al. 2020; Srichaiyo et al. 2020; Srichaiyo et al. 2020; Tippayadara et al. 2021; Vali et al. 2020; Van Doan et al. 2018; Van Doan et al. 2019a; Van Doan et al. 2019b; Van Doan et al. 2019c C. Wang et al. 2020)
						(Abarike et al. 2018; Doan et al. 2017; Ghafarifarsani, Hoseinifar, Aftabgard, et al. 2022; Hoseinifar, Ahmadi, et al. 2017; Hoseinifar, Khodadadian Zou, et al. 2017; Kuebutornye et al. 2020; Mansouri Taee et al. 2017; Mirghaed et al. 2018; Modanloo et al. 2017; Mohammadi, Rafiee, and Abdelrahman 2020; R. Safari et al. 2017b; R. Safari, Hoseinifar, Van Doan, et al. 2017; Van Doan et al. 2018; R.-F. Wang et al. 2022)
				0.45 µm		(Giri et al. 2020, 2021)
					X	(Mohammadi, Adorian et al. 2020)

(*Continued*)

Table 1. Continued.

Tool	Homogenization Solution	Centrifugation	Filtration	Lyophilization	References
		1792.12 g, 10 min, 4 °C			(Hajirezaee and Hossein Khanjani 2021)
		4192.5 g, 10 min, 4 °C			(Hajirezaee et al. 2020)
		1500 rpm, 10 min, 4 °C			(Qamar et al. 2020; Sherif et al. 2022)
		450 g, 10 min, 4 °C			(Jakab Sándor et al. 2018)
		5000 g, 10 min, 4 °C			(Mohammadi, Rafiee, El Basuini, Abdel-Latif, et al. 2020; Mohammadi, Rafiee, El Basuini, Van Doan, et al. 2020)
		4000 g, 4 °C			(Faheem et al. 2020)
		3000 g, 10 mi, 4 °C			(Jasim et al. 2022)
		Non-specific			(R. Safari, Hoseinifar, Dadar, et al. 2020)
					(Adorian et al. 2019, 2020; Hoseinifar, Hosseini, et al. 2019; Hoseinifar, Jahazi, et al. 2020; Hoseinifar, Shakouri, Yousefi, et al. 2020; Jahazi et al. 2020; Karimi et al. 2020; Mohammadiazarm and Maniat 2021; Zeynali et al. 2020)
	3.33 mL of 50 µM NaCl				(Hasan et al. 2018)
	1 mL of PBS 1X				(Nhu et al. 2019)
	8 mL of 50 mM NaCl				(Oroji et al. 2021)
	2 mL of 50 mM NaCl				(Zarei et al. 2021)
		1600 g, 15 min, 4 °C			(Abdollahi et al. 2019)
	2 mL of 50 mM of NaCl	1500 g, 10 min, 4 °C			(Lumsangkul et al. 2021; Sridhar et al. 2021b; Van Doan et al. 2021; Van Doan et al. 2021a; Van Doan et al. 2021b; Van Doan et al. 2021c; Van Doan et al. 2021d)
		1500 g, 30 min, 4 °C	0.45 µm		(H.-P. Zhang et al. 2020)
		1500 rpm, 10 min			(Vazirzadeh et al. 2019)
	NaCl (50 mM; 5 mL/g fish)	1500 g, 10 min, 4 °C			(Ahmadniaye Motlagh et al. 2020; O. Safari et al. 2019; O. Safari and Sarkheil 2018)
	10 mL of 50 mM NaCl for freshwater and 100 mM NaCl for seawater	1500 g, 10 min, 4 °C		X	(Sridhar et al. 2021c)
		1500 g, 10 min, 4 °C			(Doan et al. 2018; Rufchaei et al. 2021; Subramanian et al. 2007)
					(Subramanian et al. 2008)
	100 mM NaCl				(Chinnadurai et al. 2020, 2021)
	10 mL of 50 M NaCl	1500 rpm, 10 min			(Ahmadniaye Motlagh et al. 2020)
	5 mL of Tris-buffered saline (10 mM Tris base, 0.5 M NaCl pH 7.5)	4000 g, 15 min			(Hoare et al. 2017)
	5mL of 100 mM ammonium bicarbonate (NH_4HCO_3) pH 7.8	2730 g, 15 min, 4 °C			(Ezatrahimi et al. 2019; Ross et al. 2000; Van Doan et al. 2020b)
		2730 g, 15 min, 4 °C	0.22 µm		(Midhun et al. 2018; Ross et al. 2000)
		12,000 g, 15 min, 4 °C	0.22 µm		(Midhun et al. 2017)
	PBS buffer [10 mM Na_2HPO_4/NaH_2PO_4 (pH7.2), 205 mM NaCl, 1.5 mM EDTA, 1 mM DTT]	20,000 g, 20 min, 4 °C & 7500 g for 90 min at 4 °C (after fitration)	Microsep 3K filter		(Mori et al. 2021)

