REVIEWS IN Aquaculture

REVIEW OPEN ACCESS

Welfare Indicators for Aquaculture Research: Toolboxes for Five Farmed European Fish Species

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ABSTRACT

Refining approaches to measuring, monitoring and appraising animal welfare in aquaculture research is key to (i) protecting and optimizing it, (ii) documenting the severity of how and when it deviates, and (iii) ensuring good scientific quality, reliable results and reproducibility, amongst other factors. However, different fish species and life stages can have varying welfare needs and assessing their welfare can be challenging. An array of welfare indicators (WIs) can be utilized when documenting fish welfare, and there is currently little consensus on which WIs are most applicable to the key fish species used in European aquaculture research. The aim of this review is to propose updated, fit for purpose and comprehensive WI toolboxes for aquaculture research involving Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*), and the common carp (*Cyprinus carpio*). Where possible, these toolboxes will also include life-stage considerations. It also provides information on utilizing WIs in deciding humane end-points as well as information on how to sample different types of indicators. The review closes with information on how digitalization can affect the collection, collation and analysis of WI data in aquaculture research, including both practical and theoretical considerations. The toolboxes incorporate a range of WIs that go beyond those required for legally safeguarding fish welfare in both laboratory and operational experimental facilities in the current European 2010/63/EU Directive on the protection of animals used for scientific purposes and its amendment, the Commission Delegated Directive (EU) 2024/1262.

1 | Introduction

1.1 | Documenting Animal Welfare

To understand and document the welfare of fish used for scientific purposes we first need to define what welfare means and ways to measure, monitor, audit, or assess it [1]. There are different views on how animal welfare ought to be defined, and in this review we will define welfare as 'the quality of life as perceived by the animal itself' [2]. This definition is clear, concise and intuitive and is increasingly adopted for fish, first by [3] and later by others [4]. It also dovetails well with the definition of the World Organisation for Animal Health (WOAH formerly OIE), which states welfare 'means the physical and mental state of an animal in relation to the conditions in which it lives and dies' [5]. Under these definitions, the welfare state of an animal is the sum of its positive and also negative feelings, its conscious subjective experience, at a given moment in time [6–9]. The needs, or requirements, that are monitored by the emotional and cognitive systems in the brain are termed welfare needs, and while the list of possible welfare needs for fish is long, for simplicity they can be grouped into four domains: (1) nutrition, (2) physical environment, (3) health, and (4) behavioural interactions, which then contribute to a fifth domain, (5) the mental state of the animal. This is termed the Five Domains Model [6].

Welfare indicators (WIs) are the tools we use to document welfare and are defined as all parameters that can be measured or observed that give information regarding the fulfilment, or change in fulfilment, of a single or numerous welfare needs [4, 8, 9]. A welfare monitoring and documenation plan should include enough WIs to cover all welfare domains (Figure 1). While the list of welfare needs for a number of species studied in aquaculture research is quite comprehensive, this is not the case for all species [10, 11], and the range and diversity of welfare needs are constantly evolving as more knowledge becomes available [8].

There are many ways to categorise and classify WIs, one of which is to divide them according to whether they are input- or outcome-based, [4]. Input-based WIs include descriptions of the resources and environment the fish are exposed to and are also termed indirect, resource-based measures (RBMs) [12]. Outcome-based WIs, also termed direct, animal-based measures (ABMs) [12] outline how welfare needs are met. As an example, environmental parameters such as dissolved oxygen saturation and water temperature are input-based indicators influencing the need for an appropriate water environment, while reduced appetite, growth, gill health or even increased mortality are the result, or outcome, of this need not being fulfilled.

Outcome-based WIs can also be individual or group-based. Individual-based indicators describe, for example, the physiology, health status, behaviour or physical appearance of each fish. Group-based WIs are applicable at the population/group level, for instance schooling behaviour, population mortality or how much feed the fish consume each day as a group. Negative results for an outcome-based WI can be the result of a multitude of underlying welfare problems, and not only a single issue. Such indicators are sometimes called iceberg indicators to underline this point [9]. An example of an input-based iceberg indicator is dissolved oxygen saturation levels, which can, for example, decrease due to a variety of reasons such as increases in fish metabolism or algal blooms. Examples of outcome-based iceberg indicators include various fish injuries which can be driven by exposure to various pathogens or contact with equipment or other fish. Another approach for sorting WIs is to classify them as either Operational Welfare Indicators (OWIs), or so-called Laboratory-based Welfare Indicators (LABWIs). OWIs are easy and practical for experimental and farm use, while LABWIs are more complex, requiring further analysis or processing once the sample is collected [4].

Any framework for monitoring animal welfare should include both input- and outcome-based WIs. In cases where the aim of an experiment is to document how an animal responds when being subjected to potentially challenging conditions, monitoring can be used to document this in a scientifically robust and transparent way and also help in both actively and retrospectively assessing the severity of the procedure [13, 14]. Robust and applicable WIs are also essential for regulatory purposes. Specifically, in the European Union, Directive 2010/63/EU provides a legal framework that mandates the ethical treatment and welfare documentation of animals, including fish, used in research.

1.2 | The Importance of Documenting Fish Welfare in Research and Directive 2010/63/EU

Animal research in the EU is governed under Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes [15], which took full effect on the 1 January 2013. The Directive has recently been amended by Commission Delegated Directive (EU) 2024/1262 of 13 March 2024 with regard to the requirements for establishments and for the care and accommodation of animals, and with regard to the methods of killing animals [16]. The Directive 'represents an important step towards achieving the final goal of full replacement of procedures on live animals for scientific and educational purposes as soon as it is scientifically possible to do so' (text underlined by the current authors and not those who compiled the directive) and

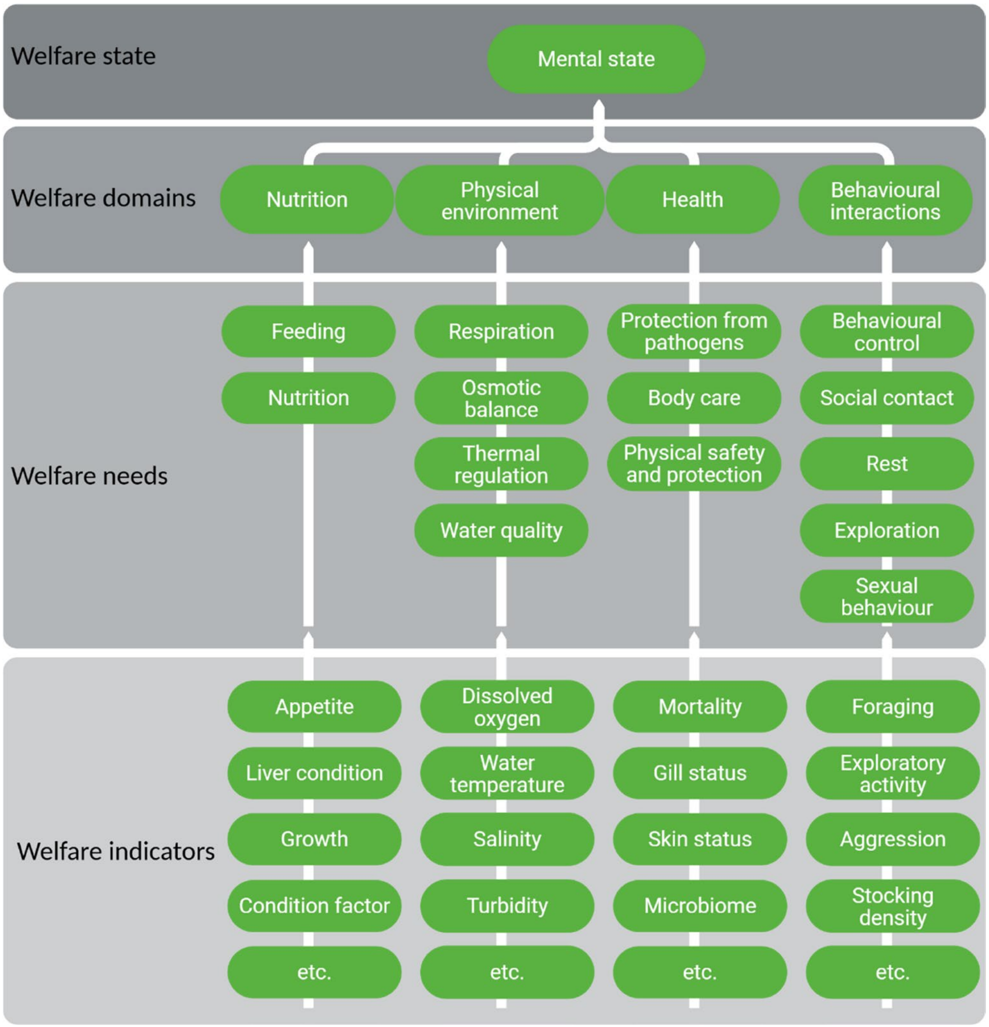

FIGURE 1 | Outlining the links between welfare indicators, welfare needs, [3, 4], welfare domains, [6], and the welfare state of an animal. Welfare indicators are the tools used to get information on the fulfilment, degree of fulfilment or changes in the fulfilment of welfare needs, which can be grouped into welfare domains. The level of fulfilment, or changes in fulfilment, of these needs is monitored by the animal's brain, and the sum of these feelings is the animal's welfare state at a given moment in time. *Source:* Linda Tschirren, Gunhild S. Johansson, Chris Noble and Lars Helge Stien.

acknowledges that 'while it is desirable to replace the use of live animals in procedures by other methods not entailing the use of live animals, the use of live animals continues to be necessary to protect human and animal health and the environment' [15]. The Directive also aims to reduce, safeguard and protect those animals that are being utilized in experimental studies and procedures and requires an awareness and examination of the level of suffering the animals are potentially subjected to. This is where the interplay between welfare, WIs and the Directive comes into focus. The European Commission has also produced seven Guidance Documents which are endorsed by the Member States that address and provide further information on, for example, severity assessment frameworks [14].

Ambitions to improve and optimise animal welfare under scientific procedures have a long history in several scientific disciplines. The Universities Federation of Animal Welfare (UFAW) in the UK commissioned a project in the 1950s to investigate how animal suffering in research could be minimised by replacing animal use altogether, reducing the number of animals used and how the quality of research which still needed animals might be optimised. The findings of this work were described in detail in a book called 'The Principles of Humane Experimental Technique' first published in 1959 and re-issued in 1992 [17]. The principles included what has become known as 'the 3Rs': Replacement, Reduction and Refinement. The 3Rs have subsequently been adopted by legislation in many countries worldwide and their principles are central to the EU Directive 2010/63/EU (see Recital 11 of the Directive [15]). To support the application of the 3Rs, the EU Directive 2010/63/EU provides a comprehensive list of recitals, articles and annexes that regulate, amongst others, standards for the scientific facilities and obligations for participants regarding scientific procedures [18]. These provisions include guidelines on the environmental parameters, feed and nutrition, rearing system and housing conditions as well as care, husbandry and transportation practices required to meet the welfare needs of the animals (see, e.g., Recital 34 [15]). There have been numerous attempts to interpret or update these guidelines in light of advances in Laboratory Animal Science (LAS), with a list of relevant references available at [19].

In addition to such 3R developments, advances were also made elsewhere. For example, one of the aims of a 1975 symposium was to investigate whether it was possible to replace the use of animals with in vitro methods and computer models [20]. Speakers included an American biomathematician, Carol Newton, and a Canadian veterinary pathologist, Harry C. Rowsell. In his presentation, Rowsell stated 'To the three R's we may add Carol Newton's three S's: good science, good sense and good sensibilities' [20]. To the authors' knowledge, Newton herself never wrote about the 3S concept, but its relevance to Laboratory Animal Science has been examined in an article by Smith and Hawkins [21]. Other developments have taken place in relation to the quality of animal experiments, especially during the design phase and when data are analysed. Poor quality can lead to poor data validity, which may take one of three forms: poor construct validity, poor internal validity or poor external validity (the 3Vs). Hanno Würbel coined the 3Vs phrase to draw attention to this, and to ways in which validity can be improved [22]. Much recent focus has also been placed on what has become known as harm-benefit analysis (HBA). The concept is grounded in an expectation that the potential harms to animals used for scientific purposes are minimised and potential benefits from their use are maximised [23]. Brønstad et al. [23], define 'HBA as a systematic, transparent way to assess and compare harms, benefits and how they are balanced' and their article gives guidance on how ethical dilemmas can be approached when deciding whether or not to allow animal experiments [23, 24]. Such an analysis is an important part of the ethical evaluation of plans for animal studies.

Responsible research practices are essential, and all animal experimentation should undergo strict ethical approval processes before being allowed to take place. Some experiments and scientific trials do not expose the animal to potential suffering and pain, and therefore fall below the threshold for regulation under Directive 2010/63/EU, see [25, 26]. These sub-threshold procedures are numerous and include but are not limited to 'Behavioural studies that do not involve any other regulated procedures' [25]. However, all experimentation that involves a procedure, which is defined as 'any use, invasive or non-invasive, of an animal for experimental or other scientific purposes, with known or unknown outcome, or educational purposes, which may cause the animal a level of pain, suffering, distress or lasting harm equivalent to, or higher than, that caused by the introduction of a needle in accordance with good veterinary practice' is covered by Directive 2010/63/EU [15]. For the ethical evaluation of these experiments, the severity of the impact on the animal is essential. The Directive's Annex VIII classifies procedure severity in terms of the 'degree of pain, suffering, distress or lasting harm expected to be experienced by an individual animal during the course of the procedure'. Severity is then classified as (i) non-recovery 'performed entirely under general anaesthesia from which the animal shall not recover consciousness', (ii) mild 'animals are likely to experience short-term mild pain, suffering or distress, as well as procedures with no significant impairment of the well-being or general condition of the animals', (iii) moderate 'animals are likely to experience short-term moderate pain, suffering or distress, or long-lasting mild pain, suffering or distress as well as procedures that are likely to cause moderate impairment of the well-being or general condition of the animals' and (iv) severe 'animals are likely to experience severe pain, suffering or distress, or long-lasting moderate pain, suffering or distress as well as procedures, that are likely to cause severe impairment of the well-being or general condition of the animals' [15].

The Directive includes species-specific sections in Annex III, including a brief part for fish and their use for scientific purposes (Table 1). This has also been recently amended by Commission Delegated Directive (EU) 2024/1262 of 13 March 2024. It offers a general overview of a restricted number of WIs, which are mostly input-based. However, there is no species- or life-stage specific information that fish researchers and other interested parties can use in their welfare documentation practices, aside from that provided for zebrafish [16]. The directive states that some input-based WIs should be appropriate/optimal/adapted to each relevant species and acknowledges the need for species-specific information also for fish, without providing extensive information on it in the Directive. Previous articles have outlined how the Directive can be applied to Atlantic salmon, rainbow trout, European seabass, gilthead seabream and the common carp [27, 28], Atlantic lumpfish (*Cyclopterus lumpus*), ballan wrasse (*Labrus bergylta*), Nile tilapia (*Oreochromis niloticus*), three-spined stickleback (*Gasterosteus aculeatus*), goldfish (*Carassius auratus*), guppy (*Poecilia reticulata*) [28] and zebrafish (*Danio rerio*) [27]. These papers also consider handling practices, transport and an array of husbandry procedures, outlining how you can apply WIs to these operations. However, while existing frameworks and legal guidelines provide a foundation, there is a need for more applicable, standardised, and species-specific indicators to effectively assess fish welfare in aquaculture research.

1.3 | Aims and Objectives

In this current article, we aim to utilize and build upon the work that has already been conducted and outlined earlier [27–29], and provide an updated and more detailed WI toolbox for documenting fish welfare in aquaculture research involving five teleost species (i) the Atlantic salmon (*Salmo salar*), (ii) rainbow trout (*Oncorhynchus mykiss*), (iii) European seabass (*Dicentrarchus labrax*), (iv) gilthead seabream (*Sparus aurata*) and (v) the common carp (*Cyprinus carpio*). These toolboxes will expand the range of WIs beyond those defined in Directive 2010/63/EU and its amendment, Commission Delegated Directive (EU) 2024/1262 [15, 16], and provide additional focus on outcome-based WIs at the group and individual level. We will further direct the reader to the latest scientific state of the art on how each WI can be documented using existing and emerging welfare monitoring technologies. This will provide an enhanced knowledge base that scientists, animal care staff, regulatory bodies and policymakers can use to inform and steer their decisions regarding the application and delivery of Directive 2010/63/EU and its amendment, Commission Delegated Directive (EU) 2024/1262 [15, 16] in aquaculture research. Much of the article's content has practical applications, but there are also more theoretical aspects with regard to sampling approaches, the potential for digitalisation in relation to measuring and monitoring welfare, and also in relation to managing and processing data. In addition, while the focus of the article is on aquaculture research settings,

TABLE 1 | Summary of the welfare indicators to be considered when conducting scientific procedures on fish, according to Annex III of the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010, on the protection of animals used for scientific purposes [15] amended by the Commission Delegated Directive (EU) 2024/1262 of 13 March 2024 with regard to the requirements for establishments and for the care and accommodation of animals, and with regard to the methods of killing animals [16].

Input-Based Operational Welfare Indicator (OWI)	**What Annex III of the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 Amended by the Commission Delegated Directive (EU) 2024/1262 of 13 March 2024 States in Relation to Fish**
General text regarding water quality	Adequate water supply of suitable quality shall be provided at all times. Water flow in re-circulatory systems or filtration within tanks shall be sufficient to ensure that water quality parameters are maintained within acceptable levels, according to the characteristics of the husbandry system, to the species and life stage requirements. Water supply shall be filtered or treated to remove substances harmful to fish, where necessary. Water-quality parameters shall at all times be within the acceptable range that sustains normal activity and physiology for a given species and stage of development. Appropriate measures shall be taken to minimise sudden changes in the different parameters affecting water quality. Appropriate water flow and water level shall be ensured and monitored.
Oxygen	Oxygen concentration shall be appropriate to the species and to the context in which the fish are held. Where necessary, supplementary aeration of tank water shall be provided, depending on the husbandry system.
Temperature	Temperature shall be maintained within the optimal range for the fish species and their stages of development and kept as stable as possible. Changes in temperature shall take place gradually.
Nitrogen compounds	The concentrations ... of nitrogen compounds, namely ammonia, nitrite and nitrate, shall be kept below harmful levels.
Carbon dioxide	The concentrations of carbon dioxide ... shall be kept below harmful levels.
pH	The pH level shall be adapted to the species and monitored to be kept as stable as possible.
Salinity	The salinity shall be adapted to the requirements of the fish species and to the life stage of the fish. Changes in salinity shall take place gradually.
Lighting	Fish shall be maintained on an appropriate photoperiod.
Noise and vibration	Noise levels shall be kept to a minimum and, where possible, equipment causing noise or vibration, such as power generators or filtration systems, shall be separate from the fish-holding tanks. For aquatic animals, equipment causing noise or vibration, such as power generators or filtration systems, shall not adversely affect animal welfare.
Stocking density	The stocking density of fish shall be based on the total needs of the fish in respect of environmental conditions, health and welfare.
Water volume	Fish shall have sufficient water volume for normal swimming, taking account of their size, age, health and feeding method.
Water flow	The water flow shall be appropriate to enable fish to swim correctly and to maintain normal behaviour.

Note: Table formulated using text directly reproduced from the Directive 2010/63/EU and Commission Delegated Directive (EU) 2024/1262, acknowledging its copyright, and with permission. Text is selectively highlighted by the authors of this paper, not those who compiled the Directive.

we acknowledge that some experimental settings can be full commercial scale. There is also a shared knowledge conduit between research and commercial farming, meaning relevant information sources from industry settings also have utility and are included in the WI toolboxes.

1.4 | Why the Focus on These Five Species?

The five selected species dominate European farmed fish production in terms of production volume. Marine cold-water species spearhead total production, followed by marine Mediterranean and freshwater species [30, 31] see Figure 2.

Atlantic salmon is the dominant European species when including all the EU, EFTA countries and the UK (Figure 3A). However, when Norway and the UK are excluded, it constitutes only 10% of the production (Figure 3B). Rainbow trout then constitutes about 31%, common carp around 11%, European seabass around 15% and gilthead seabream about 16% of a total production of 637 thousand tonnes of live weight.

Each of these species differs in their biological needs and requirements, which can also vary with life stage. There are various existing sources that offer detailed information on the ecological needs of each of these species and we direct the reader to the following relevant articles for further information [28, 33–37]. However, we will summarise some key similarities and differences here. Atlantic salmon, rainbow trout, European seabass and gilthead seabream are carnivorous, eurythermal and euryhaline, while the common carp is a eurythermal, benthivorous freshwater fish [38]. Atlantic salmon is found mainly around the northern Atlantic coast and tributaries, but is also farmed elsewhere, such as on the west coast of Canada, Chile and Tasmania. It dominates aquaculture across the EU, EFTA region and the UK (see [39] and Figure 3). Juveniles are primarily raised in hatchery tanks, while ongrowers are mostly farmed in marine net pens, but the range of production systems is diversifying both on land and at sea [4]. The rainbow trout is originally from North America but is now farmed in North America, Europe and elsewhere. It is mostly farmed in hatchery tanks, ponds, raceways and also freshwater or marine net pens [40]. The European seabass is found mainly in the Mediterranean and Black Sea but also around the eastern Atlantic coast from Morocco to Norway [41]. It is a central farmed species across the Mediterranean region. It is mostly farmed in hatchery tanks as juveniles and marine net pens as ongrowers but can also be farmed in raceways and coastal ponds [42, 43]. Gilthead seabream is one of the most widely farmed finfish in the Mediterranean. It is able to occupy diverse habitats including estuaries, lagoons, and coastal waters [44] and juveniles are mostly farmed in land-based hatchery tanks and ongrowers in marine net pens [45]. The common carp is mainly produced by a traditional and cost-effective method in semi-intensive earthen ponds, where most feed is provided by the pond's natural production, which is enhanced by fertilisation, and various grains (e.g., barley, wheat or maize) are added to provide the energy source [46, 47]. This traditional method can be intensified by using formulated feeds, to increase yield and reduce production times [48, 49].

2 | WI Toolboxes for Aquaculture Research

2.1 | Factors to Consider When Assembling a WI Toolbox

There is a growing focus on the collation and standardisation of WI frameworks for numerous captive species [50], including fish [27] and this has several benefits:

- Firstly, a wide-ranging review of the WIs included in the Directive 2010/63/EU of the European Parliament will ease its application in scientific procedures.
- Secondly, a better and more comprehensive overview of the range of WIs that are both applicable and practicable for aquaculture research will support stakeholders—including researchers, care workers, national competent authorities and their inspectorates and others—to go beyond the Directive 2010/63/EU when they monitor and document the welfare of their fish.
- Thirdly, frameworks that harmonise the way stakeholders approach their welfare monitoring tasks, while considering the species-specific needs of each life stage, strain, genotype and species, will allow for better documentation and comparative evaluations for different experiments and treatments within and between laboratories and facilities. This can also be an essential step in the standardisation process [51] as it increases the utility of data sharing and thus increase sample sizes for auditing the effects of potential treatments or interventions without the need for using additional animals [50]. It can also aid the comparison of research outputs from differing research facilities [51].

The requirement for species-specific knowledge and guidelines on the welfare and health of fish used in scientific procedures has long been recognised [51] and species-specific evidence and knowledge have grown over the years [27, 28]. Species-specific information will help stakeholders better understand the welfare of their fish, including the repercussions of the decisions they make regarding their husbandry. This information also increases the knowledge foundation that assessments, recommendations, opinions and standards are built upon and allows for more precise application of these in practice. There is also an acute need for this knowledge to be actionable in order to advance its transition and transformation into policy actions [11]. Further strain or genotypic factors could also be considered by the stakeholder as they may have differing welfare needs at the same life stage. An example of this is related to triploid Atlantic salmon, which have differing nutritional requirements and exhibit a poorer tolerance of elevated water temperatures and reduced dissolved oxygen levels than their diploid counterparts [52]. Although information on this is currently limited for a number of species, it is something that may need to be considered in future revisions and applications of the Directive in aquaculture research involving fish.