(*Continued*)

Table 1. Continued.

Tool	Homogenization Solution	Centrifugation	Filtration	Lyophilization	References
	1 mL of Phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4)	4000 g, 10 min			(Mai et al. 2021)
	1 mL of sterile phosphate-buffered saline (PBS, pH = 7.4)	10,000 g, 10 min, 4 °C			(J. R. Khan et al. 2018)
	1 mL of phosphate-buffered saline (PBS, pH = 7.4)				(Cohen et al. 2018)
	10 mL of sodium phosphate buffer (PBS, 40 mM, pH = 7.4, 50 mM NaCl)	2860 g, 30 min, 4 °C		X	(Abolfathi et al. 2020)
	1 mL of 0.1 M Phosphate-Buffered Saline (PBS) buffer, pH 7.4 with 5 mM sodium azide, 0.1 mM phenylmethylsulphonyl fluoride and 20 mM 2-mercaptoethanol	15,000 g, 10 min, 4 °C			(Tarnawska et al. 2019)
	0.2 mL of sterile PBS + 1 mM phenylmethylsulfonyl fluoride (PMSF)	2000 g, 15 min, 4 °C			(Kole et al. 2019b, 2019a; Qadiri et al. 2019)
	0.5 mL of protease inhibitor buffer (1 × PBS, containing 1 mM phenylmethylsulfonyl fluoride and 0.5% bovine serum albumin, pH 7.2)	12,000 g, 15 min			(Sheng et al. 2019)
	One volume of chilled sterile PBS (pH = 7.4)	10,000 g, 10 min, 4 °C			(Roux et al. 2019)
	5 mL of PBS mixed with protease inhibitors	400 g, 10 min, 4 °C			(Etayo et al. 2022)
	5–10 mL ice-cold sterile formulated water (FW)	800 g	1.2, 0.8, 0.45 and 0.22 µm		(Shoemaker et al. 2018)
	10 mL ice-cold sterile physiological saline	800 g, 5 min, 4 °C			(Dhowlaghar et al. 2018; Dhowlaghar et al. 2018)
	10 mL sodium salt	800 g, 5 min, 4 °C			(Hoseinifar, Shakouri, Doan et al. 2020)
		10 min			(Van Doan et al. 2020a)
					(Dhowlaghar et al. 2018)
	15 mL of physiological serum	3500 g, 10 min			(Rashidian, Abedian Kenari, et al. 2021)
	50 mL of physiological serum	10,000 g, 5 min, 4 °C			(Rashidian, Lazado, et al. 2021)
	9 mL of sterilized seawater				(Minniti et al. 2019)
	No Solution	18,000 g, 15 min			(Shibuya et al. 2019)
		1600 g, 10 min, 4 °C			(Ghafarifarsani, Hoseinifar, Sheikhlar, et al. 2022)
		12,000 rpm, 4 °C	0.22 µm		(Zhou et al. 2021)
		1500 g, 10 min, 4 °C			(Sridhar et al. 2021a)
				X	(Landeira-Dabarca et al. 2019)
					(D. M. Patel and Brinchmann 2017; Syed Salman et al. 2020)
Beakers/ Flasks (Bagging)	5 mL of 100 mM ammonium bicarbonate buffer (pH 7.8) for 10 min	12,000 g, 15 min, 4 °C	0.22 µm		(M. I. R. Khan et al. 2021; M. I. R. Khan et al. 2021)
	10–50 mL of sterile PBS for 20 min		Five filters: 1- and 0.22 µm		(Carda-Diéguez et al. 2017)
			0.8 and 0.45 µm		(Guo et al. 2019)

2020) and lyophilization (Reyes-Becerril et al. 2017a; Alijani Ardeshir et al. 2020). Levipan et al. (2020) used 0.22 µm filters to sterilize the mucus samples, testing 10 µL on trypticase soy agar and sheep blood plates to check for any bacterial contamination. de Mattos et al. (2019) used lyophilized mucus and resuspended it in 1:5 of 50 mM carbonate–bicarbonate buffer, pH 9.6, for total IgM analysis. In addition, other procedures are adopted according to the tool, such as collecting the mucus from a sponge by placing it into a syringe cylinder and compressing the barrel to extract it. Then, the collected mucus is centrifuged at 2000 g for 10 min (Carbajal et al. 2019). Leng et al. (2022) have adopted the placement of the collected mucus in a boiling water bath for 10 min before centrifugation to inhibit proteolysis. Some authors suggest storing the mucus at −80 °C until analysis to avoid protein degradation and bacterial growth (Kelly et al. 2017; Gholamhosseini, Adel et al. 2020).

For the bagging method, the most common solution was 50 mM of NaCl with volumes between 2 and 15 mL added to the bag (Rashmeei et al. 2020; Oroji et al. 2021; Zarei et al. 2021). Alternative solutions explored for solubilizing mucus were PBS buffer [10 mM Na_2HPO_4/NaH_2PO_4 (pH7.2), 205 mM NaCl, 1.5 mM EDTA, 1 mM DTT], 100 mM ammonium bicarbonate (NH_4HCO_3) with a pH of 7.8, and protease inhibitor buffer (1 × PBS, containing 1 mM phenylmethylsulfonyl fluoride and 0.5% bovine serum albumin, pH 7.2). A protease inhibitor is essential to prevent protein degradation and ensure sample integrity. Another aspect to consider, reported by Rashidian, Lazado, et al. (2021), is guaranteeing to carry out the procedures on ice. Once the mucus is homogenized, centrifugation settings of 1500 g for 10 min at 4 °C were the most commonly used, followed by storage of the mucus sample at −80 °C (Hoseinifar et al. 2019).

In some studies, cotton wipes were placed in tube filters with 0.22 or 0.45 µm pore sizes after absorbing the mucus and centrifuged at 13,000 g or 500 g for 10 min at 4 °C. The filtered mucus was then stored at −80 °C until further use (Ivanova et al. 2018; Fæste et al. 2020; Tartor et al. 2020).

Returning to Table 1, one can see that the analysis highlights an inconsistency in reporting specific conditions and procedures. The significant heterogeneity in the applied procedures adds a layer of complexity in comparing outcomes across these investigations. This lack of standardization presents a substantial challenge to the field, impeding direct comparisons between studies and potentially affecting the reproducibility of results. A standardized protocol for fish mucus collection would contribute substantially to the field by enabling more consistent outcomes, facilitating comparison across studies, and ensuring that results could be accurately replicated in various labs.

Mucus volume

Another essential issue is knowing the collection volumes to guarantee enough mucus for the planned studies. For instance, the volumes obtained by Bahamonde et al. (2019) using a spatula to scrape the mucus were between 0.1 and 0.3 mL per individual for *Percilia irwini* specie. Other researchers scraping the mucus with a glass slide collected nearly 2 mL for *Solea senegalensis* and *Sparus aurata* (Fernández-Alacid et al. 2019a; Firmino et al. 2021). These volumes depend on the fish size and the total surface available for collection. The above volumes were collected from juveniles with a body weight range of 40–105 g. In another example, Gholamhosseini, Hosseinzadeh, et al. (2020) collected 0.6 mL of pooled mucus from six *Rutilus frisii kutum* fish weighing approximately 6 g with a spatula.