Overall, this approach can (i) improve the documentation of welfare impacts as the effects of key stressors or procedures are more effectively documented, therefore enhancing transparency, (ii) help identify potential problems at an early stage, providing the stakeholder with an opportunity to minimise

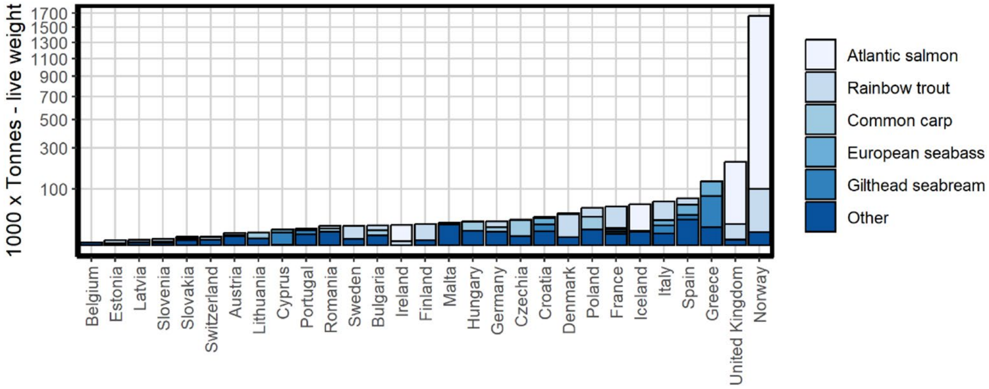

FIGURE 2 | Tonnes live weight of Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and other fish species including Atlantic bluefin tuna (*Thunnus thynnus*), Senegalese sole (*Solea senegalensis*), Arctic char (*Salvelinus alpinus*), Meagre (*Argyrosomus regius*), Bighead carp (*Hypophthalmichthys nobilis*), Red porgy (*Pagrus pagrus*), North African catfish (*Clarias gariepinus*), Turbot (*Scophthalmus maximus*) and more produced in the EU and EFTA countries + United Kingdom in 2021 [32].

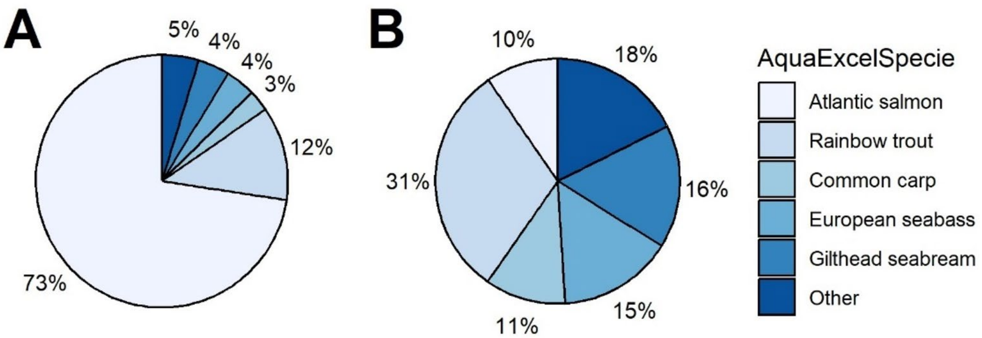

FIGURE 3 | Percentage of live weight produced of Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*), including Norway (A) and excluding Norway (B) [32].

potential harm if mitigation measures and humane end-points are available, (iii) encourage development of mitigation procedures if they are lacking and (iv) facilitate future improvements through information sharing in peer-reviewed publications, reports [14] and professional forums such as the Federation of European Aquaculture Producers, FEAP or the European Aquaculture Technology and Innovation Platform, EATIP.

2.2 | The Proposed WI Toolbox

In its simplest terms, the WI toolbox will include WIs that are linked to at least one or more welfare needs, while some welfare needs may also have one or more corresponding WIs. WIs will be categorised according to whether they are input- or outcome-based and an update on the scientific state of the art will be presented in relation to each indicator, outlining its impacts upon one or more welfare domains. The toolbox will also go beyond OWIs and introduce the reader to some key LABWIs that can be useful additions to the toolbox. We acknowledge the risk of expanding the toolbox to incorporate time- and labour-intensive redundancies in data acquisition, but hope this risk is offset by the need to generate high utility knowledge on how welfare needs are met or impacted upon during an experiment; a particularly pertinent objective when we consider species and life stage requirements within a welfare documentation framework. We also recognise the potential additional financial resources this would involve and recommend that this is considered by funding bodies in their financial allocations and the resources they make available for projects. Lastly, we are aware of the ongoing development of many indicators and future advances, and therefore, we will draw attention to some existing conflicting data or if challenges to measure or to develop thresholds exist.

The general toolbox structure and framework (Figure 4) breaks down the toolbox into (i) those indicators that are already in Directive 2010/63/EU and its amendment, the Commission Delegated Directive (EU) 2024/1262, (ii) additional input-based indicators and (iii) additional outcome-based indicators at the group and individual level that may be appropriate to consider in relation to a given experimental design.

3 | Key Input-Based WIs

Input-based WIs can have a discernible impact on fish welfare [33], and can be used as WIs for all fish species in both experimental and applied settings. They may either be the focus of a specific study or associated with other welfare-impacting factors within the study. A stakeholder may wish to control input-based WIs and set them to constant conditions or may allow for, or focus upon, their variability. In addition to the study focus, the length of exposure to input-based WIs is also relevant, and fish might be exposed to both acute and chronic changes, which can affect their welfare state. This section will consider the potential effects of a range of input-based WIs upon the fulfilment of welfare needs and outline examples of known key interrelationships between specific indicators and how these can have an additive and/or interactive effect on fish welfare.

3.1 | Challenges With Applying Thresholds to Input-Based WIs

Annex III of the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 and its amendment Commission Delegated Directive (EU) 2024/1262 of 13 March 2024 includes some general text on water quality parameters before outlining key requirements for a selection of input-based WIs. This text states '**Adequate** water supply of **suitable** quality shall be provided at all times. Water flow in recirculatory systems or filtration within tanks shall be sufficient to ensure that **water quality parameters are maintained within acceptable levels, according to the characteristics of the husbandry system, to the species and life stage requirements**. Water supply shall be filtered or treated to **remove substances harmful to fish**, where necessary. Water-quality parameters **shall at all times be within the acceptable range that sustains normal activity and physiology for a given species and stage of development**. Appropriate measures **shall be taken to minimise sudden changes in the different parameters affecting water quality**. Appropriate water flow and water level shall be ensured and monitored' [15, 16] with text selectively highlighted by the authors of this review. While species and life-stage provisions are mentioned in the text of the Directive, a mixed terminology, for example, adequate/suitable/acceptable regarding water quality levels is used [15]. Further, the definition of optimal and suboptimal is equally difficult to establish with regard to welfare as it depends on the species, life stage, experimental setting, and also what the research involves. Therefore, while adequate for high-level regulations, one could consider addressing the terminology in order to increase its utility for applied guidelines.

These considerations may come in the form of detailed semantic descriptions, scoring systems or, in the case of water quality, numeric thresholds. The latter, however, may cause problems if certain aspects are not taken into account. For example, in farm settings fish are exposed to variable conditions and a range of biological, environmental, and production-related factors that could interact with each other and have antagonistic or synergistic effects [33]. Important factors may be missed if an experiment is set up to document and monitor only one or two input-based indicators, or if the indicators are viewed in isolation and potential interactive combinations are ignored. This means that thresholds for different parameters reported in various studies may vary substantially for a species and even within a life stage. Some of this variation may be due to, amongst others, the measure that has been used to set the threshold, the health status of the fish or the quality of the study [53]. However, some water quality parameter thresholds are highly dependent on other parameters, such as temperature or pH. Therefore, relevant thresholds may be impossible to set if data on correlated parameters are not considered. Consequently, when considering potential welfare thresholds for water quality parameters in aquaculture research, there are a number of challenges and criteria that should be considered.

- Firstly, thresholds should be based upon a broad knowledge base, in addition to being audited and applicable to each life stage, species and experimental setting. However, numerous sources and experiments that have studied water quality may be designed to audit the effects of a given parameter upon, for example, production performance rather than welfare per se and the original experimental objectives may not be a good fit regarding wider welfare considerations [54]. Interpolating how these thresholds impact on welfare can therefore be challenging.
- Secondly, there are still numerous knowledge gaps on how certain water quality parameters, either alone or in tandem with others, can impact on fish welfare.
- Thirdly, there are various ways in which thresholds can be set and applied. MacIntyre et al. [55] stated that ranges rather than specific limits should be introduced, an approach which has been utilized in a number of research works that attempt to define thresholds for water quality in experimental [27] and applied settings [56]. As an example, one can consider setting thresholds for thermal tolerances, which is challenging due to methodological factors and also to those inherently tied to uncertainties in the physiological responses of fish. To begin with, a unified definition of thermal tolerance thresholds is generally missing, and the choice of tolerance indicators may differ across studies ranging from the transition to low-performance conditions, the activation of anaerobic metabolism, the point of denaturation of proteins, the onset of spasm or even death [57]. While all these constitute important tolerance thresholds, they hinder intra- and inter-species comparisons. Furthermore, these species-specific thresholds are also influenced by several factors, such as the presence of other stressors, developmental stage, animal size, health status or previous acclimation history [58, 59]. Additionally, a crucial element in understanding welfare in relation to thermal limits is time, since survival under extreme temperatures is time-dependent, with fish generally withstanding substantially higher and lower temperatures under acute thermal stress compared to prolonged exposure [60, 61]. Dynamic methods for elucidating thermal limits such as the determination of CT_{max} and CT_{min} (Critical Thermal maximum and minimum, respectively) rely on the former and are generally preferred to static methods like UILT and LILT (Upper and Lower Incipient Lethal Temperatures, respectively) from a welfare and 3R perspective, since the latter subject the fish to prolonged, lower intensity gradients of temperatures with death as the end-point. It is therefore evident that absolute thermal limits are hard (and likely not possible) to define and should only be taken as a reference for thermal tolerance under the appropriate context [62, 63].
- Fourthly, most aquaculture studies utilize and select animals that have good health and welfare at the outset, and do not push the organism to their limits unless this is the aim

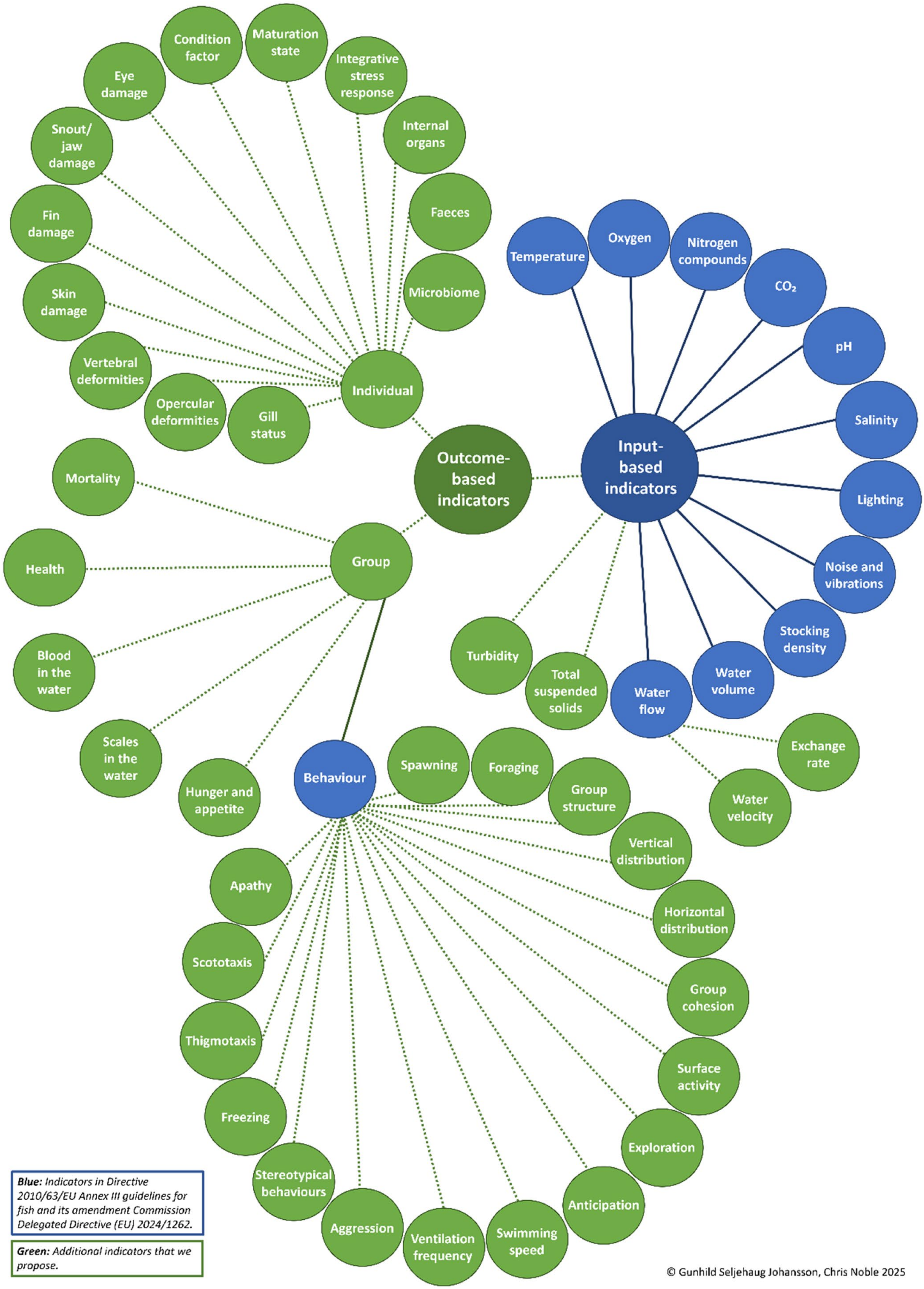

FIGURE 4 | Legend on next page.

FIGURE 4 | Outline of the range of welfare indicators in our proposed WI toolboxes. Indicators in blue are those addressed in Directive 2010/63/EU Annex III guidelines for fish and its amendment Commission Delegated Directive (EU) 2024/1262. Indicators in green are additional indicators that we propose, broken down into additional Input-based and Outcome-based indicators (at either the individual or group level). Some group-based indicators can be utilized at the individual level, for example, appetite, behaviour or health. Image copyright: Gunhild S. Johansson & Chris Noble, Nofima.

of the study, and almost no studies include compounding stressors, see [64].

We therefore propose and acknowledge that it is not always appropriate to attempt to impose thresholds (or threshold ranges) upon water quality parameters, especially if a range of connecting and interrelated factors needs to be considered. Where it is appropriate to do so, various examples will be provided for each species within each WI section.

As stated above, acclimation (the length of time that a fish has to acclimate to the conditions it is subjected to), in addition to the actual level of the parameter and speed of change, can have a striking influence on a fish's welfare state. The need for fish to acclimate to a potentially new or updated rearing environment is considered in Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 via the following: 'Fish **shall be given an appropriate time for acclimatisation and adaptation to changes in water-quality conditions**' [15, 16] where text is selectively highlighted by the authors of this paper. The prior and ongoing health and welfare state of the fish also affects how a range of input-based WIs affect welfare as they have compensatory processes (e.g., molecular, biochemical) that can help them offset the potentially detrimental effects of the water qualities they are subjected to [33, 65]. This process may take a long time (lasting up to months), and this time period must be considered when auditing how acclimation may impact the effects of each WI upon the welfare state of the fish [65, 66]. Therefore, both acclimation time and the temperature to which the fish is acclimated can have a marked impact upon thermal tolerances in fish [33, 65, 67]. Similar patterns have been observed for other water quality parameters such as salinity, where acclimation may take a number of weeks during which time the physiological capacity of the fish to cope with stress is compromised, resulting in lower tolerance to other stressors [68–71]. Social factors such as previous group or tank size can affect the ability or time needed to acclimate to experimental conditions. For instance, salmon smolts transferred from large to smaller tanks can have lower feed intake and much higher mortality than smolts transferred to tanks of the same size [72]. Atlantic salmon also show plasticity when exposed to constant sublethal amounts of ammonia and nitrite [73, 74] where negative effects on growth were observed after 3 weeks but were absent after 105 and 84 days, respectively. In both cases, the protective molecular and physiological mechanisms were activated and remained until the end of both studies. For example, there was an increase in brain glutamine (Gln) concentration and increased branchial transcription levels of ammonia and urea transporting proteins in response to ammonia exposure, or up-regulation of the cystic fibrosis transmembrane conductance regulator (cftr)-1 gene in the gills [73, 74].

Each of the sections below introduces the reader to an updated state of the art on the utility of using each WI to document the fulfilment of welfare needs, with some knowledge examples broken down for each species and, where available, each life stage.

3.2 | Temperature

Annex III of the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 and its amendment in Commission Delegated Directive (EU) 2024/1262 state 'Temperature shall be maintained within the optimal range for the fish species and their stages of development and kept as stable as possible. Changes in temperature shall take place gradually.' [15, 16]. This text introduces species and life stage, specific considerations for ensuring *optimal* temperature ranges for the chosen species. Interestingly, some studies suggest relatively narrow optimal rearing temperatures for certain species, and limiting rearing temperatures to only *optimal* ranges could preclude exposing fish to temperatures that they have both adapted to and are regularly exposed to in open-rearing systems that subject the fish to natural variations in water temperature. We also know from recent studies on fish temperature preferences that constant temperatures are not the most favourable choice for most fish species. It has been demonstrated that fish follow circadian rhythms of preferred temperatures in nature but also in artificial environments [75] as well as choosing different temperatures depending on their internal health and emotional states [76, 77].

Most fish are classified as ectotherms, meaning their metabolic heat production and retention mechanisms are insufficient to increase their body temperature. Consequently, water temperature has a major impact on their metabolism and other body functions, and influences swimming capacity, growth, sexual maturation, immune response and more [78, 79]. Temperature tolerance and preferences can vary markedly between species and life stages [80] and individuals within each species/life stage in relation to, for example, their internal emotional states [76], health status [77] or acclimation [37, 81] and references therein. Tolerance and preferences can also vary in relation to differing genetic backgrounds [37, 82]. While the temperature in tanks is usually uniform throughout the water volume, water in net pens can be vertically stratified. Strong temperature preferences may lead to increased fish densities at some depths that are much greater than the mean stocking density [83], which in turn can lead to reduced dissolved oxygen saturations [84] and references therein. Pen-held fish that move vertically through stratified water bodies of different temperatures may experience very sudden temperature shifts [85]. In many situations, changes in water temperatures occur gradually due to its high specific heat capacity [37] and rapid fluctuations in temperature can be demanding for a fish [36, 86, 87]. Further, temperatures outside the tolerance range, which are therefore life-threatening, can be potentially painful and result in strong responses [87–90].

Temperature also has several additional effects on fish welfare. Other water quality parameters, in particular oxygen solubility and the oxygen requirements of the fish, are highly dependent on temperature. Parasites, for example, salmon louse [91], other pathogens, for example, winter ulcers [92], algal blooms [93] and jelly fish [94] are also affected by temperature, and thus, the threats from these are temperature dependent.

Due to these numerous direct and indirect effects on welfare, water temperature is an important WI and primarily impacts upon welfare needs under the following domains: physical environment and health. The text below highlights the variability in temperature preferences and tolerance ranges that have been reported in some example case studies, which may be linked either to one or a multitude of the drivers outlined at the start of this section.

3.2.1 | Atlantic Salmon

Depending upon the study and its context, fry have a reported tolerance range of 0°C–20°C with a preference for 12°C–14°C in some settings, see [33]. Parr and smolts have a tolerance range of either 3°C–18°C or 2°C–22°C [95–97] and a respective preference range for either 12°C–14°C or 13°C–16°C [95, 96]. Post-smolts have a tolerance range of, for example, 7°C–17°C, 3°C–18°C or 1°C–18°C and they have a preference for, for example, 13°C–18°C, 16°C–18°C or 16°C–17.5°C in some settings [4, 33, 85, 98] and references therein. Broodstock have a tolerance range of 8°C–12°C or 1.5°C–12°C and they have a preference for 5°C or 6°C–8°C when spawning, see [33, 99], and references therein.

3.2.2 | Rainbow Trout

Depending upon the study and its context, fry/fingerlings have a reported tolerance range of, for example, 3°C–15°C, 4°C–15°C, 0°C–22°C, 7°C–17°C, 8°C–20°C, 13°C–19°C and 14°C–19°C and a preference for, for example, 7°C–13°C, 11°C–13°C, 16°C–18°C, 13°C, 16°C and 17°C in varying settings, see [37, 100–105]. There is a massive overlap between ranges across different studies and settings. Ongrowers have a tolerance range of, for example, 0°C–22°C, 1°C–25°C and 7°C–18°C and a preference for, for example, 10°C–16°C, 12°C–18°C and 16°C–18°C in varying settings [37, 106, 107] and references therein. Broodstock have a tolerance range of 0°C–22°C and they have a preference for either 16°C–18°C or 10°C–13°C when spawning, see [37] and references therein.

3.2.3 | European Seabass

Juveniles have a reported tolerance range of, for example, 8°C–32°C and a preference for, for example, 17°C–24°C in varying settings [36, 65, 108, 109]. Ongrowers have a tolerance range of, for example, 8°C–28°C and a preference for, for example, 18°C–24°C in varying settings [36, 108, 110]. Depending upon the study and its context, broodstock has a tolerance range of 8°C–28°C or 9°C–16°C and a preference for 13°C–16°C when spawning [36, 108, 111].

3.2.4 | Gilthead Seabream

Juveniles have a reported tolerance range of, for example, 8°C–30°C and a preference for, for example, 17°C–22°C in varying settings [36, 112]. Ongrowers have a tolerance range of, for example, 8°C–30°C [36]. Broodstock have a tolerance range of 13°C–20°C and they have a preference for 15°C–17°C when spawning [36].

3.2.5 | Common Carp

Fingerlings have a reported tolerance range of, for example, 2°C–38°C and a preference for, for example, 20°C–28°C in varying settings [35, 113]. Ongrowers and broodstock have a tolerance range of, for example, 2°C–36°C and a preference for, for example, 20°C–28°C when spawning [35, 113].

3.3 | Oxygen

Annex III of the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 and its amendment in Commission Delegated Directive (EU) 2024/1262 state 'Oxygen concentration shall be appropriate to the species and to the context in which the fish are held.' [15, 16]. The Annex introduces species- but not life-stage specific considerations into ensuring appropriate dissolved oxygen (DO) levels, in addition to considerations in relation to the holding conditions such as the given rearing systems, husbandry practices and procedures the fish are subjected to. Inter-relationships with other water quality indicators, for example, temperature, could also be considered here. The Annex and its amendment further state that 'Where necessary, supplementary aeration of tank water shall be provided, depending on the husbandry system' [15, 16], giving scope to proactively or reactively respond to potential drops in DO concentrations.

Oxygen availability is essential for fish, and most fish absorb oxygen from the water rather than from the air. They do this by gulping large amounts of water through the gills, where the gill filaments absorb the dissolved oxygen and transport it into the bloodstream. The availability of oxygen for fish depends on its solubility in water, which is affected by both temperature and salinity. As water temperature rises, oxygen solubility decreases, meaning that the concentration of oxygen (mg/L) at a given saturation (percentage of air saturation) drops with higher temperatures. Similarly, salinity also reduces oxygen solubility. Fish absorb oxygen through their gills primarily via diffusion, which is driven more by oxygen saturation than by concentration. Therefore, oxygen saturation is the more relevant factor when assessing oxygen availability [3]. Moreover, temperature not only influences oxygen solubility but also affects the fish's metabolic rate, increasing their oxygen demand as the temperature rises. Thus, temperature and oxygen availability are closely interlinked, and DO_{maxFI} (the threshold for oxygen saturation required for maximal feed intake) and LOS (the limiting oxygen saturation or the level below which routine metabolism cannot be sustained) are both highly dependent on temperature [114]. In addition to low dissolved oxygen saturations, chronic or periodic exposure to elevated oxygen saturations can occur in experimental settings due to the addition of pure oxygen to the rearing water [115]. This can be harmful to fish due to either oxidative stress, reviewed by [116], total gas pressure which can cause gas bubble trauma [117, 118] or diverse effects on ventilation, blood physiology and performance [119]. Oxygen saturation levels should, therefore, be measured at the same time and place as temperature and be monitored frequently, particularly when the risk of oxygen drop is elevated during stress, high densities and high temperatures. For all these reasons, oxygen saturation levels are a key WI that primarily impact upon welfare needs under the following domain: physical environment.

3.3.1 | Atlantic Salmon

An oxygen saturation above 80% is usually sufficient to maintain good welfare for all life stages. However, at temperatures above 15°C higher saturation values may be needed to maintain welfare for stressed or weakened individuals [4]. As far as the authors are aware, for fry and parr, detailed knowledge is not on hand, but [120] have reported a limiting oxygen saturation of 39% for parr held at 12.5°C and [33] suggests a preferred saturation of > 70%. For salmon post-smolts, oxygen saturation demand increases with temperature and for maximal feed intake it is approximately 42% at 7°C, 53% at 11°C, 66% at 15°C and 76% at 19°C, whereas limiting oxygen saturation is approximately 24% at 7°C, 33% at 11°C, 34% at 15°C and 40% at 19°C [114]. The impacts of hypoxia on post-smolt metabolism and activity vary with fish size, with smaller fish being more vulnerable than larger fish [121]. For broodstock, EFSA [35] suggests a preferred saturation of > 70%. Moderate supersaturations of oxygen have been shown to have positive effects on the growth of Atlantic salmon, but levels that are too high can lead to several welfare problems and even high mortalities [119, 122, 123].