Likewise, Fazio et al. (2021) collected 3–5 mL from fish *Labeo rohita* weighing 435 g at the beginning of the experiment using a plastic spatula. Comparing Fazio et al. (2021) with previous studies, it becomes evident that larger fish usually translate into larger mucus volume collected. All these previous studies measured the volumes of collected mucus without the homogenization solution. Volume variations may also relate to stress levels, as more stress could lead to more mucus secretion and the number of scrapes during collection. In some studies, the researchers pooled the mucus from two to three fish and collected it ten times a day at regular intervals to have enough mucus for their analysis (Kumari et al. 2019; Torrecillas et al. 2019; Herrera et al. 2020). In addition, fish skin physiology can also affect the volume of mucus. The fish *Anguilla anguilla* is an example since it has skin with the absence of macroscopic scales and a thick layer of mucus (Carda-Diéguez et al. 2017), unlike *Salmo salar*, whose skin is composed of a superficial scaly layer (Fæste et al. 2020).

Last remarks: scraping, bagging, and absorption

Previous studies comparing different methods already concluded that the mucus collected by the scraping generated more than twice the mucus volume (Tartor et al. 2020) and higher concentrations of proteins (Fæste et al. 2020) compared to the absorption method. The proteomic analysis also revealed a higher number of proteins identified for each sample in the scraping (961 proteins) than in the absorption method (747 proteins). To improve these yields, Ivanova et al. (2018) demonstrated that it was

Table 2. Prior to mucus collection some authors subjected the fish to a starvation period, to reduce contamination by excretions, followed by the administration of different anesthetic concentrations.

Anesthetic	Concentration (ppm)	Starved	Overdose/killed	References
2-phenoxyethanol	0.6		X	(Hernández-Contreras et al. 2021)
	1000		X	(Herrera et al. 2020)
				(Escribano et al. 2020; Guardiola et al. 2019)
	2000	48 hr		(Sanahuja et al. 2020)
	100	24 hr		(Ordóñez-Grande et al. 2020; Sanahuja et al. 2018; Sanahuja et al. 2019; Zarei et al. 2021)
	200			(Fernández-Alacid et al. 2019b; Ferreira et al. 2019)
	0.5			(Uren Webster et al. 2020)
	300		X	(Mohammadiazarm and Maniat 2021)
				(Igarashi et al. 2017)
			X	(Monteiro dos Santos et al. 2019; Ramos-Pinto et al. 2021)
Tricaine methanesulfonate (MS222)	100		X	(Benktander et al. 2021; Carbajal et al. 2019; Espinosa et al. 2017, 2019, 2020; Espinosa-Ruiz et al. 2021; García Beltrán et al. 2020; Hamed et al. 2019; Reyes-Becerril et al. 2017b; Xu et al. 2021)
		24 hr	X	(Guardiola et al. 2018)
		24 hr		(Doan et al. 2018; Subramanian et al. 2007, 2008)
				(Abbas et al. 2020; Conforto et al. 2021; Cordero et al. 2017; Giri et al. 2020; Gobi et al. 2018; Guardiola et al. 2014; Ibarz et al. 2019; Pérez-Sánchez et al. 2017; Rahimnejad et al. 2018; Richards et al. 2017; Roux et al. 2019; Saleh et al. 2018, Shakoori et al. 2019, 2021; Tapia-Paniagua et al. 2018; Yildirim-Aksoy et al. 2018; Zoral et al. 2018)
	150			(Fernández-Alacid et al. 2021; Firmino et al. 2021; A. A. N. Nurul et al. 2020; Reyes-López et al. 2020)
	120			(Nor et al. 2020)
	50	24 hr	X	(Jakiul Islam et al. 2021)
	100,000	24 hr	X	(Guardiola et al. 2017)
	125,000			(Ross et al. 2000)
	80			(Djordjevic et al. 2021; Hajirezaee and Hossein Khanjani 2021; Weththasinghe et al. 2021)
	30			(Bulfon et al. 2020)
	50 MS-222 + 200 $NaHCO_3$			(Louvado et al. 2021)
	70			(D. M. Patel and Brinchmann 2017; Rajan et al. 2017)
	500		X	(Terova et al. 2021; Zhi et al. 2020)
	300			(Hu et al. 2021)
	>200			(Shiry et al. 2020)
	200			(Hoseinifar, Hosseini, et al. 2019; Mori et al. 2021; Xiong et al. 2020)
		24 hr		(Abdollahi et al. 2019)
		24 hr	X	(Cid García et al. 2020)
	180			(De Mercado et al. 2018)
	50 MS-222 + 1500 $NaHCO_3$			(Ruiz-Jarabo et al. 2020)
	50 MS-222 + 1000 $NaHCO_3$		X	(Ikert et al. 2021)
	400			(Bulloch et al. 2020)
	0.001			(Oliveira et al. 2018)
	0.1			(Nhu et al. 2019; Soltanian and Gholamhosseini 2019)
	15			(Djordjevic et al. 2021)
			X	(Americus et al. 2020; Fernández-Álvarez et al. 2019; Franco-Martinez et al. 2019; Galbraith et al. 2018; Leis et al. 2018; Minniti et al. 2019; Winter, Nolan, et al. 2019; Winter, Nyqvist, et al. 2019; D.-X. Zhang et al. 2018)
				(Fazio et al. 2021; Giri et al. 2021; Heydari et al. 2020; Y. Jiang et al. 2019; Lange et al. 2018; Lange and Webster 2017; Midhun et al. 2017; Nurhikmah et al. 2022; Parida et al. 2018; Ross et al. 2000; Sheng et al. 2019; Tilley et al. 2020; Van Doan et al. 2020b; H.-P. Zhang et al. 2020)
Benzocaine	40,000 in acetone			(Espinosa-Ruíz and Esteban 2021)
	2000			(Valero et al. 2019)
	30			(Levipan et al. 2020; Levipan and Avendaño-Herrera 2021)
	200			(Fæste et al. 2020; Ivanova et al. 2018)
	100	18 hr		(Goulart et al. 2018)
	50	24 hr		(L. Yin et al. 2020)
	100	24 hr		(Rosli et al. 2019)
		48 hr		(Klemetsen et al. 2019)
			X	(Almeida et al. 2019; Almeida et al. 2019)