3.3.2 | Rainbow Trout

Previous authors have reported that optimal and tolerance ranges for oxygen saturation for rainbow trout fry at 17°C–19°C are 81%–100% and 30%–80%, respectively [124], with fry showing elevation of plasma cortisol during 40% hypoxia but not after return to normoxia [125]. For juvenile and adult rainbow trout, the optimal range has been described as 80%–120%, while 60%–80% and 120%–160% (supersaturation) are within tolerance ranges, but the temperature was not specified [56]. A summary of optimal oxygen concentrations by life stage was given by [126], listing optimal ranges for fry and all other stages at 11 and 8 mg/L, respectively, while referring to variables that may alter the oxygen demand, such as high water temperatures or swimming speeds.

3.3.3 | European Seabass

Feed intake and growth of juvenile seabass are reduced at oxygen saturations below 40% at 22°C [127], while [128] found feed intake and growth of juveniles to be reduced in long-term exposure to oxygen saturations below 80% at 22°C.

3.3.4 | Gilthead Seabream

In the temperature range of 12°C–20°C the LOS of fed, swimming and undisturbed fish of ~400 g increased exponentially and was 17% at 12°C, 22% at 16°C and 36% at 20°C [129]. At levels below the LOS, seabream reduced their activity level and responded less to other stressors [129]. Feed intake and growth can also be impaired at oxygen saturations below 40% [36], and saturations of 40%–60% can lead to higher levels of gill injuries and higher haematocrit levels [130].

3.3.5 | Common Carp

For fingerlings, ongrowers and broodstock, anything > 20% has been reported to be within the tolerance range of carp, and anything > 60% has been reported to be optimal for ongrowers and broodstock [35], but there is little consideration of temperature in this context.

3.4 | Nitrogen Compounds

Annex III of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 and its amendment in Commission Delegated Directive (EU) 2024/1262 states, 'The concentrations … of nitrogen compounds, namely ammonia, nitrite and nitrate, shall be kept below harmful levels' [15, 16]. There are no species- or life-stage-specific considerations regarding appropriate concentrations in the Annex. There is also no text regarding potential interactive and cumulative effects between different water parameters in relation to the potential toxicity of some nitrogenous compounds.

3.4.1 | Ammonia

For most fish species, ammonia is a product of protein catabolism and is expelled via, amongst others, the gills into the adjacent water [131]. Water-borne ammonia exists in two forms: unionised ammonia (NH_3) and ionised ammonia or the ammonium ion (NH_4^+). The ratio between more toxic unionised ammonia and the less toxic ionised ammonia/ammonium will increase with increased pH and temperature and reduced salinity.

Ammonia is toxic for fish [132, 133] as it impairs the central nervous system, membrane and enzyme stability, gill status and function, swimming activity, feeding, and can bring about a loss of equilibrium and mortality [134]. Additionally, chronic exposure to ammonia can lead to reduced growth, vigour and fertility [134]. When water exchange is limited or where water treatment is not adequate in certain production systems, ammonia can accumulate in the water, which in turn prevents its excretion via the gills or causes its uptake from the surrounding environment. In those cases, ammonia can accumulate in the fish blood, leading to convulsions and death, unless the species in question has developed strategies to avoid ammonia toxicity, reviewed in [131].

In recirculating aquaculture systems (RAS), ammonia is converted to nitrite by ammonia-oxidizing bacteria, which is then oxidized to nitrate via nitrite-oxidizing bacteria [135]. This two-step process of nitrification is an integral element of the RAS water treatment process that can be disrupted by changes in water quality, production conditions and operations [136–138]. Water-borne ammonia levels primarily impact upon welfare needs under the following domain: physical environment.

3.4.1.1 | Atlantic Salmon. For Atlantic salmon fry, 96-h LC_{50}-values of 0.17–0.28 mg NH_3-N/L have been observed [139]. For parr, physiological adaptations have been observed at around 0.02–0.03 mg NH_3-N/L, while adverse effects on growth rate and gill health have been observed from 0.03 mg NH_3-N/L and changes in blood chemistry from around 0.05 mg NH_3-N/L [73, 140, 141]. For post-smolts reared in full-strength seawater 48-h LC_{50}-values ranging from 0.24 to 0.34 mg NH_3-N/L and 24-h LC_{50}-values of 0.15 mg NH_3-N/L have been found, with increases in gill ventilation frequency observed from 0.14 mg NH_3-N/L [142, 143]. Changes in blood chemistry, including plasma chloride levels and osmolality, have been observed from 0.04 to 0.08 mg NH_3-N/L [144–146]. EFSA [33] recommended target values of < 0.02 mg NH_3-N/L for all life stages.

3.4.1.2 | Rainbow Trout. In a study of rainbow trout, with fish from fry to the adult stage, 96-h LC_{50}-values ranging from about 0.13 to 0.90 mg NH_3-N/L were observed, with a reduction in sensitivity from the yolk sac fry stage to the juvenile stage, and an increase thereafter [147]. Growth and developmental challenges have been observed in fertilised eggs and yolk sac fry continuously exposed to 0.05 mg NH_3-N/L, with damages to the gill lamellae occurring at continuous exposure to 0.19 mg NH_3-N/L [148]. Behavioural effects and reduced growth have been observed in juveniles (6–10 g) exposed to 0.09 mg NH_3-N/L. Recommended safe levels for rainbow trout fingerlings generally range from below 0.01 to 0.03 mg NH_3-N/L [37, 55, 107, 149–153], yet 0.001–0.005 mg NH_3-N/L has also been proposed [154, 155]. Becke et al. [156] found no adverse effects on ongrowers exposed to up to 0.05 mg NH_3-N/L. For ongrowers and broodstock, recommended safe levels generally range from 0.01 to 0.05 mg NH_3-N/L [37, 55, 154].

3.4.1.3 | European Seabass. For European seabass juveniles and ongrowers, 96-h LC_{50}-values ranging from 0.97 to 2.30 mg NH_3-N/L have been observed [157]. For ongrowers, reductions in feeding activity, feed intake, and growth have been seen during chronic exposure over 2 months to concentrations exceeding 0.13 mg NH_3-N/L, yet fish exposed to concentrations up to 0.50 mg NH_3-N/L were able to adapt and recover from these effects [158, 159]. A recommended safe limit for European seabass fingerlings is 0.05 mg NH_3-N/L [36]. For juveniles and ongrowers, the proposed limits range from 0.06 to 0.26 mg NH_3-N/L [36, 158, 160].

3.4.1.4 | Gilthead Seabream. For gilthead seabream juveniles and ongrowers, observed 96-h LC_{50}-values range from 0.80 to 2.73 mg NH_3-N/L [157, 161, 162]. Wajsbrot et al. [163] found growth to be affected in juveniles exposed to concentrations exceeding 0.5–0.7 mg NH_3-N/L, with liver damage occurring in juveniles exposed to higher concentrations.

3.4.1.5 | Common Carp. For common carp fingerlings and juveniles, the observed 96-h LC_{50}-values range from 1.74 to 2.33 mg NH_3-N/L [164, 165]. For ongrowers, a proportional increase in catecholamine levels in various organs was observed with increasing concentrations from ~0.16 mg NH_3-N/L onwards, with more severe physiological symptoms being apparent from 1.00 mg NH_3-N/L [166, 167]. Recommended safe limits range from 0.05 to 0.40 mg NH_3-N/L for fingerlings and from 0.05 to 0.50 mg NH_3-N/L for juveniles, ongrowers and broodstock [35, 165, 168].

3.4.2 | Nitrite and Nitrate

Water-borne nitrate and nitrite are products of nitrification, during which ammonia is oxidised by microorganisms. Like ammonia, nitrite can be toxic, depending on the fish species, life stage as well as environmental conditions [169, 170]. Blood-borne nitrite reacts with iron from haemoglobin, changing it to methaemoglobin or ferrihaemoglobin, which lacks the capacity to bind oxygen and transport it to tissues [170]. Atlantic salmon are particularly sensitive to nitrite in freshwater, where this compound can compete with chloride for uptake in the gills, causing chloride depletion and affecting ion regulation, oxygen transport to tissues and several cardiovascular, excretory and endocrine functions [169, 171]. Water-borne chloride can have protective effects when water nitrite concentrations increase. For Atlantic salmon parr, it is suggested that the ratio of 108:1 Cl:NO_2-N [74] should have a protective effect, while for catfish, tilapia and tench this ratio is above 83:1 Cl:NO_2-N [171]. For common carp, a ratio of 5:1 Cl:NO_2-N was suggested [165].

Nitrate is the ultimate product of nitrification and can build up in RAS if the water exchange levels in the production system are low. However, in comparison to ammonia and nitrite, it is less harmful and higher concentrations that can occur under experimental conditions can be tolerated even by more sensitive species like Atlantic salmon [172, 173]. Nevertheless, concerns have been raised about nitrate or nitrite acting as endocrine disruptors in fish and other vertebrates, particularly affecting steroidogenesis, with potential effects on reproductive outcomes [174, 175]. It is suggested that high nitrate levels can be used to mitigate the potential build-up of hydrogen sulphide [176], which is highly toxic for the fish and can cause mass mortalities during production, also in experimental studies. Water-borne nitrite and nitrate are therefore good input-based WIs for aquaculture research, primarily impacting upon welfare needs under the following domain: physical environment.

3.4.2.1 | Atlantic Salmon. Data on the tolerance levels of Atlantic salmon to nitrite and nitrate are lacking for most life stages. At a stable water chloride concentration of 200 mg Cl^-/L, Atlantic salmon parr showed no effects on survival or gill histology from exposure to nitrite levels up to 10 mg NO_2^--N/L. Post-smolts exposed to nitrite levels fluctuating between 0.003 and 9.3 mg NO_2^--N/L, in a commercial production setting with chloride concentrations around 11 to 15 mg Cl^-/L, may have acquired hyperkalemia as a result [177]. For post-smolts, no negative effects on growth or welfare were seen for nitrate levels up to 100 mg NO_3^--N/L [172]. No clear effects of nitrate concentrations up to 100 mg NO_3^--N/L on endocrine functioning in parr or post-smolts have been observed [178, 179].

3.4.2.2 | Rainbow Trout. For rainbow trout fingerlings, nitrate 96-h LC_{50}-values from 1050 to 1355 mg NO_3^--N/L have been observed, with the lower value being for 15 ppt salinity and the higher value being for freshwater [180]. For juveniles, nitrate concentrations at 80 to 100 mg NO_3^--N/L were shown to reduce growth and survival, and to affect behaviour [181]. For nitrite, LC_{50}-values adjusted to 96-h and normalised to a water chlorinity of 20 mg Cl^-/L range from 1.8 to 4.1 mg NO_2^---N/L, for fingerlings through to ongrowers [182]. In this case, the 96-h LC_{50}-values recorded at chlorinity levels < 0.35 mg Cl^-/L ranged from 0.15 to 0.70 mg NO_2^--N/L [182]. Kroupova et al. [183] observed hyperplasia on gill lamellae and elevated plasma glucose in ongrowers exposed to 0.1 mg NO_2^--N/L. Recommended safe limits for rainbow trout fingerlings range from 5.6 to 16.9 mg NO_3^-N/L and 0.001 to 0.009 mg NO_2^--N/L [37, 56, 107, 180]. The recommended safe limit for ongrowers and broodstock is 11.3–33.8 mg NO_3^-N/L and 0.003 mg NO_2^--N/L [37, 56].

3.4.2.3 | European Seabass. For ongrowers, no effects on growth or feed conversion ratio of nitrate concentrations up to 500 mg NO_3^-N/L were observed, yet a reduction in the hepatosomatic index and daily feed intake was observed as nitrate levels exceeded 125 mg NO_3^-N/L [184]. For nitrite, 96-h LC_{50}-values for juveniles ranging from 274 to 154 mg NO_2^--N/L were observed for temperatures between 17°C and 27°C [185]. A reduction in haemoglobin levels was observed in ongrowers at 50 mg NO_2^--N/L and higher [186]. Suggested safe limits for European seabass are < 0.2 mg NO_2^--N/L and < 0.2 mg NO_3-N/L for larvae, < 0.5 mg NO_2^--N/L and < 0.5 mg NO_3^-N/L for fingerlings, and < 2 mg NO_2^--N/L and < 2 mg NO_3^-N/L for adults, yet it is unclear how these values were obtained [187].

3.4.2.4 | Gilthead Seabream. For larvae, a 48-h LC_{50}-value of 1.4 mg NO_2^--N/L was observed [188]. For juveniles, 96-h LC_{50}-values of 370.8, 619.5 and 806.3 mg NO_2^--N/L were recorded for salinities of 10, 20 and 30 ppt respectively [161]. For fingerlings through to adults, recommended safe limits are < 0.02 to 0.06 mg NO_2^--N/L and < 50 mg NO_3^-N/L [36, 188].

3.4.2.5 | Common Carp. For larvae and fry, 96-h LC_{50}-values of 2.55–48.70 mg NO_2^--N/L were observed for chloride concentrations from 1 to 45 mg Cl–/L [165] while no effects were observed for levels up to 2.13 mg NO_2^--N/L [189]. Recommended safe limits for all life stages are 0.05 mg NO_2^--N/L and 80 mg NO_3^-N/L [168, 190].

3.5 | pH

Annex III of the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 and its amendment in Commission Delegated Directive (EU) 2024/1262 states, 'The pH level shall be adapted to the species and monitored to be kept as stable as possible' [15, 16]. While there are species-specific considerations regarding appropriate pH levels in this excerpt of the Directive, life-stage specific requirements are not taken into account, but they are covered in the general text regarding water quality. Further, no attention is drawn to the potential interactive or cumulative effects of pH levels on other water quality indicators and their possible toxicity, although the need for stability in pH levels is acknowledged. Both extreme as well as fast-changing pH levels can occur in aquaculture and impair fish welfare, which makes pH a crucial parameter when auditing fish welfare. Abrupt or intense changes in pH can be detected in RAS or in net pens with poor circulation due to fouling. Such pH fluctuations may pose substantial welfare challenges for fish. For example, it has been shown that an acute reduction in pH from 8.4 to 6.3 impairs communication and social interactions between fish with wider implications on various individual and population processes such as mating and shoaling [191]. Further, pH affects the equilibria of multiple compounds with different toxicity (e.g., ammonium/ammonia, carbon dioxide/bicarbonate), particularly in systems with low water exchange. Additionally, both decreases and increases in pH can affect the speciation of metals, for example, Al and Fe, toward more toxic forms that can precipitate on the gills and cause mass mortality events [192–195]. The exact pH values at which adverse speciation effects are seen will depend on other water quality characteristics, such as oxygen availability or the presence of organic matter, see [196], (chapters 3, 6, and 7 for details) in the inlet and/or system water. In intensively fed aquaculture systems with oxygen supplementation and insufficient water exchange and pH control, the accumulation of CO_2 is a common cause for system water acidification. One of the main consequences of low pH on fish is the disruption of their chemosensory abilities, which in turn interferes with behavioural cues such as foraging and predator avoidance. Further, for marine fish, acidified seawater seems to negatively affect ontogeny in a stage-specific way, with the preflexion and flexion stages being more resilient than the juvenile stage [197]. Moreover, low seawater pH (pH = 7.5) has been associated with low hatching and larval survival rates tied with increased occurrence of malformations in fingerlings as well as reduced appetite and growth in adults [198]. Seawater is often used to detoxify acidic freshwater containing inorganic aluminium (LAI) by increasing its pH and ionic strength [199]. However, if the inlet water is rich in LAI and humic substances, remobilisation of metals from organic materials may occur when mixing freshwater with seawater [200]. While most of the species outlined in this review can cope without signs of distress with wide-ranging pH values between 5.5 and 8.5 [33, 36, 37], the complex interactions and effects of pH make it a good input-based WI for aquaculture research, primarily impacting upon welfare needs under the following domain: physical environment.

3.5.1 | Atlantic Salmon

Fry have a reported optimal pH range of 6.5–7 or even 6–8.5 in certain settings with values below 5 or 5.4 being considered outside the tolerance range [4, 33]. Parr have the same optimal reported pH range with values below 5.4 falling outside the tolerance range [4, 33]. The optimal reported range is 6–8.5 for smolts and 7–8.5 for post-smolts, while values below 5.4 are considered outside the tolerance range for both stages [33].

3.5.2 | Rainbow Trout

Fingerlings have a reported optimal pH range of 5.5–8.5 but are able to tolerate a pH of 4–5.5 in certain settings while values below 4 and higher than 9 exceed the tolerance range [37]. For the ongrowing stage, optimal pH may be anywhere from 7–7.5

[56] to 5.5–8.5 [37] with values below 6 and higher than 8.5 [56] or below 4 and above 9 [37], exceeding the tolerance. Similar thresholds have been reported for broodstock, with optimal pH being 5.5–8.5 and values of 4 and 9 being considered the lower and upper tolerance thresholds respectively [37].

3.5.3 | European Seabass

Seabass have a reported optimal pH of around 8 for fingerlings and around 8.2 for adults [36]. Conversely, pH values below 6.5 and above 8.5 are considered outside the tolerance threshold in fingerlings and juveniles as well as adults [36].

3.5.4 | Gilthead Seabream

For gilthead seabream fingerlings, juveniles, and adults, a pH of 8 is considered optimal, while the reported lower and upper tolerance thresholds are 7.5 and 8.5 respectively [36].

3.5.5 | Common Carp

Carp fingerlings have a reported optimum pH of 7.5–8 and are able to tolerate values as low as 5.9 and as high as 9.5 [35, 201, 202]. The reported optimum range of juvenile carp is slightly wider at 7–8, while values of 5.5 and 10 are considered the lower and upper optimum tolerance thresholds respectively [35, 201, 202]. Adults and broodstock have the same reported optimum and tolerance range as juveniles [35, 201, 202].

3.6 | Carbon Dioxide

Considerations in relation to dissolved carbon dioxide (CO_2) concentrations are not covered in Annex III of the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 [15] but are addressed in its amendment, the Commission Delegated Directive (EU) 2024/1262 of 13 March 2024 [16]. The amendment states 'The concentrations of carbon dioxide … shall be kept below harmful levels' [16]. Elevated CO_2 concentrations have been shown to negatively affect feed intake, digestion, feed conversion and growth rates [203], and are therefore relevant for aquaculture research. As a product of aerobic metabolism, CO_2 can accumulate in closed culture systems without sufficient water exchange or the use of removal mechanisms such as aeration. Aquaculture ponds typically see diurnal variations in CO_2 concentrations, with levels peaking at dawn or after phytoplankton die-offs [204], and natural waters may also, at least temporarily, exhibit CO_2 concentrations that can be detrimental to aquatic life [197, 205]. Dissolved CO_2 in water and blood contributes to the carbonate system, thereby interacting strongly with pH, and it seemingly has its main physiological effect through its action on acid–base homeostasis in fish. Elevated dissolved CO_2 levels cause an increase in plasma CO_2 levels, which in turn leads to respiratory acidosis, that is, a reduction in blood and tissue pH. The fish will compensate for this by actively excreting H^+ and simultaneously increasing blood HCO_3^- concentrations [206, 207]. There is however a metabolic cost and a limit to the compensatory abilities of the fish [208]. The limit will be influenced by several physiological and environmental factors, such as the fish's activity level or the water's ionic strength [207]. If a reduction in blood pH persists, blood oxygen transport capacity may be reduced through the Bohr effect, reducing haemoglobin's affinity for O_2, and for some fish also through the Root effect, reducing haemoglobin's O_2 carrying capacity. Persistently elevated CO_2 levels have further been shown to have behavioural effects on some fish species, including negative effects on foraging behaviour, boldness and activity levels as well as sociability. These effects are associated with the alteration of certain neurotransmitter receptors, predominantly GABA, but possibly also impairments to the sensory capabilities of the fish, including the functioning of their olfactory organs [209–211]. However, detailed knowledge of how elevated CO_2 affects fish, especially at the intracellular level, is still lacking [207]. There is seemingly still a lack of published data to support any conclusions regarding safe operating CO_2 concentrations for the species considered here, except for Atlantic salmon. Nevertheless, dissolved CO_2 is a relevant input-based WI for aquaculture research, primarily impacting upon welfare needs under the following domains: physical environment and behavioural interactions.

3.6.1 | Atlantic Salmon

Studies on Atlantic salmon parr have shown negative effects on growth occurring when dissolved CO_2 concentrations increase beyond 15 mg/L [212], while [213] found reduced growth at 10 mg/L in juvenile salmon kept in RAS. In post-smolts reared in brackish water in RAS, Mota et al. [214] found reduced growth from 12 mg/L CO_2 upwards. Recommended safe limits range from below 10 and 15 mg/L [192, 203].

3.6.2 | Rainbow Trout

For rainbow trout, growth depression has been observed at concentrations from about 34.5 mg/L and upwards, but not at concentrations below this level [215, 216]. Recommended safe limits are 9–30 mg/L for fry and fingerlings [55, 107, 217, 218] and 5–30 mg/L for ongrowers [56].

3.6.3 | European Seabass

For European seabass, lethargy and skin hyperpigmentation have been observed at 75 mg/L following a graded increase in CO_2 concentration, and LC_{50}-concentrations have been documented at 115.5 mg/L following 48 h of exposure and at 104.8 mg/L following 120 h of exposure [219, 220]. A recommended safe limit is 40 mg/L for all life stages, although it is unclear how this value was obtained [187].

3.6.4 | Gilthead Seabream

For juvenile and adult gilthead seabream, growth depression has been observed at concentrations exceeding 20 mg/L [221].

3.6.5 | Common Carp

For fingerlings, ongrowers and broodstock, concentrations <25 mg/L are reported to be within the tolerance ranges of carp [168].

3.7 | Salinity

Annex III of the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 states 'The salinity shall be adapted to the requirements of the fish species and to the life stage of the fish. Changes in salinity shall take place gradually' [15]. Both species- and life-stage-specific considerations regarding salinity levels are addressed in this part of the Directive. There is no requirement for salinity levels to be optimal, just that they are adapted to the needs of the fish. The need for stability in salinity levels is also acknowledged; however, certain species and life stages can be exposed to a range of salinities in nature and, for example, in open rearing systems.

Depending on their habitat, fish have evolved different strategies to maintain their inner water and salt balance. Freshwater fish live in a hypoosmotic environment and take in large amounts of water through their gills and skin. They do not drink and excrete large volumes of dilute urine through their kidneys. In contrast, saltwater species, living in a hyperosmotic environment, compensate for their water loss by drinking, and their urine is much more concentrated [222–224]. While euryhaline fish tolerate a broad range of salinities during parts or all of their lifecycle, stenohaline fish are dependent upon a narrow and relatively constant span of salinities of the surrounding water [225]. The anadromous and euryhaline Atlantic salmon and rainbow trout undergo smoltification, involving preparatory physiological changes prior to seawater transition [226, 227]. Research on anadromous fish that spans over the smoltification period often involves seawater challenge tests to determine the seawater readiness of the experimental fish [228], whose age and size should be considered. Overall, salinity is a relevant input-based WI for aquaculture research, primarily impacting upon welfare needs under the following domain: physical environment.

3.7.1 | Atlantic Salmon

Euryhaline. Seawater tolerance is limited before completing smoltification and after the onset of maturation [229] so salinity should be adjusted accordingly [3]. The tolerance range from fry to smolts is reported to be 0–10 ppt [33, 230, 231], although Handeland and Stefansson [232] found reduced growth and post-smolt performance in parr and pre-smolts exposed to salinities of 6, 13 and 20 ppt. Salmon post-smolt cope well with salinities in the range from freshwater to full seawater, but growth, metabolic rate and stress regulation are better at moderate, more isosmotic salinities than at full seawater [233, 234]. Ongrowing Atlantic salmon cultivated in freshwater RAS systems have more incidences of precocious males than ongrowers kept in marine net pens [235]. Broodstock (prior to and during spawning) have a reported optimal salinity of <8 ppt and <10 ppt, respectively [33]. Further, wild landlocked Atlantic salmon mature at a smaller size compared to their anadromous conspecifics [236]. A possible mechanistic link between salinity and sexual maturation has however not been established yet.

3.7.2 | Rainbow Trout

Euryhaline. The osmoregulatory capacity of rainbow trout is body weight dependent, and seawater tolerance increases from 50 to 150 g [237]. Ongrowing protocols in both fresh water and brackish/seawater are tolerated, but salinity acclimatisation protocols are recommended for this species [238] if transferred to higher salinities. Even though features of smoltification are present in rainbow trout, photoperiod as a zeitgeber seems to play a less important role in their smoltification [226] than other signals, such as a gradual increase in salinity [239] or the use of transition feeds [240], which can be used to acclimatise rainbow trout to seawater. For broodstock, it has been demonstrated that too low concentrations of sodium, chloride and potassium in freshwater (0.2 mg L^{-1}) negatively impact egg quality in rainbow trout [241]. Seawater rearing is not recommended for sexually maturing broodstock as it reduces both broodfish and egg survival, whereas brackish water (10–13 ppt) resulted in the best survival [242].