(*Continued*)

Table 2. Continued.

Anesthetic	Concentration (ppm)	Starved	Overdose/killed	References
Eugenol		24 hr	X	(Hoare et al. 2017)
	100	24 hr		(Reyes-Becerril et al. 2021; Taheri Mirghaed et al. 2020; M. Yousefi et al. 2021)
	200			(Yokoyama et al. 2019)
	30			(Cai et al. 2020)
	50			(da Silva et al. 2021)
	10		X	(Guo et al. 2019)
				(H. Jiang et al. 2017)
			X	(Sun et al. 2020; Sutili et al. 2020)
Metomidate	12.5			(Padra et al. 2019)
Clove powder/oil	100			(Abolfathi et al. 2020; Cámara-Ruiz et al. 2021; Hoseinifar, Ahmadi, et al. 2017; Reyes-Becerril et al. 2017a; Vaz Farias et al. 2020)
	20			(Ceballos-Francisco et al. 2020; Chen et al. 2020; Chen et al. 2020; Hosseini Shekarabi et al. 2021)
	40			(Guardiola et al. 2017)
	5			(Chirapongsatonkul et al. 2019; Faheem et al. 2020; Hoseinifar, Sohrabi, et al. 2019)
		24 hr		(Mansouri Taee et al. 2017; Soltanian and Gholamhosseini 2018)
	500	24 hr		(Ahmadniaye Motlagh et al. 2020; Hoseinifar, Shakouri, Doan et al. 2020; O. Safari and Sarkheil 2018; R. Safari, Hoseinifar, Dadar, et al. 2020)
		48 hr		(Ahmadniaye Motlagh et al. 2020)
				(Hoseinifar et al. 2021; Hoseinifar, Khodadadian Zou, et al. 2019; Hoseinifar, Safari, et al. 2017; Hoseinifar, Shakouri, Yousefi, et al. 2020; Karimi et al. 2020; Khodadadian Zou et al. 2016; Modanloo et al. 2017; Mohammadi et al. 2020; Van Doan et al. 2020a; O. Safari et al. 2019; R. Safari, Hoseinifar, Van Doan et al. 2017; Shakoori et al. 2019; Vali et al. 2020; Zeynali et al. 2020)
	5000			(Bisht et al. 2020; Doan et al. 2017; Doan et al. 2020; Doan et al. 2020; Ezatrahimi et al. 2019; Fernández-Montero et al. 2021; Fernández-Montero et al. 2021; Hosseini et al. 2020; Srichaiyo et al. 2020; Tippayadara et al. 2021; Van Doan et al. 2018; Van Doan et al. 2019c)
		24 hr		(Kurian et al. 2020)
	3000		X	(Phusantisampan et al. 2020)
	50,000		X	(Mehrinakhi et al. 2021)
				(Khoei 2021; Maldonado-Garcia et al. 2019)
	80			(Charoenwai et al. 2021)
	15			(C. Wang et al. 2020)
	50			(M. I. R. Khan et al. 2021; M. I. R. Khan et al. 2021; Mousavi et al. 2021; Qadiri et al. 2019)
		24 hr		(Ahmadniaye Motlagh et al. 2020; Mansour et al. 2018; Rashidian, Lazado, et al. 2021)
	150,000	24 hr		(Rashmeei et al. 2020)
	300	24 hr		(Ponce et al. 2021)
	250	24 hr		(Mirghaed et al. 2018; Rufchaei et al. 2021; R. Safari, Hoseinifar, Imanpour et al. 2020; R. Safari et al. 2017b)
	200	24 hr		(Ghafarifarsani et al. 2021; Hoseinifar, Jahazi, et al. 2020; Oroji et al. 2021)
				(Mai et al. 2021)
	1000 (1:9 clove oil to 95% ethanol)			(Schorno et al. 2018)
	100 with 70% alcohol (1:1.5)			(Tang et al. 2017)
				(Lumsangkul et al. 2021; Srichaiyo et al. 2020; Van Doan et al. 2021a; Van Doan et al. 2020a; Van Doan et al. 2021b; Van Doan et al. 2021c; Van Doan et al. 2021d)
Formalin			X	(S. Liu et al. 2019)
Ethylene glycol		24 hr		(Lazado and Skov 2019)
		24 hr	X	(J. R. Khan et al. 2018)
Nika Transmore			X	(Chieng et al. 2020)
Carbon dioxide				(Burbank et al. 2017)
Blunt trauma method				(Alijani Ardeshir et al. 2020; Y. Jiang et al. 2019; Klemetsen et al. 2019; Minniti et al. 2019; Padra et al. 2019)
			X	(D. M. Patel and Brinchmann 2017)
Cold			X	(Adorian et al. 2019, 2020; Qamar et al. 2020)
				(Honda et al. 2018; Nigam et al. 2017, 2019; Srivastava et al. 2018)
		24 hr		(Rashidian, Boldaji, et al. 2021)

(*Continued*)

Table 2. Continued.

Anesthetic	Concentration (ppm)	Starved	Overdose/killed	References
Non-specific anesthesia				(Hasan et al. 2018; Jakab Sándor et al. 2018; Sarhadi et al. 2020)
		24 hr		(Arani et al. 2021; Chinnadurai et al. 2021; Dawood et al. 2017; El Basuini et al. 2021; Hajirezaee et al. 2020; Hoseinifar et al. 2018; Kumari et al. 2019; Mohammadi, Adorian et al. 2020; M. Patel et al. 2020; Sridhar et al. 2021a; Sridhar et al. 2021b; Sridhar et al. 2021c; Syed Salman et al. 2020; Zaineldin et al. 2018, 2021; Y. Zhao et al. 2017)
		48 hr	X	(Papadopoulou et al. 2017)
		48 hr		(Shoemaker et al. 2018)

Table 3. Overview of each property of the method: volume, contamination, dilutions, stress, and lesion.