3.7.3 | European Seabass

Euryhaline. For juvenile seabass, mortality increased with increased salinity above 30 ppt, while no mortality occurred in the range of 3–30 ppt [243]. Ongrowers have a reported optimal salinity of 30 ppt, and tolerance ranges of 0–60 ppt depending on strain and conditions [244–246]. To the authors' knowledge, no published data on the salinity tolerances or preferences of European seabass broodstock in aquaculture is available. Field studies on wild European seabass indicate that spawning takes place at salinities above 30 ppt around the Irish coast [247] and that a salinity around 35 ppt was preferred for spawners in the English Channel [248].

3.7.4 | Gilthead Seabream

Euryhaline. Juveniles have reportedly a better growth at 12 ppt versus either 6 or 38 ppt [249]. For ongrowers, only small differences in metabolic rate were observed for salinities ranging from 5 to 30 ppt [250].

3.7.5 | Common Carp

Stenohaline, freshwater. Fingerlings are reported to have an optimal salinity of 0.5–2.5 ppt and a tolerance range of 2.5–7.0 ppt, while ongrowers and broodstock have a reported tolerance range of <6.0 ppt [251–253]. In juveniles, salinity levels from 10 ppt and above have been found to result in reduced feed intake and growth, increases in plasma glucose levels, and a failure to uphold osmoregulatory homeostasis, increasing the risk of mortality [254–256].

3.8 | Lighting

Annex III of the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 states in Section A: general information that '(a) Where natural light does not provide an appropriate light/dark cycle, controlled lighting shall be provided to satisfy the biological requirements of the animals and to provide a satisfactory working environment. (b) Illumination shall satisfy the needs for the performance of husbandry procedures and inspection of the animals. (c) Regular photoperiods and intensity of light adapted to the species shall be provided. (d) When keeping albino animals, the lighting shall be adjusted to take into account their sensitivity to light'. When specifically considering fish, the Annex states 'Fish shall be maintained on an appropriate photoperiod' [15]. The fish-specific text does not provide additional requirements for species or life stage considerations beyond what is already outlined in the general text. Furthermore, it does not address other aspects of light, such as its colour spectrum, although light intensity is covered in the general information section above.

The light environment has widespread effects on biological processes in most animals, including fish. In natural fish habitats, both the intensity (quantity) and colour (quality) of light depend on the depth and turbidity of water [257]. While outdoor systems are self-reliant as they receive natural illumination, lighting needs special consideration when fish are kept in indoor systems. The three components of lighting include the quantity (intensity measured in lux), quality (spectrum and distribution measured in nm wavelength) and periodicity (photoperiod mostly indicated in hours) [257, 258], and the application of artificial lighting in indoor systems requires an appropriate combination of these three components [259]. Additionally, the vision and spectral receptivity of each fish species are strongly adapted to their natural habitat and behaviour [259–261]. Consequently, appropriate light conditions are highly dependent on the species and developmental stage of fish [257]. For example, a minimal light intensity is necessary for achieving normal feeding behaviour, and thus for appropriate development and growth for most fish species [259, 262, 263]. However, a light intensity that is too intense can be stressful or lethal [257]. Similarly, the wavelength (quality) of light, which includes the colour of rearing tanks, can affect growth [258, 259, 264–266], behaviour [267] and physiological status [265, 266]. Further, the photoperiod can be a zeitgeber for the circadian (diel) and circannual (seasonal) rhythms of fish [257, 258, 268]. Common photoperiod protocols are simulated natural photoperiod, continuous light, continuous darkness, or a combination of darkness and light (e.g., a 12:12 light–dark cycle). A number of physiological functions, such as smoltification in salmonids [269–271], muscle growth [272], immune response [273] and sexual maturation [274] are under photoperiodic control.

When fish are kept under an artificial light–dark cycle, one should avoid any swift adjustments in lighting as this may be stressful for the fish [275] and the stress response is only partly habituated [276]. One fundamental limitation for creating standardized appropriate light environments in aquaculture research is insufficient characterisation of species-specific light perception in combination with light measuring practices based on the human visual perception of light (lux). For mammalian research, it has recently been recommended to use a new metrology, α-opic irradiance, to quantify how light is experienced by the animal [277]. Correspondingly, knowledge about species-specific light perception in relevant aquaculture species is expected to see advances in the near future. Given its far-reaching effects, lighting is a relevant input-based WI that primarily impacts upon welfare needs under the following domains: behavioural interactions and nutrition.

3.8.1 | Atlantic Salmon

In the wild, Atlantic salmon are classified as mostly diurnal, but parr can be nocturnal at water temperatures below 10°C [278], and smolts often undertake their migration to sea at night, to, for example, avoid predation [279]. During egg incubation, it is recommended to keep the eggs in darkness [280]. This recommendation can be ascribed to an older study that demonstrated adverse effects of early light exposure on sockeye salmon [281]. Rearing under 24-h continuous light is common practice, for example in Atlantic salmon smolt production. Yet, an increased change in daylength is required to initiate smolt development and this is commonly achieved by subjecting the fish to a short photoperiod, followed by a period of approximately 350 degree-days with long photoperiod [282]. Recently, it has been suggested that early male maturation in post-smolts can be prevented by including a 3-h scotophase during long-day exposure [283]. Continuous exposure to light without a period of shortened daylength can interfere with smolt development and later seawater performance [284, 285]. The absence of a daily light–dark cycle can inhibit the ability of salmon gills to respond to oxidative stress [286]. Continuous high light intensities can cause retinal damage in post-smolt Atlantic salmon [287]. In marine net pens, light intensity is a major driver of swimming depth [84], and artificial underwater lighting can affect salmon swimming depth [288, 289] and growth [290]. The use of continuous artificial light during the first winter at sea inhibits sexual maturation in Atlantic salmon and other salmonids [291]. On the other hand, exposing salmon to a decreasing daylength during summer may advance maturation and is a common protocol used in broodfish production [290, 292, 293]. Lights with a low enough intensity to not affect maturation may still be sufficient to attract salmon to differing swimming depths [294].

3.8.2 | Rainbow Trout

Wild rainbow trout are classified as mostly diurnal, but juveniles can be nocturnal in winter when water temperatures drop below 8°C [295]. Egg incubation should be done in darkness [296]. An immunosuppressive effect of continuous 24-h light has been reported in juvenile rainbow trout [297]. Increasing daylength promotes seawater adaptation in juvenile fish [298], but other drivers for adaptation should also be considered, such as salinity and water temperature [226]. Sudden transitions from light to dark can lead to panic in trout [275]. Surface light during the night can promote swimming behaviours that are similar to those seen during the day in ongrowers [299], and changes in daylength can be a driver for maturation and spawning in marine-reared ongrowers [300].

3.8.3 | European Seabass

The species is classified as mostly diurnal but can become nocturnal as water temperature decreases [301]. In European seabass larvae, 24-h continuous light results in high survival but also a relatively high prevalence of swim bladder problems and jaw deformities; therefore, a light–dark cycle is recommended for early rearing of this species [302]. Continuous high light intensities can cause elevated cortisol levels and retinal damage in seabass [287].

3.8.4 | Gilthead Seabream

The species is classified as mostly diurnal but can become nocturnal as water temperature decreases [303]. The use of continuous light to enhance growth and delay sexual maturation is common in gilthead seabream, yet it has not been resolved whether rearing under continuous light conditions favours the development of skeletal deformities [45].

3.8.5 | Common Carp

In the laboratory, demand-fed carp can select feed throughout a 24-h period, irrespective of whether it is during the light or dark period, although they adopted a 60:40 preference for feeding during the dark phase [304]. Many sources have suggested a 12:12 light–dark cycle for all life stages of carp in the laboratory [27, 258, 305, 306].

3.9 | Noise

Annex III of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 states in Section A: general information that '(a) Noise levels including ultrasound, shall not adversely affect animal welfare. (b) Establishments shall have alarm systems that sound outside the sensitive hearing range of the animals, where this does not conflict with their audibility to human beings. (c) Holding rooms shall where appropriate be provided with noise insulation and absorption materials'. With specific regard to fish, it states 'Noise levels shall be kept to a minimum and, where possible, equipment causing noise or vibration, such as power generators or filtration systems, shall be separate from the fish-holding tanks' [15]. The 2024 amendment of the Directive [16] added a further point '(d) For aquatic animals, equipment causing noise or vibration, such as power generators or filtration systems, shall not adversely affect animal welfare' [16]. The Directive and its amendment contain good, brief guidance on setting up a holding or experimental facility to (i) minimise exposing the fish to noise that affects their welfare, (ii) reduce unnecessary noise and (iii) create an alarm system that is not within the audible range of the fish. It does not provide further information on what thresholds to consider, and species or life stage considerations are not referred to in the text, beyond what was already covered in the general text on water quality.

There are several definitions of noise [307, 308], and for the purpose of this review, we define noise as any unwanted sound that has a detrimental effect on the fish. Noise can alter normal behaviour, decrease the detectability of biologically relevant sounds (masking), induce loss of hearing (temporally or permanently), cause physical injury, physiological changes, or, in the worst case, death, depending on its duration and amplitude [309]. Due to the differences in hearing capabilities between different species, a given sound could be detrimental to one species, whereas it may not even be detected by another species. All fish species can perceive the particle motion constituent of sound (the kinetic energy), while only some species can perceive the sound pressure constituent of sound (the potential energy) [310, 311]. Hearing thresholds that describe which sounds can be detected have been determined for several species, such as Atlantic salmon [312, 313], rainbow trout [314], and common carp [315]. However, thresholds that distinguish between detectable sound and detrimental noise are mostly unknown. Furthermore, even though particle motion is what most species detect, and particle motion and sound pressure are not proportional in rearing environments such as tanks [316], most studies investigating noise in aquaculture have focused on sound pressure. Typical sound pressure levels found in aquaculture have been described for a range of different production settings, such as tanks of different sizes and designs [317–319], raceways [317], ponds [317, 319] and net pens [319]. Both sound pressure and particle motion should be measured to determine whether an environment is noisy for a given fish species, and as the Directive 2010/63/EU states, it is advisable to keep noise at a minimum. We would also like to suggest that, where possible, noise is kept at similar levels across all experimental units, at least within a given experiment, to reduce unnecessary variability between, for example, replicates within a treatment, and to enhance the potential reproducibility of the results. Johansen et al. [51] suggest that fish can become acclimated to background noise, so noise levels should be kept similar throughout the duration of the experiment. We would also like to suggest that attempts are made to keep levels similar to those experienced prior to the experiment to aid acclimation. Given its potential effects, noise is a relevant input-based WI, primarily impacting upon welfare needs under the following domains: physical environment and behavioural interactions.

3.9.1 | Atlantic Salmon; Rainbow Trout; European Seabass; Gilthead Seabream; Common Carp

As far as the authors are aware, there is no data available on thresholds for different noise levels with regard to sound pressure and particle motion in relation to their impacts upon welfare. Yet, there is some evidence of abnormal behaviour (sudden dives or direction changes) caused by thunderstorms in farmed gilthead seabream [320].

3.10 | Stocking Density

Annex III of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 states 'The stocking density of fish shall be based on the total needs of the fish in respect of environmental conditions, health and welfare' [15]. This information outlines the need to holistically consider density in relation to water quality and the fish's health and welfare, allowing for the inclusion of further indicators, for example, behavioural and morphological, in the decision matrix when deciding which stocking densities are appropriate or not.

Stocking density, calculated as kg/m^3 can be defined as fish density at any given moment within a specific production system [321]. Its utility as an input-based WI is under evaluation as the application of a given stocking density should be driven by the outputs of a number of other WIs [4]. Indeed, EFSA's scientific opinion on the welfare of farmed trout states 'stocking density per se should not be used as an indicator for good welfare as it is difficult to set appropriate levels of stocking densities, the monitoring of the conditions of the fish should be regarded as a preferred option' [37]. This is because a simple biomass/volume basis for auditing and safeguarding welfare does not consider fish behaviour, water quality parameters [37] and other variables (e.g., the injury status of the fish). We therefore consider that guidelines for optimal/within/outside tolerance would be inappropriate.

However, it has been documented that high stocking density in fish systems may negatively impact numerous welfare needs including the fish's need for behavioural freedom. It may also impair health by causing skin and fin damage [322], likely caused by the resulting decreased water quality [323] and contact and interactions with other fish, the production system and equipment within the system [4]. Additionally, high stocking densities may cause elevated primary stress response (cortisol) that, in turn, leads to elevated secondary (lactate, glucose and magnesium, amongst others) and tertiary stress responses, such as loss of appetite, weight loss [324] and in worse cases, mortality. Further, previous work points out the strong link between density and environment and that high densities may cause stress for the fish even if other environmental factors are within acceptable limits [51]. The degree of harmful effects of high stocking densities depends on numerous considerations, for example, the species, life stage, duration of exposure to high densities, their intensities in terms of kg/m^3 and water quality.

Similarly, densities that are too low may also impair welfare [51] causing, for example, physical damage or impaired physiology and condition [325]. Atlantic salmon, rainbow trout and brown trout juveniles seemingly exhibit more territorial and aggressive behaviour at lower rearing densities [326–328], although some findings contest this [329]. Johansen et al. [51] also point out that low densities may cause stress, especially in shoaling fish.

Stocking density, in addition to its complex interactions with fish welfare, is a critical parameter in aquaculture operations and is included in many farm protocols, industry guidelines, and national regulations. This makes it a relevant consideration in aquaculture research, especially when experiments are scaled up to densities relevant to industry practices. When trials are conducted in industrial settings or on farms, existing welfare standards, local regulations, and laws may apply. As a result, we acknowledge stocking density as an important input-based welfare parameter in aquaculture research, primarily impacting upon welfare needs under the following domains: physical environment and behavioural interactions.

3.10.1 | Atlantic Salmon, Rainbow Trout, European Seabass, Gilthead Seabream, Common Carp

With regard to the considerations and confounding factors outlined above, the authors do not wish to make recommendations on numerical thresholds for different stocking densities in relation to their impacts upon welfare. For aquaculture research, if densities are not a specific objective of the experiment, they should be defined in relation to water quality, fish health and other WIs including behavioural considerations and injury levels and a focus should be on monitoring and documentation. For more information on the wide range of species-specific densities that have been studied there are various reviews, articles and reports available for (i) Atlantic salmon [4, 33, 324, 330] (ii) rainbow trout [37, 321, 326, 331, 332] (iii) European seabass [36, 333–336] (iv) gilthead seabream [36, 130, 337–339] and (v) common carp [35, 340–342]. Many of these references also contain general recommendations on stocking densities for each species.

3.11 | Water Volume, Water Velocity and Water Exchange Rate

Annex III of the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 considers both the dimension and the setting up of the rearing system in relation to (i) water volume, stating 'fish shall have sufficient water volume for normal swimming, taking account of their size, age, health and feeding method' and (ii) water flow, stating it 'shall be appropriate to enable fish to swim correctly and to maintain normal behaviour' [15]. In the Annex, the water volume and flow auditing parameters address how these impact the behaviour of the fish, focusing on swimming and the maintenance of normal behaviour, without specifically outlining how these indicators should be examined in detail. Further, water flow appears to address both water exchange rate and water velocity, which will be addressed separately in this review.

3.11.1 | Water Volume

The appropriate water volume will depend on the fish number, species and life stage. In many instances, the rearing systems used in experimental settings are smaller than those used in commercial facilities, to satisfy needs related to improved control, replication or economic considerations [72]. However, the applied research done on a small scale should often aim to be applicable or representative of commercial farming [72]. Whatever the scale of the system, the rearing unit must have enough water volume to ensure normal swimming behaviour [15].

3.11.2 | Water Velocity

A good water velocity in a tank-based aquaculture system is important for both system cleaning and fish welfare. The velocity of the system water depends on several factors, such as system size [343], pump strength and many construction specifics. Several studies have demonstrated that velocity in this type of system is not homogeneously distributed, either vertically or horizontally, and this variability is driven by the system design, for example, the placement of the inlet and outlet pipes [344, 345]. In large-scale tank systems, techniques and methods for homogenising water velocity throughout the tank are often needed and preferred to secure uniform water speeds. Velocities that are too high or low can lead to poor welfare [72], and even though high velocities have been related to exercise, heart health [346] and increased growth [234, 347], too high velocities may have negative effects on, for example, skin health [348].

In marine net pens, water velocities are more variable in time and may change with, for example, the tidal rhythm or weather conditions, and further depend on how exposed or sheltered the site is. The highest water velocities at a site must not exceed the sustained swimming capacity (duration of hours) of the fish, as this will lead to fatigue and poor welfare [349]. Sustained swimming capacity is determined by a range of factors such as fish size, temperature, duration, and the health status of the fish [350]. Whether in tank-based systems or open net pens, water velocity is an important input-based welfare parameter in aquaculture research, primarily impacting upon welfare needs under the following domains: physical environment and behavioural interactions.

3.11.2.1 | Atlantic Salmon. There is a lack of information regarding optimal velocities for young stages of farmed Atlantic salmon. Heggenes and Traaen [351], showed that for wild salmonids, the optimal velocities just after first feeding were between 0.10 and 0.25 m/s, dependent on individual condition and water temperature. Once the fish size exceeded 40–50 cm they could handle velocities above 0.5 m/s. For post-smolts, higher levels of cortisol, reduced skin quality and more cataracts were found when reared at velocities of 1 body length per second (BL/s) compared to 0.3 BL/s in RAS [234]. Timmerhaus et al. [348] also compared velocities in RAS of 0.5, 1.0, 1.8 and 2.5 BL/s and found a positive relationship between weight and velocity; more precisely since the 0.5 BL/s resulted in a significantly lower condition factor compared to the others, and the muscle cells from the fish in the higher velocity groups deviated from the lower velocity, it was concluded that the weight was a consequence of higher muscle mass due to exercise. The higher velocity groups further resulted in inflamed muscle fibres, reduced skin health and schooling behaviour, most likely because the fish tried to gather at tank locations with lesser velocities [348]. This led to the conclusion that the overall optimum water velocity for post-smolts was 1 BL/s or slightly above. When comparing the effects of velocities of 0.2, 0.8, and 1.5 BL/s in tank-held Atlantic salmon post-smolts over 6 weeks, Solstorm et al. [352] found an increase in fin erosion in the higher velocity group yet more agonistic and structural behaviour in the lower velocity group. The sustained swimming capacity of healthy Atlantic salmon post-smolts is at least 80% of the critical swimming speed [353].

3.11.2.2 | Rainbow Trout. In fry/fingerlings, good water velocities can be around 0.9 BL/s or range from 0 to 1 BL/s or 0.75 to 1.5 BL/s [332, 354–356]. For ongrowers, Tschirren et al. [56] suggest 0.5 to 1 BL/s is optimal and 0.2 to 3 BL/s are within tolerance ranges. Other studies on juveniles and ongrowers using velocities from 0.5 to 3.0 BL/s have not been able to show differences in cortisol and haematocrit levels, hepatosomatic index, growth or survival after 9 weeks or more [357, 358].

3.11.2.3 | European Seabass and Gilthead Seabream. Larval European seabass and gilthead seabream are susceptible to vertebral deformities and opercular malformations, respectively, when exposed to high water velocities [359–361]. When exposed to minimal water movement or to water velocities of 1 or 2 BL/s over 8 months, juvenile gilthead seabream grew significantly larger at 2 BL/s but showed overall lower stress, larger hearts, and fewer lordotic deformities at 1 BL/s [362]. Juveniles exercised at 1.5 BL/s utilized nutrients more efficiently and showed greater white muscle growth [363], and similar observations were made for juveniles exercised at 5 BL/s, which additionally had higher muscle capillarisation at 5 BL/s than in still water [364]. Swimming at velocities of 1.1 BL/s, as compared with 0.8 BL/s, also affects the body shape and increases the oxygen-carrying capacity of juveniles [365].

3.11.2.4 | Common Carp. Only a few studies appear to have been conducted on the velocity tolerances of common carp, a species that naturally lives in still water and tends to avoid higher water velocities in the wild [366]. However, water velocities of up to 2.5 BL/s for 3–4 weeks have been beneficial for growth and physiological performance in carp juveniles up to 5 g [367] as well as for the density of myonuclei in the fast muscle fibres of carp weighing ca. 85–95 g [368].

3.11.3 | Water Exchange Rate

The exchange of water is essential in closed, intensively operated production units such as tanks and raceways due to the need to replenish oxygen levels, remove waste material and metabolites, and possibly control water current regimes. Additionally, the efficiency of water treatment units may be affected by water flow rates. Conversely, extensive ponds can be operated with little to no controlled water exchange due to natural processes for oxygen replenishment and waste removal, and open production units, such as floating net pens, are subject to a constant, current-driven exchange of water if appropriately placed and well maintained. For instance, certain design choices, such as applying shielding technology in the form of lice-shielding skirts or semi-closed net pens are known to limit water exchange rates in open net pens under certain conditions [369]. Water exchange in closed production units can be expressed as the volume of water flowing into and exiting the unit per unit of time (e.g., L/min) or as the percentage of the water volume exchanged per day. Alternatively, it can be linked to the biological production and expressed as the volume of water flowing into and out of the unit per kg fish per time (e.g., L kg^{-1} min^{-1}), a quantity referred to as specific water flow. A quantity closely linked to water exchange is hydraulic retention time (HRT, expressed in minutes), which is given as the volume of a container divided by the amount of water flowing through that container per time. In land-based flow-through systems, the specific water flow is commonly used and can serve as an intensity indicator. At low levels, oxygen supplementation may be necessary to secure good rearing conditions. At the same time, carbon dioxide and other substances can accumulate in the water and create risks of sub-optimal water quality. This rather complex situation may cause many different welfare problems for the fish.

Establishing target values for water exchange is complicated by the fact that both characteristics of the system water, the production unit and the biological processes occurring in it, affect the need for water renewal. Intensively operated tank-based aquaculture systems often employ unit processes for water treatment such as seawater intermixing, oxygenation, aeration (also used in shielded net pens) or recirculation loops, which all may further complicate setting target values as well as correlating water exchange values to fish welfare characterisations. Nevertheless, water exchange remains an important process monitoring and control parameter, primarily impacting upon welfare needs under the following domain: physical environment.

3.11.3.1 | Atlantic Salmon. Fivelstad et al. [370] saw reduced growth in Atlantic salmon fry reared at 0.7 $L\,kg^{-1}\,min^{-1}$, however, this could also have been the result of lower oxygen levels. Later, Fivelstad et al. [231] found no adverse effects on smolts reared in brackish water (1.6–4.5 ppt) at specific water flows down to 0.15 $L\,kg^{-1}\,min^{-1}$. Elevated stress and immune responses have been found in the skin of post-smolt Atlantic salmon reared at specific water flows at 0.3 $L\,kg^{-1}\,min^{-1}$ and below [322]. Similarly, Calabrese et al. [371] recommended 0.3 $L\,kg^{-1}\,min^{-1}$ as a lower limit for specific water flow for post-smolts in closed systems. All these studies were conducted using flow-through tank configurations and oxygen supplementation.

3.11.3.2 | Rainbow Trout. Ross et al. [372] reported that subadult rainbow trout welfare was affected by different tank designs. However, the negative effects were modified or eliminated with an increased water exchange rate (1.5 to 2.5 exchanges per hour). Good et al. [373] have reported significantly worse fin damage and subclinical skin lesions in fish raised on low water exchange rates in RAS, but a further study by Davidson et al. [374] reported that using ozone in low water exchange rates in RAS can enhance growth, condition factor and survival.

3.11.3.3 | European Seabass. For seabass (100–150 g individual weight), waste product accumulation was found to be problematic below water exchange rates of ca. 0.33 $L\,kg^{-1}\,min^{-1}$ [375]. This study was also conducted using flow-through tank configurations and oxygen supplementation.

Studies on the other species and life stages covered in this review are scarce but generally, the accumulation of metabolites becomes limiting at lower specific water flows [376]. An exception has been observed for gilthead seabream larvae, where a lack of water exchange up to 10 days after hatching was found to improve growth [377].

3.12 | Turbidity and Total Suspended Solids (TSS)

Considerations in relation to levels of turbidity and total suspended solids are not covered in Annex III of the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 [15]. However, suspended particles are known to be potentially detrimental to fish in natural waters, and both physiological and behavioural effects have been observed [378]. The physiological impact of particles on fish includes increased stress, gill lesions/abrasions, skin irritations/lesions and fin erosions/abrasions. Given those impairments on fish health and welfare, the authors consider suspended solids a relevant WI worth including in aquaculture research.

From an aquaculture systems management perspective, high turbidity can affect light attenuation in water, thereby changing the intensity and quality of light in rearing systems and affecting rates of photosynthesis and levels of dissolved oxygen [379]. High turbidity may also affect the albedo and heat flux of natural waters and can result in the stratification of lakes, even shallow ones, which may be of relevance to aquaculture ponds [380–382]. Noble et al. [4] also pointed out that the visual monitoring of fish health and feeding responses on farms will be limited under conditions of high turbidity. Further, increased suspended particle loads have been shown to adversely affect the performance of pumps [383] and several water treatment processes common in aquaculture systems, especially RASs, including UV water disinfection [378]. Additionally, elevated organic particle loads can have negative effects on biofilter performance and microbial loads, thereby increasing oxygen consumption and threatening system stability in RAS [378].

Beyond these effects on the rearing environment, solids have been shown to directly impair fish health and welfare. Studies on Sockeye salmon have shown the trapping of fine particles in the gills and increased occurrence of gill trauma at natural suspended sediment concentrations exceeding 3000 mg/L, and a higher coughing frequency at suspended sediment concentrations in excess of 200 mg/L [384, 385]. It should be noted that salmonid aquaculture systems, as reported in the literature, usually experience TSS loads no higher than 20–25 mg/L [378]. With regard to behavioural responses, Pacific salmonids in nature appear to avoid highly turbid waters [386–388] and the reactive distances of some fish (e.g., rainbow trout) decline as turbidity increases [389]. In a laboratory setting, Atlantic cod juveniles have been shown to prefer slightly turbid over clear water [390], yet larvae of the same species grew better in RAS with lower turbidity and particle load [391]. Pikeperch seemingly have lower feed intake and show higher levels of blood glucose concentrations in RASs with lower turbidity [392].

Several measures of suspended particle load exist that rely on or address different aspects of suspended particle characteristics. In aquaculture studies, total suspended solids (TSS) concentrations, that is, a mass-based measure, and turbidity, an optical measure, are often employed. Turbidity measures the refractive light scattering properties of particles in the water and could be understood as the water's 'cloudiness'. Nephelometric turbidity measurements, stated as nephelometric turbidity units (NTU, EPA Method 180.1), or formazin nephelometric units (FNU, ISO 7027), seem to be the most common in aquaculture. These compare the light scattering, at an angle up to 90° relative to the incident beam, properties of an artificial standard reference suspension, most often formazin, with a test sample [393, 394]. Total suspended solids (TSS) concentration is given by the dry weight of matter in a measured volume of water that is retained on a glass-fibre filter or cellulose membrane. Filter pore sizes employed typically range from 0.4 to 1.2 μm [395].

Concerns about the stability and characteristics of formazin solutions and variations in the design of commercially available instruments raise questions about the repeatability and comparability of readings from nephelometric turbidimeters [394, 396]. Furthermore, relating the given turbidity units to formazin concentrations occludes the link between the given turbidity values and the light intensity that is measured [393, 396]. Calibration and reporting procedures that can allay these shortcomings have nevertheless been proposed [396]. Several authors have also questioned the utility of using turbidity measurements as indicators of suspended particle content, as correlations between these measurements can be poor and vary according to several factors, such as particle size distributions and geometries [386, 394, 397]. Water clarity, measured as black disk visibility, is a more direct measure of water visibility and has been suggested to be more repeatable and to correlate better with suspended particle loads [394]. Although TSS is a more robust measure of suspended particle load, it is a crude quantitative value that does not provide important information about physical, mechanical and chemical characteristics of suspended solids [378]. Most studies on the effects of particle contents or characteristics on fish appear to have been conducted using suspended solids from or reminiscent of those found in natural water bodies. In aquaculture systems suspended particles predominantly originate from feed and faeces. A limitation of applying studies on suspended solids from natural water bodies to intensive aquaculture systems is the different characteristics of particles in these systems, especially the relatively higher mineral particle content (e.g., from clay, silt, and gravel) relative to organic particle content in most natural water bodies [378].

Despite these methodological issues, assessing turbidity and TSS remains important for aquaculture research given the far-reaching effect of solids in waters. Due to its various direct and indirect effects on fish, turbidity and TSS are relevant input-based WIs primarily impacting upon welfare needs under the following domains: physical environment, health and behavioural interactions.

3.12.1 | Atlantic Salmon; European Seabass; Common Carp

To the best of our knowledge there is not sufficient data to suggest safe operating levels for suspended solids concentrations for these species.

3.12.2 | Rainbow Trout

Some recent studies have been unable to show adverse effects of TSS concentrations up to about 60 mg/L on rainbow trout ongrowers in RAS [156, 398, 399]. Other studies have suggested safe limits being below 15 and 25 mg TSS/L for fry and fingerlings [400].

3.12.3 | Gilthead Seabream

Suggested safe limits for juveniles and adults range from below 25 and 50 mg TSS/L [401].

4 | Key Outcome-Based WIs: Applicable at the Group Level

4.1 | Behaviour

Behaviour can be affected by the physical and social environments, the rearing system, husbandry procedures and pathogens [402]. Behaviour therefore provides stakeholders with key insights into the subjective experiences of fish and is a non-invasive measure in most situations. There are several behaviours exhibited by fish in aquaculture research settings that can be indicative of their internal state, positive or negative, in real time. These behaviours include but are not limited to swimming speed and acceleration, shoaling behaviour and orientation/polarisation, horizontal and vertical distribution, foraging (exploration for food), agonistic behaviours and aggression, thigmotaxis, freezing behaviours in addition to ventilation frequency [402–404]. While many of these indicators can be monitored at the individual level, in aquaculture research settings where high numbers of fish may be held during an experiment, they are often applied at the group level.

When applied, a careful and often context-dependent examination of the indicator is important. Firstly, in addition to the behaviours themselves, significant changes in these behaviours are indicative since they have been associated with stress and disease [402]. Secondly, seasonal variations in the circadian activity of some species and the individual variation of responsiveness can be confounding factors for welfare monitoring and assessment [405, 406]. Thirdly, the behaviours are typical iceberg indicators, which can vary with both species and life stage, and the significance of each behaviour can vary from one developmental stage to the next.

Behavioural indicators can be categorised as indicators of positive welfare, negative welfare and context-dependent indicators. Examples of behaviours indicative of good welfare are synchronised swimming and exploration. Synchronized swimming activity is often associated with good welfare [402] and it has been determined that—depending on the degree of domestication of the fish species and strain—feeding schedules and the stocking density can be used as synchronizing factors of activity [407, 408]. Exploration as well as food anticipatory behaviour can be an indicator of positive welfare states [402]. Such behaviours and particularly their quality or quantity and changes over time are ideal WIs for assessing if good welfare is present and documenting it.

Conversely, there are behaviours indicative of poor welfare both at a group or individual level. For example, if a limited number of individuals begin to exhibit sickness behaviours including apathy, low appetite, reduced locomotory activity and begin isolating themselves from conspecifics [409], it can indicate a welfare problem limited to these specific fish, such as poor smoltification status in Atlantic salmon [410]. But it can further be an early warning of a potential health problem that can affect the rest of the population, for example, parvicapsulosis in Atlantic salmon [411].

Behavioural deviations require identification by experienced observers with an understanding of their potential relevance. Care is further advised when interpreting behavioural changes at the group level, and how these changes manifest in terms of welfare outcomes. For example, if fish activity increases prior to feed delivery, this could indicate feeding motivation or food anticipatory activity, both of which are positive [402]. However, if this behaviour is prolonged or is observed during and after feeding, it could be indicative of competition and/or that fish are still hungry, which would be negative [412–414]. Similarly, behaviours that are indicative of apathy, and the fish isolating themselves from the group should always be interpreted as a warning sign in the first instance. However, isolation from the group and low appetite can also be an indicator of a change in the fish's maturation state [4], which isn't a particular problem for the fish per se but will become one if the fish is anadromous and can't access freshwater to breed. Therefore, specific behaviours must always be considered in wider contexts.

The application of behaviours as WIs should also not be limited to monitoring fish stocks under routine culture conditions, as it could be extended to the study of the group response to controlled challenges. An easily reproducible test, such as a fish compression stress test, with a decrease of available space in tanks with no detriment of water quality, could be used in any infrastructure or fish species as part of a gold standard procedure for welfare monitoring in normalised conditions.

When applying a behavioural toolbox in research settings, a good overview of what behaviours the fish can exhibit in their given rearing system is important. While this can be quite complex, we would like to introduce the reader to some ethograms that have already been proposed for each species. Saraiva et al. [34] have put together a comprehensive guide for examining behavioural OWIs in the top five European aquaculture species that we focus on in this review and have kindly given their permission for us to use and adapt their ethograms for assessing behaviour in addition to how they can be indicative of negative or positive welfare states (see Table 2). We have adapted the table to consider two additional behaviours, scototaxis and freezing. We have also split 'Individual swimming behaviour' into 'swimming speeds', 'vertical distribution' and 'horizontal distribution' and removed general appetite and vacuum behaviour from the ethogram as the former is considered later in this article and the latter can be classified as a stereotypical behaviour that is already covered in the ethogram [402]. Behaviours primarily impact upon welfare needs under the following domains: nutrition, physical environment, health and behavioural interactions.

4.2 | Hunger and Appetite

The utility of using appetite as a WI has been well documented in aquacultural settings by [4, 331, 434], and this utility is equally applicable to aquaculture research. The fish's appetite system can be considered a control framework that incorporates hunger, the 'sensation which reminds organisms that food should be sought or eaten' or more succinctly 'the drive to consume', which is directed by a biological need or signal [435]. Appetite therefore addresses 'food intake, selection, motivation and preference' [436] and is 'the system that influences energy intake (food consumption) and associated motivational states such as hunger' [437]. Further factors that must be taken into account include satiation, the 'process that leads to the termination of eating' [436] and 'the control of meal size' [437] and satiety, the 'process that leads to inhibition of further eating' [436] and the 'control of the post-eating period' [437].

Appetite levels are often proxied via an audit of feed intake levels, changes in feed intake levels over time or by monitoring feeding motivation [434, 438, 439]. When using these proxies, key situations to consider are (i) when appetite drops or is lost and (ii) the time it takes for it to return. As for the first, drops or loss of appetite, key aspects are whether it is acute or long-term and where it ranges from mild to severe [440], which depends upon the driver and its severity. A reduction or complete loss of appetite is an iceberg indicator for a number of factors linked to poor welfare, including but not limited to the health status of the fish [441, 442], poor water quality [127, 443], an infestation with ectoparasites [444] or stress [438].

Appetite is often documented by visually monitoring how the fish respond to pellets when offered to them and by direct observations of their behaviour, feeding motivation [331, 438], pellet wastage [412] and food anticipatory activity (FAA) as feeding time approaches [445, 446]. The time it takes for appetite to return following exposure to a potentially stressful or harmful procedure can be used as an OWI in experimental settings, given that stress can cause a loss of appetite in fish [438, 447, 448]. Documenting drops in appetite or the time it takes to return are simple and effective OWIs and are easier to interpret when a stakeholder employs thorough feeding management practices that are appetite-driven and allow for the examination of both fish behaviour and any potential pellet wastage.

However, there are some caveats that need consideration when using appetite as an OWI. Stress may potentially lead to an increase in feed intake as well as a decrease. It can increase the expression of orexigenic factors at the central level [449, 450] and this controversial response may indicate the activation of non-homeostatic feeding stimulators by stress. In mammals, this response is linked to the activation of the reward system, which leads to decreased activity of the hypothalamus-pituitary–adrenal (HPA) axis and an increase in food intake [451]. Therefore, stress-related changes in feed intake and their implications for welfare should be carefully studied, as appetite is modulated by physiological mechanisms that, depending on the circumstances, could lead to different final phenotypes, distorting the expected outcome of stress.

Fish may also ignore feeding opportunities or lack feeding motivation simply because they have recently eaten and are full [331]. Similarly, they may choose not to eat due to other factors that are not connected to their welfare state [447] such as maturation status [434, 452]. Further, when auditing pellet wastage as a proxy for appetite, there may be high numbers of uneaten pellets simply because the fish are satiated, or being deliberately or accidentally fed to excess, or being fed too fast for the fish to consume all the pellets offered to them [434]. Furthermore, the feeding ecology of certain fish species, such as gilthead seabream, may involve high levels of feed manipulation in the oral cavity and even the spitting out of pellets [453], and this may not be related to a lack of feed motivation or appetite. Additionally, low feed intake may be related to natural variables such as certain life-history

TABLE 2 | An example of a general ethogram for use in aquaculture research involving Atlantic salmon, rainbow trout, European seabass, gilthead seabream and common carp.

Behaviour	Definition	Level	Effect upon welfare	Source data
Spawning	*Movements, actions and/or displays that lead to reproduction. May include courtship, nest building, egg releasing, fertilisation, parental care or other species-specific behaviours.*	*Group or Individual*	*Positive*	Saraiva et al. [34] and relevant for all five species
Foraging	'...a complex behaviour that ranges from detecting and searching for food, capturing prey, and determining if it should be swallowed or rejected' [415].	*Group or* Individual	*Positive*	Martins et al. [402] and relevant for all five species
Group structure	*Shoaling* (*in a group but not directional or coordinated*); *schooling* (*in a polarised, directional and coordinated swimming*) *or disperse* (*no clear group formed*). Aspects of these behaviours are further outlined below, in addition to group cohesion.	*Group*	Coordinated swimming outside feeding is generally considered positive in aquaculture research settings	Martins et al. [402] and relevant for all five species
Vertical distribution	Vertically distributed *close to surface, midwater, bottom, etc.* or throughout the rearing system.	Group or Individual	Negative and Positive	Oppedal et al. [83]; Saraiva et al. [34] and relevant for all five species
Horizontal distribution	Horizontally distributed, e.g., in the centre or periphery of the rearing system or near system features that may have utility such as feeding delivery points or tank water inlets. See also 'thigmotaxis'.	Group or Individual	Negative and Positive	Martins et al. [402] and references therein. Relevant for all five species
Group cohesion	'... describes the distance between individuals within the group' [416].	Group	Negative and Positive	e.g., Ward et al. [416] and relevant for all five species
Surface activity	Refers to the '...number of rolls and jumps the fish make' [4] where jumps (leaps) can be described 'leaping, with the whole body breaking clear of the water' and rolling which can be described as 'only the dorsal part of the body breaking the surface' [417]. Rolling has also been defined as where the surface is '...broken by the fish...rolling through with a larger body proportion' [418].	*Group*	Low can be positive but also negative if fish need to refill the swim bladder (e.g., in *A. salmon*). High can be positive if it indicates, e.g., the filling of the swim bladder or feeding motivation, but can also be indicative of stress or high parasite load	Furevik et al. [417]; Bui et al. [419]; Noble et al. [4]

(Continues)

TABLE 2 | (Continued)

Behaviour	Definition	Level	Effect upon welfare	Source data
Exploration	'...individual willingness to investigate novel environments, food items or objects' [420]. *Movements or actions that apparently serve to collect information* on new environments and objects.	*Individual*	*Positive*	Martins et al. [402] and relevant for all five species
Anticipation	*Movements or actions that precede* an occurrence *and indicate that the fish are aware of routine procedures taking place imminently. The most common is food anticipatory behaviour, where the fish* increase activity *before feeding*. Can be indicative of feeding motivation.	*Individual*	*Positive*	Martins et al. [402] and relevant for all five species
Swimming speed	*slow, regular, fast, erratic bursts*	*Individual*	Negative and Positive	Martins et al. [402] and relevant for all five species
Ventilation frequency	*Rate at which the opercula open and close, as a measure of the respiratory needs of the fish*.	Individual	High can be both *negative* if fish are gasping (poor water quality or respiratory problems), or positive if indicative of increased respiratory needs (exercise)	Millidine et al. [421]; Martins et al. [402] and relevant for all five species
Aggression	'...behaviour that actually or potentially causes harm to another animal' Huntingford and Damsgård, in [414]. *Agonistic interaction between two or more individuals. Can occur without physical engagement* (*i.e., Low Intensity Aggression: fin erection, colour changing, displays* etc.) *or including physical interaction* (*High Intensity Aggression: chasing, biting, fighting*)	*Individual*	*Negative* for the recipient	Axling et al. [422]; Carbonara et al. [423]; Duan et al. [424]; Flood [425]; Neofytou et al. [426]; Newcombe and Hartman [427]; Oikonomidou et al. [428]; Solstorm et al. [352]; Wagner et al. [298]; Øverli et al. [429] and relevant for all five species
Stereotypical behaviours	'Stereotypies are repetitive, invariant behaviour patterns with no obvious goal or function' [430]	*Individual*	*Negative*	Martins et al. [402] and relevant for all five species
Freezing	Fish cease swimming and become immobile, for example [431].	Individual	Negative	Maximino et al. [431] and relevant for all five species

(Continues)

TABLE 2 | (Continued)

Behaviour	Definition	Level	Effect upon welfare	Source data
Thigmotaxis	*Strong avoidance of open areas and preference for moving in very close proximity of the walls of the rearing environment.*	*Individual*	Mostly *Negative*, unless, e.g., a water inlet or other potentially beneficial flow dynamic parameters are close to a wall	Saraiva et al. [34] and references therein. Relevant for all five species
Scototaxis	Preference for dark instead of light substrates, for example [431].	Individual	Negative	Maximino et al. [431] and relevant for all five species
Apathy	'the animal ceases to respond to stimuli that would normally elicit a response' [432].	Individual	Negative	Browning [433] and relevant for all five species

Note: Reproduced and adapted with kind permission from Saraiva JL, Volstorf J, Cabrera-Álvarez MJ, Arechavala-Lopez P. Using ethology to improve farmed fish welfare and production. Report produced for the AAC. (2022) 67 pp + annexes https://aac-europe.org/en/publication/using-ethology-to-improve-farmed-fish-welfare-and-production-2/. Italics indicate original text from Saraiva et al. [34] and non-italic text is our adaptation of their ethogram. The top-down flow within the tables considers positive to negative welfare effects.

events or naturally low water temperatures that decrease metabolism, energy expenditure and the need to feed [414, 434] and references therein. Lastly, the negative consequences of a loss of appetite must always be considered in a wider context by the stakeholder [454]. For example, a drop in appetite can reflect the short-term costs of being exposed to a procedure such as vaccination that many stakeholders consider extremely beneficial for health and welfare in both the short- and long-term [455, 456]. In addition to all these alternative causes of changes in appetite, the time frame and assessment level (individual vs. group) must be considered. When attempting to quantify deviations from expected feed intake as a proxy for reduced appetite, one should be careful in selecting 'normal' or 'expected' appetite levels [331], as these can vary at both short and longer time intervals, also when fish are potentially satiated [434, 457]. And individuals in group-held settings may wish to feed but do not have the opportunity to do so because they are excluded from accessing a feed resource by more competitive conspecifics [458], which can be especially problematic if feed is delivered in a limiting fashion [459]. Because of, rather than despite, this complexity, appetite is a key outcome-based WI that, when put into the context at hand, provides far-reaching insights into animal health and welfare. Appetite primarily impacts upon welfare needs under the following domains: nutrition, physical environment, health and behavioural interactions.

4.3 | Scales or Blood in the Water

Fish scales are embedded in the dermis/epidermis and have numerous functions that are hypothesised to be linked to bodily defence, flow management during locomotion and the reduction and hindrance of fouling, see [460] and references therein. However, scales can be dislodged during handling [321, 461] or aggression [462] and can be seen in fish-holding water, at the water surface [403] or any potential collection points in the rearing system or operation, such as whirling collectors in tank-based systems [462] or on lice collection belts in mechanical delousing for salmonids (Noble and Stien, pers. obs.). As lost scales are an indicator of mechanical damage or trauma to the skin of the fish, they have been suggested as a potential WI in salmonid aquaculture [4] and are considered suitable as an outcome-based OWI. Unless fish are held or handled individually in the rearing system, scientific procedure or operation, the presence of scales in water bodies or collected from operational machinery must be considered a group-level OWI. The presence of scales in the water can be recorded by a simple audit of their presence/absence but where feasible they can also be collected and weighed [462].

Gill bleeding can be a sign of health challenges in farmed fish [463] and can be caused by physical trauma during fish handling and operations [464, 465] or chemical trauma [464]. This damage can manifest itself as blood in the water, also termed 'red water', and has been suggested as an additional outcome-based OWI in salmonid aquaculture by [4]. Once again, unless the fish are individually reared or handled, red water must be considered as a group-level outcome-based OWI unless the source of the bleeding can be identified. As far as the authors are aware, the presence of blood in the water is recorded by a simple presence/absence score. Due to their direct correlation with physiological damage, scales and blood loss are key outcome-based WIs, particularly during and after handling. Scales or blood in the water primarily impact upon welfare needs under the following domains: physical environment, health and behavioural interactions.

4.4 | Health

Annex III of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 states in Section A: general information that '(a) Establishments shall have a strategy in place to ensure that a health status of the animals is maintained that safeguards animal welfare and meets scientific requirements. This strategy shall include regular health monitoring, a microbiological surveillance programme and plans for dealing with health breakdowns and shall define health parameters and procedures for the introduction of new animals. (b) Animals shall be checked at least daily by a competent person. These checks shall ensure that all sick or injured animals are identified and appropriate action is taken' [15].

Health is a central welfare need in both the 'five freedoms' [466] and 'five domains' [6, 7] frameworks of animal welfare. In the five freedoms health is addressed in the 'Freedom from pain, injury or disease – by prevention or rapid diagnosis and treatment' [466] and in the five domains model it is addressed under the health domain focusing 'attention on the welfare impacts of injury, disease and different levels of physical fitness' [6]. Health has long been defined by the World Health Organisation as 'a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity' [467] and this is the definition we will apply here.

Robust veterinary and health monitoring plans are essential to the delivery of Directive 2010/63/EU as the diagnosis of a pathogenic or non-infectious disease amongst fish used in an experiment can impact upon fish welfare as well as the results. If the fish are health compromised and this challenge goes undetected, it can markedly affect the results and outcomes of the experiment, see [51]. However, the clinical and sub-clinical effects of various diseases upon fish welfare are often not clearly defined or understood, and the severity of their effects may vary in relation to the underlying existing health status of the recipient fish [447, 468]. Due to this, Pettersen et al. [469] have suggested that 'diagnosis per se, although useful for predicting future development and the need for treatment (prognosis and cure), is not of prime relevance to welfare assessment'.

Numerous earlier articles have offered guidance on health monitoring in fish research facilities [51, 470], but we would like to guide the reader's attention to a series of recent articles on health monitoring and biosecurity in experimental fish-holding facilities. Mocho et al. [471] have produced FELASA-AALAS recommendations for the health monitoring of experimental fish with a key focus on zebrafish and suggest that fish health monitoring plans should have numerous goals and objectives to (i) get an overview of the latest pathogenic and non-infectious disease status of the fish in the experiment, (ii) safeguard scientific robustness and integrity, (iii) promote the secure movement and sharing of fish and (iv) protect staff. They describe how to establish a routine screening programme based on environmental samples and the fish themselves using indicator-based and diagnostic tools, how to manage infection pressure, and how to report the results of this work. They also highlight that '…results should be interpreted with the understanding that detection of a microorganism alone does not define it as a pathogen and alternatively, the lack of apparent clinical signs does not rule out biochemical and host-microbiotic alterations that could affect research projects' [471]. In an accompanying article, Mocho et al. [472] further outline FELASA-AALAS recommendations for biosecurity measures in fish research facilities with sections on (i) managing and preventing risks from zoonotic pathogens, (ii) setting up biosecurity barriers and maintaining a biosecurity program and (iii) guidelines for introducing new fish to a facility including the setting up of quarantine barriers, screening and treatment of these fish. In a further article, Mocho and von Krogh [473] conducted a survey on health monitoring and biosecurity in a range of global fish laboratories revealing a lack of fish health monitoring systems in some facilities, a high variability in when and how often fish health was monitored and a heterogeneity of which pathogens to screen for and the methods used for screening. Environmental samples were often used for screening, as were PCR and histopathological tools for the fish themselves. They also highlight the need for standardized guidelines for ensuring smooth delivery for driving changes at the laboratory level [473] which will also ensure a smooth delivery of the health monitoring objectives of Directive 2010/63/EU.

Segner et al. [468] have written a thorough review of how health status can be used as an aquacultural WI, and their article also has high value for aquaculture research settings. They highlight how poor health status can impact upon a fish's resilience in relation to adapting to stressful situations, homeostasis and its ability to thwart disease, leading to poor welfare. They also underline how poor welfare, where fish may be experiencing stress-related suppression of immune competence, can in turn lead to poor health [468].

Many outcome-based WIs can also be indicative of health problems amongst the fish. At the group level, an increase in mortalities can be driven by health challenges [474] and monitoring absolute mortality levels and changes in these levels on a daily basis should be central to a health monitoring framework (see next section). Further, the potential cause of the mortalities should be investigated by using a visual inspection of the external and internal status of the dead fish or by health diagnostic tools [4, 469]. Health problems can also manifest themselves behaviourally, in the form of 'sickness behaviour', where the fish become immobile [402] and apathetic or isolate themselves individually or in small groups from the rest of the rearing group [411]. Further, reduced appetite can be symptomatic of underlying health problems [441]. At the individual level, outcome-based WIs can include a variety of injuries or conditions affecting the eyes, skin, fins, gills (respiratory health), heart (cardiac health) and other abdominal organs, which may result from pathogenic or non-infectious challenges. Additionally, deformities, signs of emaciation and skin colour changes can also indicate underlying health issues, see [27, 51, 469] and references therein. Further, pathogen load or burden can be used [4] as can faecal consistency [51, 475], see also the later section in this article. However, it must once again be noted that the majority of the above can be iceberg indicators, and the monitoring framework must consider this when investigating potential health-related drivers for deviations in these parameters. This challenge can be circumvented with the ongoing development and application of diagnostic tools and complex LABWIs relevant for auditing fish health status, such as relevant omics-based approaches, see [476–478]. Health status primarily impacts upon welfare needs under the following domains: nutrition, physical environment, health and behavioural interactions.