	Methods	Volume	Contamination	Dilution	Stress	Lesion
Scraping	Cotton, sponge, medical wipes	+	−	−	++	+
	Spatula, cell scraper, scalpel	+	+	−	++	++
Bag		++	++	++	++	+
Absorption		−	−	−	+	−

+: favorable; ++: more favorable and −: unfavorable.

possible to perform repetitive mucus collection in short intervals with the absorption method since it was less invasive. It is essential to be careful and avoid continuously scraping the surface of the fish body, which leads to more mucus but can cause skin lesions and contaminate the samples with blood and epidermic cells. In addition, the use of gentler tools, such as cotton or medical wipes, can also minimize this issue (Lange et al. 2020; Mousavi et al. 2021). Furthermore, to avoid dilution by seawater, the collection of mucus should be performed without re-wetting the fish, which is frequently complex because to guarantee the fish survival, the process must be completed in the shortest time possible (Fernández-Alacid et al. 2018).

Collecting mucus by placing the fish in a bag with a saline solution might not be the most effective method for retrieving mucus. This could lead to the mucus being contaminated with other substances and diluted in solution. To minimize the risk of contamination by excretions, some authors starve the fish for 24-hr (Ghafarifarsani et al. 2021), as shown in Table 2. This procedure, however, may also interfere with the mucus bioactivity tests. Soltanian and Gholamhosseini (2019) demonstrated that 20 days of starvation significantly reduced bioactivities such as enzymatic (lysozyme and alkaline phosphatase), total immunoglobulins level, and bactericidal activity. An overview of each methodological property of the volume, contamination, dilutions, stress, and lesion is shown in Table 3.

Anesthetizing is an option to reduce the stress imposed on the fish being handled. Common anesthetics used in fish are clove oil, eugenol, ethyl 3-aminobenzoate methanesulfonate (MS-222), and 2-phenoxyethanol (2-PE), as shown in Table 2 (Van Doan et al. 2019; Guardiola et al. 2019; Saleh et al. 2019; Sutili et al. 2020). It is important to note that adding anesthetics to the water may contaminate mucus samples and interfere with bioactivity tests (Soltanian and Gholamhosseini 2019). Soltanian and Gholamhosseini (2018) studied the effects of 2-PE (0.2 mL/L), MS-222 (50 ppm), and clove oil (25 ppm) in some immune parameters, revealing that 2-PE had depressive effects on the mucus immune parameters, namely, in the decrease of total IgM levels and lysozyme activity. Clove oil was shown to increase lysozyme activity, while MS-222 did not affect immune parameters (Soltanian et al. 2018). This information can be valuable to standardize anesthesia procedures for mucus collection.

A brief procedural guide

Figure 2 displays a diagram to support the review objective. This is a suggested guide for a more optimal mucus collection procedure, considering factors such as sample dilution, contamination, fish lesions, and stress. The procedure would ensure the procurement of high-quality mucus samples suitable for subsequent bioactivity tests. The selection of the scraping method would allow the obtention of a more concentrated mucus sampling, and if performed with a gentler tool such as cotton, medical wipes, or a sponge, there would be less risk of skin lesions. The next step would be