4.5 | Mortality and Survival

The Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 states 'The methods selected should avoid, as far as possible, death as an end-point due to the severe suffering experienced during the period before death. Where possible, it should be substituted by more humane end-points using clinical signs that determine the impending death, thereby allowing the animal to be killed without any further suffering'. In addition, 'Where death as the end-point is unavoidable, the procedure shall be designed so as to: (a) result in the deaths of as few animals as possible; and (b) reduce the duration and intensity of suffering to the animal to the minimum possible and, as far as possible, ensure a painless death' [15].

The utility of using mortality as a WI has been previously reviewed by [474]. However, it is sometimes shunned as a WI since it is too late for the individuals in question. Being able to survive can, except for in some very special cases, be said to be the minimum welfare requirement. If an animal in an experiment is not able to survive, one can reasonably assume that its welfare has not been optimal, either over the short- or long–term before this ultimate outcome. In addition, mortality by itself does not provide information on the reasons driving it, how long the animals have suffered or how much.

However, in a fish population, there will typically be a distribution from strong healthy individuals to compromised individuals with less tolerance for stressors and a poor environment. Mortality does therefore have utility as a WI in numerous experiments comparing the effects of, for example, different production systems [479], vaccinated and non-vaccinated populations [480] and outcomes after some kind of stressful and/or risky treatment [481]. Although it is relatively straightforward to use mortality as a WI comparing the outcome for two groups, the fact that experiments often are conducted with relatively few fish can create artefacts. For example, if one has a population of 20 fish where 3 fish die due to some undiagnosed defect or disease, and these fish have been divided into two equal treatment groups before the mortality occurs, the random chance of all three fish being put into the same treatment group is as high as 25%, giving this group a mortality ratio of 30% compared to 0% in the other.

Mortality is often reported as the total accumulated mortality for the experiment [482], but in many cases, it is of interest to report if the mortality has been high throughout the period or is driven by short-term incidents and isolated events [481]. It is important to monitor mortality throughout an experiment and investigate the underlying reason in case of an unexpected increase [481]. This adds value to the experiment and ensures that the experiment is not compromised by some unrelated pathogen or other unknown factor. Similarly, if mortality amongst the source groups was high before the experiment started, it can be an indicator of a problem, either with the fish population or with the conditions the fish were subjected to. Setting general thresholds for what is considered high, normal or low mortality for a fish population is difficult. In a sufficiently large population, there will almost always be weak individuals and some mortality is to be expected, depending on the species and life stage. To set thresholds, it is therefore necessary to have large datasets of previous generations for the same species and life stage, for example, based on industry data [483]. An alternative to reporting mortality is to report the data as a percentage of survival. This is often done in studies monitoring the survival of hatched eggs to later life stages [484]. Mortality primarily impacts upon welfare needs under the following domains: nutrition, physical environment, health and behavioural interactions.

5 | Key Outcome-Based WIs: Individual Level

Most individual-focused outcome-based WIs can be measured as continuous data using various measures and units, one of many examples being the 'Fin Index', where the lengths of individual fins are manually measured against standard fish lengths in cm [485]. However, such manual measurements are often time-consuming and problematic from a welfare point of view when evaluating large numbers of individuals and/or many indicators, at least on fish that are to be kept alive. Therefore, the use of scoring levels to describe how much an indicator deviates from what is expected of an injury-free fish makes manual evaluations faster. As an example, various scoring schemes have been developed to audit the external injury status of fish in aquaculture and aquaculture research settings [4, 486, 487]. However, if different schemes are used to audit similar types of injuries within and between species, they can lead to unnecessary obstacles and bottlenecks with regard to comparing, examining, evaluating and benchmarking their results within and between species and experimental settings. Additionally, such scores are subjective to some degree, and the resolution of the data is reduced compared to continuous data. Nevertheless, scoring systems are suitable for manual auditing in operational settings and therefore widely used for welfare evaluations [3, 4].

In aquaculture research, during initial (primary level) welfare evaluations where no particular deviation is expected, broad indicator categories make it possible to rapidly examine many indicators on each individual. If the primary evaluation reveals elevated frequencies of one or more of these broad categories, the next step may be a secondary-level evaluation focusing on these problems in greater detail. For example, a broad category like 'fin injury' can be divided into 'splits', 'erosion' and 'haemorrhaging' [4], or each fin can be scored separately rather than one score being assigned for the whole individual. If an even more detailed analysis is required the evaluation can be done at a tertiary level, going beyond category-based scores and using more sophisticated methods, including LABWIs [485]. For scientific studies where effects on some indicators are more expected or relevant than others, it may be natural to move directly to the secondary or tertiary level. This stepwise primary, secondary and tertiary scoring framework for welfare evaluation is described in [4, 8], and is applicable in both industry and research.

5.1 | An Example of Using an Adapted 0–3 Scoring Scheme in Aquaculture Research

In 2018, various authors of this current review proposed a primary level, unified 0–3 categorical scoring scheme for auditing external injuries in Atlantic salmon in aquaculture settings as part of the FISHWELL project [4]. This scheme utilized a combination of pictorial examples of various injuries accompanied by explanatory text to aid scoring and attempt to reduce any potential intra- and inter-observer variability when subjectively scoring fish. The FISHWELL scoring scheme was an amalgamation of previously published [3] and unpublished (C. Noble unpubl., J. Kolarevic unpubl., and J.F. Turnbull unpubl.) injury scoring schemes that were in use in the Atlantic salmon farming and research community at that time. The FISHWELL scoring scheme categorised 13 different WIs into levels ranging from level 0 'little or no evidence' of an injury or deviation to level 3 'clear evidence' of an injury or deviation problem [4]. In 2022, following testing and auditing of the scoring scheme, a new version was developed in a follow-up project called LAKSVEL. The LAKSVEL scoring scheme had a more extensive pictorial guide to each injury and updated the injury levels; it also had more informative and extensive text describing each injury and injury level [488]. LAKSVEL is primarily aimed at only one species and life stage: Atlantic salmon post-smolts during production in open marine net pens. LAKSVEL can still be considered a primary-level scoring scheme, as it is manual and its aims are to be operational and user-friendly for auditing the injury status of fish in a variety of procedures and settings.

While the description of each indicator level is more extensive in the LAKSVEL than in the FISHWELL scoring scheme, and the pictorial guide hopefully improves its ease of use, there are some limitations that one must consider if LAKSVEL, or LAKSVEL-like scoring schemes, are to be used in research settings. For ease of use out on commercial farms, LAKSVEL simplifies the scoring of each injury and in some cases bundles a series of mixed injury traits to a specific body part or appendage within one scoring category, for example, by bundling various types of fin damage and the fins affected into a single 0–3 scoring scale [488]. While this is understandable for its application in commercial settings, only 4 levels and a combined score for all fins may be considered too coarse for many scientific studies. One possibility is to refine the scoring by, for example, adding plus or minus to the scoring to indicate a strong or a weak level and thereby get increased precision. This does, however, in practice introduce new levels, and different researchers may have different opinions on what constitutes a minus or a plus for a given level. Further, a scoring scheme for injuries in an aquacultural research setting should still be comparable to what is increasingly being used in aquacultural settings. This would allow for comparing and contrasting welfare outcomes in experimental studies with industrial settings and also allow for levels of benchmarking if required. Another possibility to increase detail is to split the broad WIs in LAKSVEL into more specific WIs, for example, one for each fin. In this way, more detailed data can be provided, while still preserving the levels from 0 to 3. We therefore propose that splitting the WI into more detail should be the preferred method if more resolution is needed and that this can be done along the lines specified in Table 3 and Figure 5 below.

5.2 | Gill Status

The gills have evolved into a multifunctional organ that performs vital functions in the exchange of gases, osmoregulation, ionoregulation, hormone regulation, acid–base balance, ammonia excretion, immunity and the modification of circulating metabolites [223, 492]. They are rich in vascularisation, and their anatomical specializations arguably make them have the most complex circulation found in any vertebrate organ [493]. Numerous lamellae arise from the filament body and provide a massive respiratory surface area and contact points. With an estimated surface area of 0.1–0.4 m^2/kg body weight, the gill surface is considered the largest organ-specific surface with a close interaction with the aquatic environment [494]. Furthermore, there is only a short distance between blood and water, only two to three layers of cells, that is, a mean of 6 μm in rainbow trout lamellae [494]. As a mucosal organ, gills are covered with a mucus layer secreted mainly by mucus cells found in both the filament and the lamellae [495]. Because of their dynamic nature, the changes in size and number of mucus cells are often used to provide indications of gill mucosal responses to different production-related changes, including but not limited to, water quality, stress and infection status, diets [496] or injury as the mucus cells in the gills can be emptied when fish are subjected to fin clipping in some species, such as Nile tilapia [497]. Additionally, the biochemical characteristics of mucus make it an essential component for defence in intra- and inter-specific behavioural interactions, see [498]. Even though the gills are a complex biophysical barrier, their intimate contact with the rearing water makes them highly susceptible to environmental changes and a port of entry for noxious materials and pathogens [494, 499, 500]. Therefore, compromised gills can influence the health of fish at the organismal level.

The emergence of numerous and complex gill diseases and disorders in recent years in global marine and freshwater finfish aquaculture emphasises the need to proactively address the issue [501, 502]. In Norway alone, gill health problems in Atlantic salmon are on the rise and are currently considered the topmost concern with regard to poor welfare on farms [503]. Gill diseases and disorders can be caused by infectious and non-infectious agents and based on one principal causal agent or insult, there are presently seven distinct types of gill disease including but not limited to amoebic gill disease (AGD), parasitic gill disease, viral gill disease, bacterial gill disease, zooplankton (cnidarian nematocyst)-associated gill disease, harmful algal gill disease and chemical/toxin-associated gill disease (listed in [504]). Further, sub-optimal water quality and residuals from water treatment, especially in RAS, can affect gill structures and impair basic physiological functions [505, 506]. It is important to state that these agents and challenges are often presented simultaneously in complex aquaculture environments. Hence, gill problems in recent years are predominantly complex or multifactorial in nature and are often termed proliferative (PGD) or complex (CGD) gill disease.

Evaluation of gill health status on farms and under experimental conditions can be both macroscopic and microscopic. Gross pathology scoring through visual inspection often includes scoring with a scale from 0 to 3 or 5, with 0 indicating good health status and increasing scores denoting increasing cases of alterations or lesions; interestingly, the majority of these approaches were developed for salmon farming. For instance, the Taylor system for scoring the white protruding gill lesions is the most widely used system in assessing amoebic gill disease severity in Atlantic salmon [507]. These scores are used in deciding when to treat the infection, and often it is advisable that treatment is done between gill scores 1 and 2 [508, 509]. A new total gill scoring system has been developed for auditing gross pathological changes in the gill. It includes presumptive or healed AGD in addition to other kinds of gill lesions and can be a good way to document severe proliferative pathologies [510]. In another newly developed scheme for Atlantic salmon which only addresses AGD, five key lesions are scored including (i) white areas (presumed hyperplasia), (ii) haemorrhages, (iii) loss of gill tissue (i.e., shortened filaments, swollen, thickened gills), (iv) yellow discolouration, and (v) fusion of filaments [511]. In theory, gross gill scoring is a simple method; however, it requires substantial experience in order to properly differentiate the pathologies and gauge their severity. It is important that only a small group of trained personnel do the scoring throughout the trial or production to ensure uniformity and reproducibility. During gross gill scoring with no intention of terminal sampling, fish are often subjected to handling-related stress which can be a welfare issue if the process is done several times during production or experimental trial. Therefore, new technologies that will allow high-throughput evaluation of gross gill scores, with high predictability and reproducibility, must be developed.

Minimally invasive techniques have been employed to assess gill health status and some of these focus on the biochemical properties of gill mucus. Currently, there are no standardized methods for mucus collection and the approach is often dependent on the different downstream analyses, but the use of highly absorbent swabs is a common strategy. For example, changes in the proteomic profile of gill mucus have been used to assess the impacts of repeated treatments for a gill disease [512]. Moreover, changes in the gill mucus glycome have been used to assess responses of salmon gills to AGD [513]. Although *N. perurans* can be quantified using gill swabs, no specific markers have yet been identified in gill mucus that reliably indicate the severity of infection or effectively complement traditional gill scoring.

Besides proteomics, molecular biology techniques can be used to investigate gill health. For example, a multigene expression assay, ImCom, has been developed to assess the immune competence of Atlantic salmon, based on observations of immune suppression in smolts that persists for several months after seawater transfer [514, 515]. ImCom includes immune and stress genes selected by expression profiles in numerous experiments and field samples of farmed salmon, as well as markers of smoltification and reference genes [516, 517]. The assay can be used to monitor gill health and a minimally invasive gill biopsy, requiring only a few milligrammes of tissue, is sufficient for gene expression analysis. The assay has been tested on a large number of clinically healthy high-quality fish to establish the normal range of gene expression in the gill, resulting in a comprehensive reference data set. Screening smolt batches with varying performance levels has identified two main characteristics. First, intermediate mortality is associated with immune suppression, characterised by the downregulation of many genes. Second, high mortality is linked to the upregulation of gene markers of acute inflammation and stress, including *il-1, cathelicidin, serum amyloid A5*, and *matrix metalloproteinases 9 and 13*. ImCom has been utilized in longitudinal studies of farmed salmon to evaluate production methods and facilities [518, 519] and its utility for other species can also be examined. Gill status primarily impacts upon welfare needs under the following domains: physical environment, health and behavioural interactions.

5.3 | Opercular Deformities

The operculum plate is a structure comprised of four bones, found attached to both sides of the head in fish, covering the gills and sealing the opercular and buccal cavities. Opercular deformities have been described in all the species discussed in this paper and have been noted as a particularly frequent problem in seabream aquaculture, where they are among the most commonly observed skeletal deformities [4, 360, 520–525]. Among surveyed fish farmers in Norway, opercular deformities are cited as the 3rd most important cause for reduced welfare in the freshwater phase of both Atlantic salmon and rainbow trout farming [503]. They have also been described in wild fish stocks as well [526–528]. Opercular deformities can occur as a partial or complete shortening of the operculum or as curling or folding of the operculum either inwards, toward the gill arches, outwards, or a combination of these. Localised shortening of the operculum, affecting only the ventral or dorsal edges, can also occur and the deformities can either be unilateral or bilateral. Studies on seabream, Atlantic salmon, and common carp indicate that unilateral deformations are more common [360, 522, 525]. Further, some studies on seabream indicate that opercular deformities occur with similar frequencies on either side of the head [359, 529]. However, other research on seabream indicates a higher frequency of left-side opercular deformities [530], as have been observed in studies on tank-reared Atlantic salmon and the larvae of seabass [520, 522, 528], likely indicating different aetiologies.

A complete assessment and description of the aetiologies behind opercular deformities is not yet available, but possible causes addressed in the literature include genetic factors [531–534], nutritional deficiencies [535–540], adverse husbandry practices and environmental conditions during early development, including the teratogenic effects of pollutants [526]. Further, in Atlantic salmon, individual fish nipping on the posterior edge of other individuals' opercula, either aggressively or accidentally during feeding, has been suggested as a common cause of opercular shortening [522]. Moreover, Atlantic salmon parr reared in RAS were shown to have a lower incidence and severity of shortened opercula than parr reared in flow-through systems, although the driver behind this is unknown [479]. Elevated CO_2-levels, however, were not shown to affect the opercula in post-smolt Atlantic salmon [214]. In seabream, opercular deformities are first seen 17–23 days after hatching and mechanical loading from high water flow velocities or feeding is thought to adversely influence opercular bone development or cause adaptive remodelling in

TABLE 3 | Outlining an example of a potential injury scoring scheme for aquaculture research that can be integrated within existing operational scoring frameworks.

OWI		Further breakdown
Skin	Scale loss left side	Scored 0–3 following [488], but can then be broken down into: different sides of the fish and dorsal or ventral of the lateral line or posterior/anterior of the dorsal fin, according to established subsections as the Scottish Quality and the Norwegian quality cut (NQC), see Figure 5 and, e.g., [489].
	Scale loss right side	
	Haemorrhaging left side	
	Haemorrhaging right side	
	Wounds left side	
	Wounds right side	
Spine	Vertebral deformity	Scored 0–3 after [488] in the first instance
Snout	Snout damage	Scored 0–3 after [488]
	Colour change	Scored 0/1 presence/absence after [482]
Nostrils	Haemorrhaging	Scored 0/1 presence/absence after [482]
Upper Jaw	Deformity	Scored 0–3 after [488]
	Injury	Scored 0–3 after [488]
Lower jaw	Deformity	Scored 0–3 after [488]
	Injury	Scored 0–3 after [488]
Eye damage	Exophthalmos	Scored 0–3 after [4]
	Cataract	Scored 0–4 after [490]
	Keratitis (corneal inflammation)	Scored 0–3 after [488]
	Haemorrhaging	Scored 0–3 as [4]
Operculum	Erosion/deformity	Scored 0–3 after [488] but separately scored on each operculum to allow for auditing of unilateral or bilateral damage
	Haemorrhaging	Scored 0/1 presence/absence for each operculum
Gill	Gill injury	Scored 0–3 after [488] but separately scored on each gill to allow for auditing of unilateral or bilateral damage
	Gill paleness	
Fin damage	Healed injury	Healed injury, Active splitting and Active erosion should be scored 0–3, and haemorrhaging scored 0/1 presence/absence after {Citation}Each of the medial fins should be scored separately. Paired fins should also be scored separately to allow for auditing of unilateral or bilateral damage.
	Haemorrhaging	
	Active splitting	
	Active erosion	

Note: This scheme is based upon the scoring of morphological OWIs in research settings for Atlantic salmon, can be integrated with the existing LAKSVEL scoring scheme for scoring injuries in commercial settings [488] and which is central to the Norwegian Standard 9417 'Salmon and Rainbow Trout – Terminology and Methods for Documentation of Production' [491]. See [488] for descriptions of each OWI. The concept is also relevant for the other species addressed in this review.

response to these mechanical loads, leading to malformations during early development in this species [359, 360, 529, 541].

A result of these opercular deformities is that the opercula do not properly enclose the opercular cavity and gills [522]. In the case of inwardly curled opercula, the operculum may also directly interfere with the gills [360, 529]. In either case, the known and hypothesised functions of the operculum are impaired: the opercular/buccal pump is unable to maintain adequate pressure gradients, thereby inhibiting gill ventilation, forcing the fish to increase its swimming activity to adequately exchange water in the buccal cavity, leading to an increased energy expenditure associated with ventilation [360, 542–544]. Further, insufficient protective covering of the gills may increase the risk of pathogenic infection or infestation [360, 545]. Additionally, the occurrence of opercular deformities has been shown to impair growth in seabass and common carp [520, 525] and to significantly increase the risk of mortality following handling in Atlantic salmon [546]. Opercular deformities primarily impact upon welfare needs under the following domains: nutrition, physical environment and health.

5.4 | Vertebral Deformities

The fish vertebral column plays a crucial role as a body-supporting structure and in facilitating and controlling movement, being an important attachment site for muscles. The column is comprised

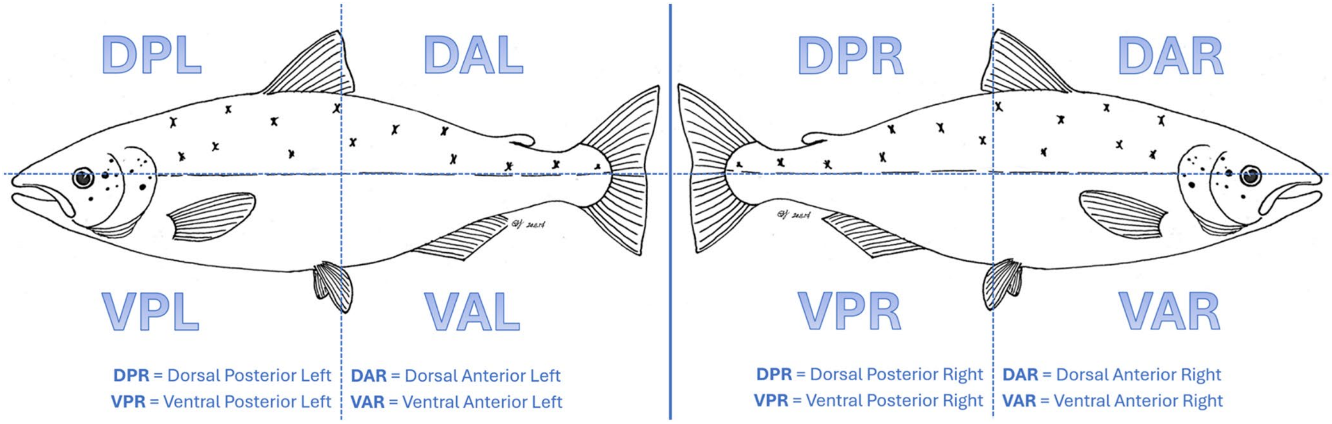

FIGURE 5 | Outlining an example of how skin damage (scale loss, haemorrhaging, wounds) can be subdivided according to the left or right side of the fish and into dorsal or ventral side of the lateral line or posterior/anterior of the dorsal fin, or according to established subsections as the Scottish Quality and the Norwegian quality cut (NQC) see [489], Image copyright: Gunhild S. Johansson, Nofima.

of a series of vertebrae, structures composed of bone or cartilage, with the notochord running through the whole vertebrae making up the intervertebral regions. The central part of the vertebra is normally amphicoelous, meaning both the anterior and posterior surfaces are concave or inwardly rounded. Supported by the intervertebral regions, this structure allows for flexibility and adeptness in handling bending loads during swimming. The shape and dimensions of the vertebrae can vary along the vertebral column [547], and the morphology and regional variation of the vertebral column are intricately linked to the mechanical demands of different environments and swimming modes in fish [548]. On the ventral and dorsal sides of the vertebrae there are protrusions known as the haemal and neural arches that, respectively, enclose blood vessels and the spinal cord. Different sections of the vertebral column are joined with the head and the ribs or give support to the dorsal and caudal fins.

Vertebral column anomalies are common in fish in aquaculture, occurring in all the species discussed in this review [549, 550], and can have marked consequences for welfare, restricting the ability of the fish to control its movements and thereby its ability to, for example, swim or feed. Anomalies affecting the haemal or neural arches of the vertebrae may also affect blood flow or neurological functioning [550]. Thus, vertebral column anomalies may cause pain and stress to the affected fish [447, 551] and are therefore a relevant outcome-based WI. Vertebral column anomalies can occur as the deformation of one or two vertebrae or intervertebral regions, with little apparent impact on the vertebral column, or as clear morphological changes to or deformations of the vertebral column, possibly resulting from severe deformations of multiple vertebrae [550]. Witten et al. [552] sought to categorise vertebral anomalies in Atlantic salmon, using x-rays, and found 20 different types, including vertebrae compressions, fusions or ankylosis (the merging of two or more vertebrae), elongations, changes to intervertebral spacing and poorly aligned vertebrae (vertical shifts), as well as combinations of the above. Also included in this typology is the bending of the vertebral column, either ventrally (lordosis), dorsally (kyphosis) or laterally (scoliosis), which in severe cases can coincide with vertebral deformations [552]. Since this typology was proposed, other novel deformity types have been outlined, such as, for example, 'cross-stitch' vertebrae, a form of vertebral lesions accompanied by a reduction in intervertebral spacing in Atlantic salmon [553].

As indicated by the complexity and variety of vertebral column or vertebral anomalies, the aetiologies behind them are also complex, diverse and often not well understood. Additionally, the induction of a given anomaly is not always reproduced, even in laboratory studies [554]. However, the occurrence of lordosis, often observed in European seabass juveniles but also in gilthead seabream, has been attributed not only to poor water current conditions and mechanical strain from excessive swimming during early life stages but also to feeding behaviour and genetics [361, 550]. Further, the bending of the vertebral column, in general, has been attributed to neuromuscular and nutritional factors [552, 554]. Vertebral anomalies have been linked to various environmental factors, such as early-life rearing temperatures [550], excessive growth [550, 555], nutrition and especially vitamin and mineral deficiencies [550, 556], handling and vaccination [553, 557], as well as teratogens, such as heavy metals [558], or some combination of these [4, 559].

Vertebral deformity is included as a single OWI in both the FISHWELL and LAKSVEL scoring schemes for Atlantic salmon, but these do not discern between, for example, lordosis, kyphosis, scoliosis, the sometimes-observed S-shaped body shape, or truncation of the tail referred to as 'short tail' [4, 488]. Further discernment would be possible within an appropriate framework. However, a complicating factor is that several, if not most, types of vertebral column anomalies cannot, at least easily, be observed without resecting the vertebral column, possibly doing a histopathological analysis or using tools such as radiographic imaging or tomography [552]. There is therefore limited opportunity to execute refined, non-invasive clinical monitoring of vertebral column anomalies during fish cultivation [550, 554]. Vertebral deformities primarily impact welfare needs under the following domains: physical environment and health.

5.5 | Skin Damage

Covering the whole fish, the skin is the initial barrier defence system, protecting the organism from its external surroundings

[560]. Skin and its mucus layer have important roles when it comes to, sensory mechanisms, movement, chemical signalling and homeostasis [561]. Further, the function and composition of the different skin constituents, for example, tissue layer thickness, mucous positioning and its amount, can change with external and internal factors [515, 562, 563]. Therefore, functional studies and descriptions of fish skin microarchitecture are needed to understand the processes involved in skin functions, such as repair and regenerative mechanisms. Several layers of different tissues build the skin and provide it with different functional features, with the primary layers being the epidermis, dermis in addition to the hypodermis. The epidermis consists of live epithelial cells, keratocytes and mucous cells, that together make the important protective outer barrier. The dermis consists mainly of connective tissue that provides the skin with flexibility. The hypodermis is the deepest layer, consisting of adipocytes that provide insulation, but also other cell types contributing to skin pigmentation and vascularization [560]. Some species, like salmon, trout and carp, have calcified scales that act as armour against mechanical damage, while others, like lumpfish, have bone plates instead. The scales of Atlantic salmon are anchored by collagen fibres from the dermis to the scale pockets [564].

While the skin has a high capacity for healing and regeneration [322, 565], severe skin damage may be lethal to the fish. Upon wounding, keratocytes in the epidermis rapidly migrate to cover the wounded area and seal the damaged surface, thereby preventing pathogens from entering and infections occurring [566]. The skin's ability to repair after sustaining damage is determined by external factors, such as temperature and stress, as well as internal factors, such as nutrition and the age of the fish [562, 563, 565, 567]. Skin wounds can be divided into two different types: those caused by mechanical trauma and those caused by ulcer-induced diseases [561]. Mechanically induced wounds can be observed after, that is, treatment and handling of the fish, while those caused by pathogens or underlying pathologies can result from, that is, changed nutrition or altered environmental factors [562, 568, 569]. The severity of the wounds is further divided into superficial and deep wounds [560]. Superficial wounds cause damage to the epidermis but leave the dermis intact. Deep wounds extend through all the layers of the skin, down to the subcutaneous adipose tissue, making them more complex than superficial damages. The severity of the wound also determines the skin's ability to heal, which also depends on other factors like nutrition, environmental parameters like temperature, and the species; see previous paragraph.

While wounds and scale loss may easily be detected during visual inspection, micro-damage, such as lost epidermis or altered mucus production may not [515, 570, 571]. Still, micro-damage may impair skin barrier functions and reduce the overall robustness of the fish and make it more susceptible to further damage, wounds and secondary infections. Stress has for instance been shown to induce changes in the glycosylation pattern of mucus that may affect its interactions with pathogens [570]. Furthermore, environmental parameters, nutrition and production procedures (i.e., crowding) can lead to different grades of lost epidermis and scales [571, 572]. Since scale loss is energy-consuming and may lead to osmoregulatory problems, it could further reduce performance and decrease survival [4, 573]. In Atlantic salmon, this type of damage is seen after transfer to sea, but the skin does heal, and immune activity in the skin gradually recovers after some weeks. Karlsen et al. [515] suggest that the first months in the sea be considered a barrier recovery phase for salmon, where handling should be reduced to a minimum and fish should be protected as much as possible to build resilience.

Given all the crucial functions of the skin, skin damage is an important outcome-based WI, particularly since out of the top 10 causes of mortality in Norwegian Atlantic salmon farms, six are related to skin problems [503]. While external examination is one way of monitoring skin health, to reduce mortalities and increase welfare, new methods and technologies are obviously needed to better characterise skin health and to detect skin problems at earlier stages. Skin damage primarily impacts upon welfare needs under the following domains: physical environment, health and behavioural interactions.

5.6 | Fin Damage

Fins are bodily appendages that are primarily utilized in body propulsion and control. In fish, they are often comprised of jointed, bony fin rays that offer flexible and structural support to a thin layer of epithelium tissue that lacks scales [574, 575]. However, exceptions such as the adipose fin can also lack structural fin rays [576]. Muscular control of each fin ray, in addition to joints within the ray, allows for precise control of the fin size and shape, meaning fins can be utilized to both produce and influence hydrodynamic wake during propulsion [574].

Fish often have a selection of rayed median fins such as the dorsal, caudal or anal fin and rayed paired fins, such as the pectoral and pelvic fins [574]. Others, such as salmonids, also possess a median non-rayed adipose fin [576]. Each fin can have a distinct function linked to differing aspects of, for example, propulsion and manoeuvring [574]. For example, when considering their role in propulsion, the median dorsal and anal fins can generate vortex wakes that enhance the flows around the caudal fin and can also be involved in promoting stability and generating lateral forces and thrust [574, 577]. It has further been hypothesised that the adipose fin may play a role in fish swimming and propulsion [577]. Conversely, the caudal fin is often employed to generate thrust and lift during swimming [578, 579], while the paired pectoral and pelvic fins have roles in propulsion, stability, braking and turning movements as well as station holding in both the water and upon the substrate [574, 580–583].

Given their key role in locomotion, fins are structural tissues that can possess nociceptors and mechanoreceptors [497, 584]. Any damage breaches the epidermal barrier [585] and can be a conduit for pathogenic incursion and infection [586], which in turn can exacerbate the damage and negatively impact the function of the fin [575]. Furthermore, exposed fin rays can be sharp and injure adjacent epidermal tissue on the injured individual [587] or other conspecifics if they come into close contact. Further, fin-related welfare impairments can be affected by a number of species- or life-stage-specific traits as well as by the type of fin damage, which is therefore often broken down into the most relevant categories such as fin erosion, splitting, thickening or haemorrhaging [4, 575, 588] or a combination of these injury traits [4]. Previously, authors have classified fin damage as either active, and thus an ongoing risk, or healed, which can be used as evidence of a previous injury state and also a potential change or improvement in the present rearing conditions [4]. Fin damage primarily impacts welfare needs under the following domains: health and behavioural interactions.

5.7 | Snout/Jaw Damage

The snout region of fish encompasses the area from the anterior tip of the fish's head to the anterior side of the eye [589], hence, in the five fish species covered in this review, the snout region includes the mouth, jaws and nasal pit area, in addition to the lateral line, whose canals often run over the dorsal surface of the fish's head and along the length of the lower jaw [590]. This lateral line system lets the fish follow and react to changes in the movement of water around the fish and movements of prey, predators and conspecifics, in addition to assisting in schooling and orientation behaviours [33] and references therein. Right below the nostrils and sitting inside the nasal cavity are two sensory organs, the olfactory rosettes, which connect to the central nervous system (CNS) via the olfactory bulb [591]. The olfactory organ is important for olfaction guiding behaviour for mating, food selection, homing and escape from predators, among others [592]. Recently, the role of the olfactory organ in the defence against waterborne antigens in fish has been elucidated, offering new insights into the evolution of nasal immunity in vertebrates [593]. The function of the mouth in fish is primarily to ingest and consume food as well as to take in water and sometimes air. The mouth of fish incorporates an upper and lower jaw, which often have teeth and enclose a tongue [575, 594] and references therein. The oral cavity further contains tastebuds, often on the tongue, that have a chemo-sensory role during feed acquisition and feed processing within the cavity [594–596]. The jaws, tongue and mouth are therefore primarily used for capturing, transporting and processing food for ingestion purposes and transporting water through the mouth for respiratory purposes. Their morphological characteristics can vary with species, strain and even life stage [597].

Snout damage refers to injuries in the snout region that can affect the upper and lower jaw, the nasal pit region or extend beyond these areas [488]. It can be driven by, for example, mechanical trauma from handling, abrasion against rearing materials or equipment, as well as collisions with infrastructure or conspecifics [331, 598]. Further, snout damage can be exacerbated by opportunistic bacterial pathogens such as *Tenacibaculum* spp. [599]. The differing types and aetiology of snout, mouth and jaw damage, in addition to their effects on fish welfare, have been widely reviewed by Noble et al. [575], and only a brief and updated summary will be presented here. Mouth/jaw abnormalities can arise due to deformities or injuries and here we will mainly focus on mouth injuries and not deformities per se. Injuries can take the form of tissue haemorrhaging, tissue lesions, erosion or broken bones Noble et al. [575], including nasal pit haemorrhaging [482], and can lead to the exposure of underlying bones and cartilage [599].

Snout, nasal, mouth and jaw damage are a welfare issue for several reasons. First, the jaws can be rich in nociceptors [600], second, damage breaches the dermal barrier [599] which can lead to secondary osmoregulatory challenges [601], pathogenic incursions or further damage [575], and third, the damage can have a detrimental effect upon the fish's ability to capture and consume feed [551]. Damage around the mouth may also impact other mechano-sensory systems of the fish, including the lateral line, whose canals often run over the head's dorsal surface and along the length of the lower jaw [590] and nasal region. Additionally, the olfactory organ is, as a mucosal organ, constantly exposed to different stimuli, making it a portal of entry for pathogens, especially those affecting the central nervous system [591, 593]. However, we have limited knowledge of the number of pathogens hijacking the olfactory organ to gain entry because it is not often included in routine histopathological evaluation. Further, the olfactory organ has been shown to be very sensitive to husbandry-related stressors, including temperature, water disinfectants, handling, and in some cases, it is more sensitive than other mucosal organs (i.e., skin or gills) [79, 509, 602–604]. Furthermore, damage to the olfactory organ has been shown to impair fish health, welfare, performance [605] and behaviour. For instance, a recent study showed that toxicant-induced structural alterations in the olfactory organ of Atlantic salmon influenced feeding behaviour resulting in poor growth [604]. Snout/jaw damage primarily impacts welfare needs under the following domains: nutrition, health and behavioural interactions.

A basic snout damage and jaw deformity classification scheme has been suggested for salmonids in farm settings by [4, 488] and classifies snout damage in relation to lesion and haemorrhaging severity and jaw deformities based on their severity and the specific jaw that is affected [4] or their general severity irrespective of which jaw it affects [488]. Either of these may be suitable for an early-stage audit of snout and jaw damage and deformities in aquaculture research. A change in the colour of the snout (darkening of the snout) has also been observed in salmonids following warm water treatment for delousing purposes [482] and thus colour change may be suitable as an acute indicator of treatment/trauma, although the drivers and mechanisms for its expression do need further investigation.

5.8 | Eye Damage

A fish's eye consists of the cornea, the sclera, the lens and the retina. The cornea, which is the outermost layer, directs light into the eye and acts as a transparent protective barrier. The hard sclera keeps the form of the eye by acting as structural support. Fish lenses, which are spherical in shape, direct light onto the retina, which is in the rear of the eye [606]. Within the retina, there are rods and cones which are photoreceptor cells that gather and send visual information to the brain and are frequently adapted to the low light levels found in underwater environments [607].

Eye damage can take various forms depending upon both the pathology and the aetiology. Damage can be in the form of haemorrhaging (hyperemia) in and around the cornea that can be caused by, for example, parasites [411] or by mechanical, thermal or impact trauma [608]. Further, lens opacity or cataracts are a common problem for many aquacultural species [609, 610], with cataracts being either long or short-term problems depending upon the driver [331] and references therein. Other forms of eye opacity can be driven by corneal inflammation (keratitis) and in operational settings, it can be difficult to distinguish between these drivers and numerous scoring schemes simply record eye opacity without breaking it down further [488]. Additionally, eye damage can also manifest in the bulging of the eye, called exophthalmos or commonly termed pop-eye [611] or enophthalmos, called sunken eye [612]. Drivers for exophthalmos can be multifactorial, including viruses [613], parasites [614] and gas bubble disease [615], while enophthalmos can be, for example, virus-driven [612].

Eye damage may lead to blindness [610], secondary infections [469] (and its source references) and can be potentially painful as the cornea can contain nociceptors [616]. In addition to these direct impairments, partial or full loss of vision can affect, amongst others, orientation, feeding and social interactions. Studies have shown that social preferences in fish can be based on vision since seeing fish of the same age and size is important when developing social behaviour [617]. Additionally, research has shown that, in Atlantic salmon, eye colour is used as a social rank signal with lower rank individuals tending to have darker eyes than dominant ones [618]. Eye damage primarily impacts upon welfare needs under the following domains: nutrition, health and behavioural interactions.

5.9 | Condition Factor

Condition factor (K) is a morphometric index for evaluating length-weight relationships in fish and is calculated using the formula $K = 100 \times \text{weight} \times (\text{length}^3)^{-1}$. It is a well-established instrument for documenting changes in the nutritional status of animals [619] as it is generally assumed that if fish are identical in length, a heavier fish has more energy reserves than a lighter one and is in better condition [620]. However, there are exceptions to this assumption as some studies have found no clear relationship between condition factor and lipid reserves in certain species or life stages [621].

While condition factor is not directly mentioned in the EU Directive 2010/63/EU [15], a low condition factor may be indicative of malnutrition or lack of feed access [622, 623], deficiencies in the diet [624], exposure to unfavourable environmental parameters [127] or poor health [625]. A high condition factor is often used to suggest that fish are healthy [626] and are being fed a well-balanced diet [627] under an appropriate feeding regime that gives them sufficient access to food [622]. However, a high condition factor may denote some health conditions, such as the presence of vertebral deformities in species such as Atlantic salmon [628] or overfeeding. When linked to nutritional status, condition factor can also be used as an indicator of potential future performance, for example, by being one of a myriad of factors influencing the timing of maturation [629] in salmonids. In addition, it should be acknowledged that it is an iceberg indicator that is not solely affected by fish welfare [468]. For example, condition factor has been known to naturally decline during smoltification in salmonids [630, 631] and during spawning [632], neither of which is an impingement of the fulfilment of some key welfare needs. This led Folkedal et al. [631] to suggest that fish size should be included in any consideration of condition factor as an OWI, and we would like to suggest that life history events should also be reflected upon when considering the utility of using condition factor as a WI.

Condition factor is a rapid and inexpensive tool to include in a welfare audit as long as it is considered in relation to natural, for example, life stage variability, see [4] and references therein. To advance its utility as a WI, thresholds or ranges should be made available that indicate good or reduced welfare. However, due to the multitude of non-welfare-related factors that can affect condition factor, it is difficult to outline specific values or thresholds that are symptomatic of good or poor welfare [4]. Nevertheless, some authors have stated condition factors indicative of emaciation as being (i) <0.9 in Atlantic salmon [3, 631], (ii) <1.0 in rainbow trout, see [331] and references therein, (iii) <0.9 in European seabass [633] or around 0.9 when fasted for 3 months [634] and (iv) <1.4 in gilthead seabream fasted for 2 months [635]. The authors could not find a condition factor that is indicative of poor or good welfare in common carp, and this may be because there are many wild and domesticated variants of this species. Once again, these values must be applied with caution and with consideration of the other factors outlined above. Condition factor primarily impacts upon welfare needs under the following domains: nutrition and health.

5.10 | Maturation State

Sexual maturation is the process of becoming capable of reproducing (i.e., reaching maturity) and although the ultimate purpose of a fish may be reproduction, precocious sexual maturation or early puberty within both an aquaculture and aquaculture research setting can result in a number of welfare issues. Sexual maturation is a transient process that dramatically changes the physiological status of the fish, thereby influencing for example, appetite, behaviour, growth, immune response and osmoregulation; see [4, 331, 636]. While the majority of the problems regarding early maturation are observed in males [4, 636], females can also mature early, but little is currently known about this process in, for example, seabass [637]; Atlantic salmon [638]. Controlling or delaying precocious maturation is to some extent possible through selective breeding, the restriction of feed intake, photoperiod manipulation, swimming exercise [639] or sterility [636, 640].

There are multiple ways to measure or document maturation state, largely by laboratory methodologies but also by some operational techniques. External morphological traits of secondary sexual characteristics can often be observed visually. For example, sexual maturation in post-smolt Atlantic salmon males is characterised by the development of a kype (i.e., a hook-like protrusion at the tip of the lower jaw), the occurrence of skin spotting (red/brown) and a change in skin colouration towards a brown-green hue. Skin spotting has been recently shown to be highly correlated with the development of gonads and appears to be a promising marker for assessing early sexual maturation [641]. However, when these traits become visible, processes related to gonad development may be well on their way. Similarly, the use of ultrasound is a relatively non-invasive method to determine sex as well as assess gonadal development, and thereby the maturation state. It has been applied in various fish species, such as Atlantic salmon [642], and European seabass [643]. Although it requires handling, the application of ultrasound is beneficial as it quickly provides insight into the maturation state of the fish, without killing it, which is required to assess the gonadosomatic index.

Applying biochemical techniques, sex steroids such as testosterone, 11-ketotesterone, 17β-estradiol and androstenedione—which are produced during sexual maturation—can be measured in blood plasma or serum and to some extent in skin mucus [644], the latter being a less invasive method. Sex steroids can be assessed by several laboratory methods that are commonly applied in fish research, including radioimmune assay (RIA [645]), commercially available enzyme-linked immunosorbent assay (ELISA [642]) or liquid chromatography tandem mass spectrometry (LC–MS/MS [641, 644]). Further, molecular markers linked with the occurrence of early puberty have been identified in the last decade through genomic investigations and can be assessed by genotyping. For example, in Atlantic salmon, gilthead seabream and European seabass the genes vestigial-like family member 3 (vgll3) and six homeobox 6 (six6) have been associated with the age of maturation [629, 646]. These markers can be used to distinguish fish that have a higher tendency for early maturation [647].

Knowledge of the maturation state of the fish and its potential impacts should not be neglected in research studies, as they may affect the physiological response of the fish to certain procedures, and this, in turn, can affect the reproducibility of the results. Further, physiological and environmental factors can impact sexual maturation, which in turn influences the welfare state of the fish. Additionally, the diversity of reproductive strategies of fish should also be considered in aquaculture research since hermaphroditic fish species are relatively common, including synchronous, protandric (male-first), and protogynous (female-first) hermaphroditism. For instance, a highly cultured fish such as gilthead seabream is a protandrous hermaphrodite, and the progression of sex reversal is regulated by an array of factors, including those that are nutritional and genetic [408, 648, 649]. Therefore, the evaluation of sex reversal, considered as the end point of a complex cascade of developmental events, can be considered a valuable tool for the retrospective documentation of welfare in this economically important fish species. Maturation state primarily impacts upon welfare needs under the following domains: physical environment, health and behavioural interactions.

5.11 | Integrative Stress Responses, the Role of Serotonin, Cortisol, Glucose and Lactate

5.11.1 | Stress Markers, Cortisol, Glucose, Lactate and Osmolarity

Living organisms need to maintain a balance between their physiology and the environment they inhabit. Fluctuations of the external and internal body condition, out of what an animal can tolerate, can create imbalances, jeopardising the normal functioning of vital physiological processes. Changes in environmental conditions can be predictable, for instance, when triggered rhythmically, that is, transitions between day and night, tides, seasons or others [650]. However, sometimes changes can be unpredictable, generating distress and affecting the welfare of animals, such as a predator encounter in nature, or handling or net chasing in experimental settings [651]. It has therefore been an evolutionary advantage to develop physiological mechanisms and behavioural strategies that allow fish to predict and cope with environmental perturbations in an agile and flexible way, maximizing the likelihood of survival [652].

Similar to other vertebrates, fish are furnished with a complex neuroendocrine system capable of perceiving changes, either internal or external, integrating stimuli centrally, and developing an adaptive response [653, 654]. The integrated stress response, studied extensively in fish, is one of the most conserved physiological processes between vertebrates, and underlies the mechanisms involved in how this adaptive response develops. The hypothalamus-pituitary-interrenal (HPI) axis, responsible for cortisol release, and the hypothalamus-sympathetic-chromaffin (HSC) axis, responsible for catecholamine release (adrenalin and noradrenalin), often addressed as the primary stress response, are activated by external or internal stimuli that affect the animals' allostasis, triggering a cascade of processes aimed at restoring balance [655]. The activation of these neuroendocrine axes has a marked impact on the behaviour of the animals, generating a state of warning (fight or flight), which is crucial to coping with situations that endanger their survival. Secondary reactions involve metabolic shifts, including adjustments in hydromineral balance, as well as modifications in cardiovascular, respiratory, and immune functions. Examples of these secondary changes include heightened levels of glucose and lactate, alterations in antibody production and elevated production of heat-shock proteins [656]. However, the prolonged activation of these axes is known to become maladaptive and energetically unsustainable, causing changes in, for example, disease resistance and behaviour [656] leading to exhaustion and, in extreme cases, even to death [653, 655].

With regard to the HPI axis activation, the concentration of the hormone cortisol is a relevant marker for measuring fish welfare. After an acute stress episode, inter-renal cells in the head kidney create cortisol, which is then discharged into the blood and transported around the organism [657]. Cortisol action takes place through glucocorticoid receptors (GRs), which have been identified across all tissue types, including the liver, brain, gills, gonads, intestine, muscle, as well as in red and white blood cells [658]. Further, cortisol has been recognised for its ability to modify brain structure and functions [659]. Additionally, it plays a crucial role in regulating energy metabolism [660], growth [661], immune function [662], maturation and reproduction [663] as well as behaviour [664]. This underscores the key impact of cortisol on both the physiology and behaviour of fish [655, 658, 665, 666] making cortisol levels a potential marker for stress.

There are several aspects that must be considered when cortisol is adopted as a stress marker. For instance, while cortisol is widely acknowledged as an acute plasma-based stress indicator, it may not always mirror a chronic state of stress due to HPI axis desensitisation resulting from allostatic overload [657, 667]. Thus, with the aim of assessing welfare in potentially chronically stressed fish, cortisol and other plasma values should be integrated with other sources of information such as appetite, growth or swimming behaviour. During welfare assessments, basal levels of cortisol can be compared to those of stressed fish to give an indication of the latter's welfare state. However, cortisol basal levels can differ between species and even within different life stages, independently from stress. For example, during smoltification or sexual maturation, cortisol concentrations may be high without the fish being subjected to stress due to the involvement of cortisol in these physiological processes. Furthermore, given the myriad of cortisol functions on physiology, it is essential to consider the prospect that an increase in cortisol levels in fish may serve as an adaptive reaction not only to negative but also to positive stimuli/experiences [655, 668] such as arousal or excitement. Furthermore, cortisol may display a wide range of variability between fish of the same age and species, as it can be affected by the feeding state (e.g., if fish are starved or not), the sex of the fish, or variations in the neuroendocrine stress response linked to individual differences in behavioural phenotypes, social status or coping styles [669].

In addition to biological factors, sampling procedures may also affect cortisol measurements. Cortisol production and release varies over time after a stressful stimulus [670] meaning a stakeholder should carefully select a time window for sampling it [657]. The optimal window may be species-specific, as well as temperature-dependent since basal and stress-dependent cortisol release levels are highly affected by acclimation temperature both in magnitude and time [671, 672]. Additionally, a sampling plan using several time points may furnish more information on the baseline, increasing, maximal peak and decreasing timing for a given life stage and species under specific conditions. It is also strongly suggested that, where possible, an experimental control group for comparative purposes is considered. During experiments, the most common measurement of cortisol is carried out on blood plasma or serum, which quantifies circulatory cortisol concentrations when a fish is sampled. However, there are less invasive methods that can be used to collect and measure it [657]. For instance, cortisol can be measured in various mediums such as water, for example, the tank outlet [673], fish faeces, scales and mucus, extensively reviewed by Sadoul and Geffroy [657]. Nevertheless, each of these tissues provides different information on the welfare state of the fish and particularly on the timing of the stress response. Examples of cortisol levels in different farmed finfish species, as well as the levels under stress and by using different detection methods, have been outlined elsewhere [4, 331, 657, 674, 675].

Another important marker that is indicative of a stress response in fish is glucose. An increase in cortisol causes an increase in glucose concentrations via gluconeogenesis, which is the generation of glucose from non-carbohydrate sources, and glycogenolysis, which is the breaking down of glycogen [660], meaning glucose can be a reliable indicator of acute stress. However, the response is relatively slow; it has a longer duration than cortisol, and the peak in concentration occurs several hours after the exposure to the stressor [675], which allows for the assessment of stress levels of fish at different time scales. As before, other factors should be considered when using glucose levels as markers to assess welfare. For example, the feeding schedule and feed composition may induce small changes in glucose profiles, while environmental factors such as temperature may affect glucose baseline levels across species [676]. Examples of glucose levels in different farmed finfish species, as well as the levels under stress and by using different detection methods, have been outlined previously [331, 670, 677].