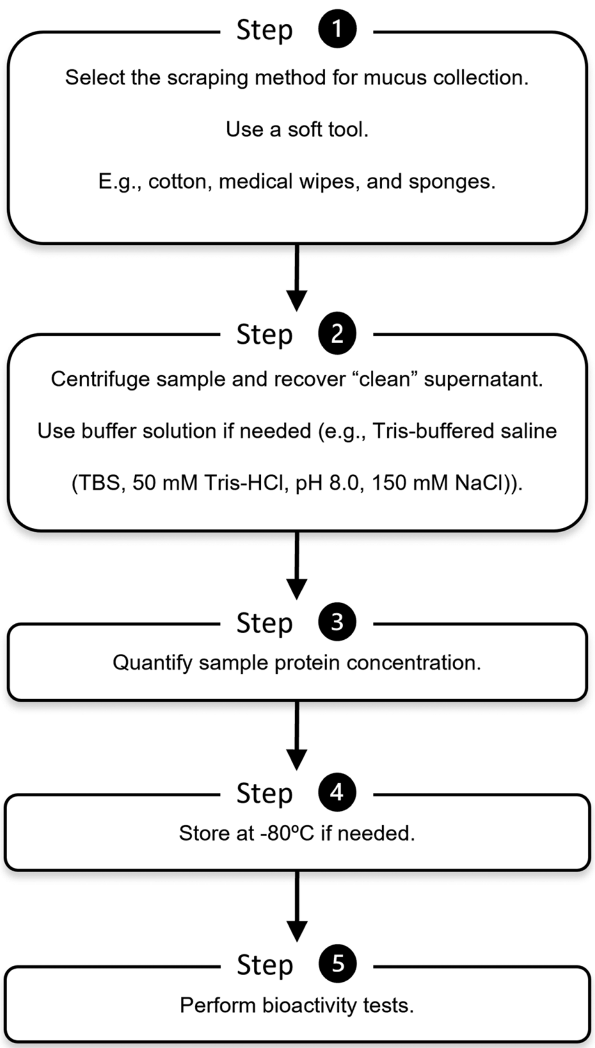

Figure 2. Schematic depiction of the suggested protocol for mucus collection, extending from the point of collection to the preparatory steps for analysis.

centrifugation. If necessary, a solution can also be added to wash the tool, improve extraction, or facilitate the homogenization of mucus. Once the supernatant is recovered, the protein concentration can be measured, indicating the mucus collection quality. To prevent degradation until the bioactivity analysis in the mucus is performed, the sample should be stored at −80 °C.

Conclusions

In undertaking this comprehensive review, the objective was to critically evaluate and compare methodologies involved in the collection and pre-processing of fish mucus samples, specifically those protocols employed until samples were adequately purified and stabilized for subsequent bioactivity analysis. An exhaustive examination of the literature provided invaluable insights concerning the diverse strategies utilized for mucus collection. These methodologies were categorized into three main categories: scraping, bagging, and absorption.

Nevertheless, a striking observation from this literature exploration was the recurrent omission of vital procedural elements. Key specific procedure conditions were frequently missing, such as the duration and temperature of centrifugation and the specifications of the type of filters used. Such lapses in comprehensive methodological reporting may significantly hinder result interpretation and reproducibility.

The scraping technique, with its attribute of a high-yield output, is a prevalent choice among researchers. Nevertheless, this study posits that applying a gentler, less invasive tool may enhance the overall effectiveness of this approach. This dual-advantage strategy potentially increases the quality of collected samples by reducing scale and epidermal cell contamination while simultaneously ensuring the well-being of the fish. It, therefore, facilitates repeated sample acquisition from captive fish or minimal interference when handling wild specimens.

Upon collection, a common practice among researchers is to homogenize the samples in a suitable solution and subsequently subject them to centrifugation to eliminate epidermal residues. Preceding mucus collection, it is imperative to judiciously select an appropriate type and dosage of anesthetic to alleviate stress in fish, ensuring the exclusion of those chemicals that may bias the bioactivity tests. An alternative approach may be handling fish during mucus collection without anesthesia, thus mitigating potential interference with the analytical parameters.

Nevertheless, it is crucial to acknowledge that selecting a suitable methodology may hinge on the specific context or research question. While identifying overarching trends and improvement opportunities, this review underscores the requirement for researchers to adopt and report methodologies congruent with their unique research objectives.

In conclusion, this review accentuates an urgent need for escalated standardization and more thorough reporting within fish mucus collection research. By addressing these pressing challenges, there is an opportunity to enhance cross-study comparisons, improve result reproducibility, and expedite advancements in this pivotal area of research.

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The authors report that there are no competing interests to declare.

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ORCID

David Gonçalves http://orcid.org/0000-0001-6851-7215
Ezequiel R. Coscueta http://orcid.org/0000-0003-4496-2001
Frederico Almada http://orcid.org/0000-0002-1389-8951
Manuela Pintado http://orcid.org/0000-0002-0760-3184
Marta Fernandez Cunha http://orcid.org/0000-0001-5935-8670
María Emilia Brassesco http://orcid.org/0000-0001-9027-4643

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