The driver for increased glucose release in the blood plasma is that fish require additional energy to cope with stress and glucose, the primary fuel for aerobic metabolism, provides this energy. In cases where aerobic metabolism is insufficient, such as under increased or prolonged activity and especially under conditions that require intense muscle activity, such as acceleration and burst swimming, the internal system of the fish resorts to anaerobic metabolism to cover energetic demands [678]. This can also occur in other situations that challenge the aerobic capacity of the fish, such as poor water quality parameters like low dissolved oxygen saturations [679–681]. The outcome of this anaerobic metabolism is lactate, which is therefore a reliable indicator of acute stress, especially in cases that involve handling, high activity levels or exhaustion. Examples of lactate level measurements in different farmed finfish species as well as under different stress levels using different detection methods, have been outlined previously [4, 331, 670, 682].

In addition to cortisol, glucose or lactate, osmolality can be considered an outcome-based WI. Osmolality is critical to survival for both marine and freshwater fish, as all fish actively maintain their ionic homeostasis by consuming energy and involving organs such as the skin, the kidney, the gut, and most of all, the gills [658]. Therefore, the individual concentrations of various ions as well as osmolality can be deemed to be a feature of the secondary stress response of fish [683] and therefore are important markers that can be used to assess stress and the overall physiological status of a fish [4]. Examples of osmolality levels in different farmed finfish species as well as under different stress levels using different detection methods have been outlined previously [4, 331, 654]. These stress markers primarily impact upon welfare needs under the following domains: nutrition, health and behavioural interactions.

5.11.2 | Serotonin's Role in Stress Responses

At the central level of the nervous system, the monoamine serotonin (5-HT) is important in the integration of information and the activation of stress axes [684, 685], for example by regulating the release of hypothalamic neuroendocrine factors such as corticotropin-releasing factor (CRF) or arginine vasotocin (AVT), both precursors of cortisol release [686, 687]. However, the role played by the 5-HTergic system is complex and multifactorial control mechanisms beyond the mere activation of this neuroendocrine axis are to be expected. 5HT appears to be involved in the assembly of the behavioural phenotype, involving high-order brain areas that give rise to the development of different styles of coping with stress [688, 689]. These in turn have a very strong component of intraspecific variability, forming the behavioural profile of each fish as a function of genotype, environment and previous experience [690].

5-HT is extensively distributed in the fish brain, with the largest clusters of cells located at the level of the hindbrain and diencephalon [691], projecting to large areas of the CNS, including the forebrain [692], where stressful stimuli have been reported to activate the system [693] and where high levels of serotonergic activity are associated with animals showing a low reactive behavioural profile [688]. Subordinate fish show high and prolonged levels of serotonergic activity following stressful stimuli, and these profiles are in turn related to phenotypes that show general behavioural inhibition, including inhibition of food intake, reduced aggression, decreased growth and reduced reproductive behaviour [694], clear indicators of compromised welfare [404]. On the other hand, dominant animals show quick and short activation of the 5-HT system after stressful stimuli, rapidly recovering baseline values once the stressful stimulus disappears [694, 695]. In contrast, low levels of 5-HT seem to be compatible with stages of anxiety-like or depression-like conditions [696–698], similar to those observed in mammals [699]. These phenotypes are closely associated with deficiencies in animal welfare and are a topic of wide interest due to the negative implications they have on animal production, such as, amongst others, loss of appetite. Indeed, 5-HT seems to play a regulatory role in feeding behaviour [700] as an inhibitory signal at the level of the diencephalon and hindbrain, acting on brain neuropeptides to promote a satiety profile [701]. Consequently, this monoamine has also been reported to be involved in feeding disorders in fish, such as obesity [702]. Furthermore, deficiencies in the serotonergic system are associated with impaired cognition, as 5-HT is closely related to neurogenesis in fish [703].

Overall, the serotonergic system of fish appears to be involved in behaviour and cognitive performance, emerging as a useful marker for assessing fish welfare in both aquaculture and aquacultural research settings. It provides information on the integrated stress response, as well as on the perceived mental state of the animals, which provides valuable information for targeting the emotional component of fish welfare. Given these far-reaching effects, the components of the serotonergic system primarily impact welfare needs under the following domains: nutrition, health and behavioural interactions.

5.12 | Optional Internal WIs If the Fish Are Being Euthanised

The health status of the internal organs is central to the health and welfare state of fish, see [56] and references therein. Some authors have stated that all fish organs should be visually inspected for severe inflammation as a primary health and welfare documentation tool in experimental settings, before progressing to histological examination only when this is feasible, appropriate or where a more in-depth audit is needed [51]. Organs, which can be potentially of interest if the fish are being euthanised, include, but are not limited to, the heart [51], liver [704], spleen, kidney, stomach and intestines [56] or visceral fat levels around the pyloric caeca [704]. Further, an audit of the buccal cavity can provide an overview of any potential internal bleeding into the body cavity [56]. In this section, we will introduce the utility of using heart shape and placement, liver score, visceral fat levels and focal dark spots in the fillets as potential WIs to include in a health and welfare monitoring plan, if the fish are being euthanised.

The heart physiology of Atlantic salmon has been researched for several decades and it is well known that the heart shapes of wild and farmed fish deviate [705]. In Atlantic salmon, wild fish usually have more triangular-elongated hearts and show differences in bulbus alignment, which leads to more efficient blood pumping. On the other hand, farmed fish tend to have more rounded hearts and show a vast variety of shape deviations [706]. Certain morphologies, such as differences in heart sizes and bulbus misalignments, have already been linked to fast growth rates of smolts due to rearing temperatures [707]. However, these differences may be life-stage specific and may disappear during the grow-out phase [708]. Additionally, methods to describe and measure heart shape often differ between studies or work groups, which makes comparisons difficult. Thus, standards are needed and Engdal et al. [706] have recently provided the first comprehensive nomenclature for salmon heart shapes. However, measurements are still performed manually, which is time-consuming and may introduce human error and bias. Due to the large variety of salmon heart shapes, high sample numbers are needed for conclusive results, such as linking shape types/morphological variables to functionality and the identification of risk factors. Thus, much about salmon heart morphology is still unknown and automized methods with faster, standardized measurements have a great potential to positively impact this field of research in the near future.

The fish liver is essential for processes such as metabolism, detoxification and immunity and is split into cranial and ventral lobes in most fish species [709, 710]. The lobules consist of hepatocytes, which are the functional centres of the liver. The liver can be scored by colour, shape and pathologies (Table 4), and the organ has been used as a health and WI for several years [56, 704, 711–716]. A common denominator for most of the scoring systems that have been used to evaluate liver status is that a dark liver is considered normal compared to livers that are paler. However, this assumption may not be correct in all cases. For example, in Atlantic salmon, a dark-coloured liver, together with other pathologies, may be a sign of health problems such as Infectious Salmon Anaemia, which causes liver haemorrhages [717, 718]. A dark liver in gilthead seabream is associated with inadequate lipid metabolism, and Fernández-Díaz and Yúfera [719] observed dark livers in gilthead seabream larvae associated with insufficient digestion of the experimental diet.

Liver colour can be scored manually (Table 4, although to our knowledge there are no scoring scales for European seabass) or by a spectrophotometer, for example, in rainbow trout [720] and European seabass [721]. Further, Candebat et al. [722] used image acquisition methods for liver colour scoring in addition to a visual assessment to check for green liver syndrome in yellowtail kingfish. Differences in liver colour can be due to, for example, diet composition, genetic differences or disease [714, 716–719]. A correlation between liver colour and fat content has been found in Atlantic salmon, with pale livers having higher lipid content than darker livers [714] and hyperspectral imaging has recently been demonstrated to be a valuable tool for measuring liver fat content in Atlantic salmon, Atlantic cod and European seabass [723]. Fat accumulation in the liver of salmon may have a negative effect on liver function and health, as seen in humans and other mammals that do not usually store fat in the liver. However, this should be further investigated. Similarly,

TABLE 4 | Examples of published liver colour scoring systems summarising the number of categories per scoring system and also some examples of the categories.

No. of categories	Examples of scores	Species	References
3	Score 0 = Normal Score 2 = *Totally discoloured*	*R. trout*	Austreng and Refstie [711]
5	Low score = *Yellow* liver High score = *Normal dark brown* liver	*A. salmon* and *R. trout*	Gjerde and Gjedrem [712]
3	Score 1 = *Normal dark red* Score 3 = *Discoloured* (*yellow/spotted*)	*A. salmon*	Hillestad et al. [713]
5	Score 1 = *Pale/yellowish* Score 5 = *Dark brown* Includes a pictorial guide	*A. salmon*	Mørkøre et al. [704]
4	Score 0 = *Inconspicuous* Score 3 = *Several discoloured and/or enlarged and/or necrosis*	*R. trout*	Tschirren et al. [56]
6	Score 0 = *Pale/yellow* Score 5 = *Normal/brown*	*A. salmon*	Dessen et al. [714]

Note: Citations, in italics, are directly reproduced from the source articles.

in lumpfish, liver colour has been correlated with lipid content and histopathology, and has also been suggested as an appropriate indicator for welfare [724]. Additionally, temperature, dietary fat and fatty acid content have been shown to influence liver fat in Atlantic salmon [716, 725, 726].

Visceral fats surrounding the internal organs are one of several fat depots in the fish body. Visceral fat stores of Atlantic salmon can be scored according to the visibility of pyloric caeca [704, 727, 728], see Table 5. Factors such as dietary fat and fatty acid content and season [729, 730] can influence abdominal fat deposition. Further, excessive fat deposition in salmon may indicate a failure in the mechanisms regulating lipid deposition and mobilisation, that can result in a low-grade metabolic inflammation and compromise fish welfare [729].

Focal dark spots (DS) in farmed Atlantic salmon fillets are the largest quality issue for the industry and affect approximately 16% of all fish produced in Norway [731, 732]. The spots are presented as mainly red or black discolourations in the fillet, primarily cranio-ventrally [733]. The aetiology of DS is unknown, but it has been suggested that rib and muscle damage are the primary causes in Atlantic salmon fillets [734]. Black DS are characterised by chronic inflammation [735, 736], and there appears to be a clear link between DS and rib abnormalities [733]. Thus, considering chronic inflammatory processes in the muscle, as well as the association with poor skeleton health, DS may present a welfare issue for farmed Atlantic salmon [4, 734], and we here propose its consideration as a WI.

DS are normally assessed visually on the processing line on a limited number of fillets. In Norway, a national scoring system was developed in collaboration with the industry, where the DS is scored based on size and intensity according to a logarithmic scale (since larger and more intense spots are more problematic) on three parts of the fillet: anterior and posterior parts of the belly, and dorsal loin [737]. Individual companies may however use other methods.

5.13 | Faecal Consistency

The consistency of fish faeces can be used as a proxy indicator of health and welfare. Poor faecal consistency can be indicative of fish going extended periods without feeding (either voluntary or involuntary fasting), which can indicate health and welfare problems [475, 738]. Further, loose faecal consistency (diarrhoea) can be indicative of poor health and welfare, indicating, for example, inappropriate feed types and constituents that lead to gastrointestinal problems and/or osmotic problems for the fish [739–741]. The collection and examination of fish faeces has previously been suggested as a suitable health and welfare auditing tool in fish research by [51]. Faecal samples are often stripped from individual fish in feeding and nutrition studies [740], or microbiome and gut health investigations [475], but may also be collected at the system level as a potential aquaculture waste reduction and solids management strategy in numerous farming systems [742]. As faecal collection by stripping can be stressful for the fish [51, 743] it is often undertaken on euthanized fish or those under anaesthesia. Earlier studies have reported that the procedure can be conducted without leading to physical injuries or damage to the digestive tract [743]. However, some fish species, including gilthead seabream and lumpsucker (*Cyclopterus lumpus*), will quickly evacuate the contents of their hindgut following anaesthesia [744], while others, including Senegalese sole (*Solea senegalensis*), may have intestines that are too fragile for stripping [745].

Measurements of faecal state and consistency have previously been rarely performed as part of a general health and welfare monitoring plan, and this may have been due to challenges in interpreting the results due to a lack of published knowledge [51].

TABLE 5 | Examples of published visceral fat scoring systems summarising the number of categories per scoring system and some examples of the categories.

No. of categories	Examples of scores	Species	References
3	Score 0 = *Little* Score 2 = *Much*	*R. trout*	Refstie and Austreng [715]
5	Score 1 = *Leanest* Score 5 = *Fattest*	*A. salmon*	Hillestad et al. [713]
4	Score = 0, *individual pyloric caecae clearly visible with no adherent fatty deposits* Score = 4, *all pyloric caecae completely obscured by visceral fat deposits*	*A. salmon*	Morris et al. [727]
5	Score 1 = Pyloric caeca *clearly visible* Score 5 = Pyloric caeca *not visible*	*A. salmon*	Dessen et al. [728]
5	Score 1 = Pyloric caeca *clearly visible* Score 5 = Pyloric caeca *not visible* Includes a pictorial guide	*A. salmon*	Mørkøre et al. [704]
3	Score 0 = *No VF* (on the heart surface) Score 2 = *Severe accumulation of VF on the heart surface*	*A. salmon*	Mørkøre et al. [704]

Note: Citations, in italics, are directly reproduced from the source articles.
Abbreviation: VF, visceral fat.

However, the state of the art has gradually increased within this field, and there are established scoring schemes now available [738] that help structure and guide evaluations in operational settings out on the farms [475]. However, it will likely remain a challenge for species that naturally have more liquid faeces and species for which faeces collection by anaesthesia and stripping is not possible. Basic scoring schemes are outlined in Table 6.

5.14 | The Microbiome

The microbiome refers to a complex ecosystem composed of an enormous number of organisms including bacteria, fungi, archaea, viruses, and yeasts in a particular environment. Bacteria are the dominant component of this group and represent the principal research focus within this topic [747]. In aquaculture settings, microbial populations are largely present in the environment as well as on and within the animals. They colonise external and internal fish mucosae, such as the gills, skin and intestine, forming peculiar niche-associated communities [748]. The influence of the microbes, especially the gut-associated microbiota, on the host physiology is largely documented and involves numerous processes, including metabolism, immunity, the absorption of nutrients, and pathogen defence [749–752]. Due to this profound connection, established through co-evolution and the ontogenesis process, the host-microbiome system, according to the hologenome theory, must be considered a distinct biological entity, the holobiont, which evolves and adapts to environmental changes [753–755]. In this regard, the microbiome and host do not only include the microbial populations that inhabit the fish mucosae, but also the environmental bacteria that inevitably interact with the holobiont and, in a broad sense, are part of it. In aquaculture, the microorganisms associated with the farm systems cannot be ignored, as they constitute a biological fraction that can strongly affect water quality, especially in RAS systems [756–758]. As a consequence, the water microbiome represents a simple route to routinely monitor and audit the environment without stressing animals and can be considered an input-based WI.

Following this concept, the evaluation of the microbiome as an indicator becomes crucial to guarantee and monitor the health and welfare of the holobiont. However, the precise definition of an ideal microbiota is difficult to determine, especially considering different and phylogenetically distant species, such as those included in this review (Atlantic salmon, rainbow trout, European seabass, gilthead seabream and common carp). Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes are widespread phyla which typically dominate the microbiota of these fish species, often maintaining comparable ecological roles and relative abundances, albeit in varying proportions [759–763]. Many authors consider them to be a core structure that can be considered a healthy profile, and a welfare marker, since a change in their equilibrium manifests the onset of a negative process, a worsening of the condition of well-being which can ultimately develop into dysbiosis [764]. However, more in-depth analyses of bacterial communities show how these fairly constant relationships are lost when considering lower taxonomic levels in which a strong inter- and intra-species variability is highlighted [765]. Numerous extrinsic and intrinsic factors have demonstrated a direct influence on microbiota in fish, such as water quality, environmental microbial populations, diet, gender, age and stress condition [749, 766–769]. In addition, the host's genetics are particularly important as they can strongly affect microbial populations, particularly the gut microbiota, both in model and farmed fish species [627, 770, 771]. Recent studies have demonstrated how an intensive selection process for fast growth in gilthead seabream and European seabass can actually co-select intestinal microbes, reducing their abundance but enhancing their plasticity at a metabolic and functional level [768, 772, 773]. Similar results were obtained in salmon, where instead of genetic selection, the life-cycle stage progression and

TABLE 6 | Examples of published faecal scoring systems with citations, in italics, directly reproduced from the source articles.

No. of categories	Examples of scores	Species	References
6 categories, 1–5 with an additional sub-level	Score 1 = *Solid* Score 3 = *Diarrhoea* Score 5 = *No faeces*	*A. salmon*	Zarkasi et al. [738]
4	*N* (*normal*), *F* (*flocculants*), *M* (*mix/intermediate between normal and flocculants*) *or E* (*empty*)	*A. salmon*	Waagbø et al. [746]

development have been shown to determine a progressive reduction of the microbial diversity in healthy adult specimens, suggesting a specification towards the most important and metabolically active taxa [774]. These results may be considered controversial, as a decrease in alpha diversity is commonly associated with dysbiosis. However, these results suggest that a more detailed analysis should be conducted to better clarify the real function of microbiota, rather than its variability. Indeed, genes and pathways are redundant and abundant across different bacterial taxa; therefore, instead of focusing on taxonomic differences, it may be valuable to identify and investigate functional changes in the microbiota [748, 775].

Given the complex interactions, the microbiome does not only represent an outcome-based WI that gives a diagnostic evaluation of the welfare state of the animal, but it can also be used as a prognostic tool to predict potential negative developments at an early stage. This approximation is currently used in human studies [776, 777], and in agricultural animal production [778, 779], however, there is an urgent need to improve the knowledge about functional and specific bacterial markers and proxies also in the aquaculture field [774, 775, 780]. In this regard, a concrete case in which a bacterium is used as a marker is represented by the genus *Mycoplasma*. This taxon generally occupies a great fraction of the bacterial population associated with both intestinal mucosa and faeces of salmonids and so, given its abundance, it has been identified as an indicator of poor health in adult Atlantic salmon, even if its function remains unclear [759]. However, for other fish species, due to the flexibility and the variety of farming conditions, microbiota populations do not offer easy and general identification of markers at the genus or species level. Addressing this aim requires moving beyond broad taxonomic descriptions and focusing on the identification of a functional core microbiota. This approach, although still in an early but evolving stage, offers a promising way to disentangle functional redundancy and highlight ecologically relevant bacterial groups that act as stable WIs. Through meta-analysis and detailed insights, by defining the core as the most consistent, abundant, and functionally influential fraction of the community, researchers can establish a reliable baseline that captures both conserved microbial traits and species-specific markers. Such a framework not only facilitates the interpretation of dietary and environmental interventions but also enhances the predictive value of microbiota-based indicators for early detection of dysbiosis or compromised welfare in aquaculture systems [781].

While the particular molecular mechanisms and relationships of the microbiota-host interactions are not yet established, numerous studies have analysed how different microbial metabolites, especially the ones produced in the gut, can impact the host functions both locally and at a systemic level [765, 767]. For instance, microbial active molecules can impact upon metabolism, digestive processes, neurotransmitters such as serotonin and catecholamines such as dopamine which can influence gastrointestinal motility, as well as feeding behaviour [782–784]. It is worth mentioning that, through these biochemical routes, microbiota play an important role in different physiological dynamics that are currently considered and measured as WIs, such as changes in fish behaviour, interacting with the microbiota-brain axis and stress-related responses [785–787]. Regarding the latter, the climate change challenge currently faced by the aquaculture sector may be an important context. Heat stress, characterised by increasingly severe and frequent waves of increased water temperature, can affect the stability and homeostasis of the animals, prompting dysbiosis and favouring pathogen proliferation [748]. In this case, the identification of microbial marker species and changes in the metabolic function of the microbiota can be valuable methods to monitor the sensitivity of the microbial populations to heat stress [788]. Several authors indicated the ratio between *Firmicutes* and *Bacteroidetes* as potential bacterial markers of heat stress, demonstrating its direct correlation with high temperatures in broilers and swine [789–791]. Additionally, the same ratio was commonly used in human studies as an inflammatory and obesity indicator [776, 792, 793]. These results are particularly important as they suggest that different stressors can determine convergent physiological responses of the holobiont system. In fish, although the dedicated literature is limited, recent studies highlighted the same correlation. A study conducted during extreme heat stress episodes evidenced that, within the intestinal microbiota populations, it was possible to associate the phylum *Spirochaeta*, which was almost completely composed of the genus *Brevinema* (abundance > 90%), with the temperature stressor. This taxon is commonly present in tropical fish microbiota [794], however, it was shown to be a strong heat stress marker in gilthead seabream and to a slightly lesser extent in Chinook salmon (*Oncorhynchus tshawytscha*) [795, 796]. Other trials have demonstrated how low levels of dissolved O_2, due to high stocking density, can modify holobiont behaviour, defining two opposite profiles (proactive/reactive fish) as assessed by AEFishBIT data-loggers, which simultaneously monitor respiratory frequency and swimming activity [407]. These results were linked with a different abundance of *Alteromonas* and *Massilia* in seabream skin mucus, making them suitable microbial welfare markers for seabream [649, 797]. These important results gain even more resonance in light of the fact that the skin's microbiota can be considered a minimally-invasive outcome-based WI as it does not require euthanasia of the animal.

Microbiota markers may be more than simple indicators; they can be used to anticipate the development of negative events, as a basis to plan corrective interventions to mitigate an environmental issue, or to modulate the physiological state of the animals as needed. In this context, high stocking density and elevated temperature represent two types of stressors which are both capable of triggering the activation of hepatic lipogenesis, increasing lipid deposition in the liver. This apparently controversial physiological response is actually a measure aimed at counteracting the decreasing level of available oxygen, storing oxidizable energy molecules instead of catabolising them, and decreasing the general demand for oxygen at the mitochondrial and cellular levels. This evidence demonstrated that even in fish various stimuli can elicit convergent physiological responses, leading to shared potential solutions. Among many, those with promising expectations include the formulation of specific diets to mitigate or alleviate the negative impact of stressors [772, 796, 798]; mild-hypoxia preconditioning to promote better performance and enlarge the tolerant range of adverse conditions treatment [680, 767, 799]; Faecal microbiota transplantation (FMT), which represents a widespread method for human disease, but which is being applied with increasing frequency in animal production to reconstruct a functional and healthy microbiota environment, providing not only the microorganism fraction, but also additional components, such as proteins, bile acids, and vitamins, helpful for the full remodulation of the whole holobiont [800–804].

Apart from the use of microbiota as a tool for a functional evaluation of complex biological phenomena or welfare markers, it is worth mentioning that this field of investigation has an equally convoluted branch that concerns the technique to obtain the data. It is clear that a solid experimental design defines the guidelines that must be suited to the purpose and necessity. Among the numerous steps that compose the process, the sample collection and the choice of sequencing technique are the main sources of variability [805]. Additionally, microbiome abundances and functional structures can be negatively affected by physiological animal features, such as the metabolic activity of the fish, due to season, water temperature and long- or short-term fasting [475, 806–808]. In addition to the sampling timing and methods, the analytical technologies are key. Nowadays, the amplification and sequencing of 16S ribosomal RNA (16S rRNA) due to the development and availability of next-generation sequencing (NGS) technologies, is the most common method for analysing microbial communities [809]. Nevertheless, the selection of the exact sequencing platform is dependent upon several factors, such as the taxonomic depth, the accuracy, the quality of the data and affordability [810–813]. The two most common types of platforms use short-read and long-read sequencing. Both these approaches present benefits and drawbacks, including the choice of primers, which may represent sources of variability, and the quality and integrity of the DNA molecules, which are particularly important for the success of long-read sequencing [811, 814, 815]. Due to their high depth and coverage, in combination with a very low sequencing error rate, short-read platforms allow for the characterisation of bacterial community structure at high taxonomical levels, giving important information about low abundance operational taxonomic units (OTUs) or microbial populations characterised by numerous unknown species, absent in common reference databases [812]. On the other hand, although with an increased sequencing error rate, long-read platforms reach a higher confidence in the taxonomic and functional assignation at species-level resolution. This entails an overall more accurate estimation of the richness of the microbiome, due to the ability to distinguish rare or phylogenetically close taxa [816–818].

In summary, due to the variability and differences among farmed fish species, including the five considered in the present review, these challenges highlight the need to define ad hoc solutions for microbiota investigations which involve the definition of a strong dedicated healthy core microbiota, since techniques, environmental variables, experimental conditions and holobiont responses may change for each species and situation they are subjected to [819]. Within this framework, the association between the core microbiota and specific microbial biomarkers represents a key step in the use of the microbiota as a strategic resource for sustainable aquaculture development. Moreover, the possibility to integrate a multi-species perspective adds an additional dimension, as the investigation of convergent ecological pressures and habitat adaptations (e.g., freshwater and saltwater phenotype) allows the identification of conserved and effective microbial traits, common to multiple organisms, focusing more on their ecological roles, relationships, and functional contributions, rather than on taxonomic variability [820]. On the other hand, the results demonstrated the importance and versatility of the microbiome as a promising WI, since although its use is still in its early stages and requires further development, it can be considered a multi-purpose tool for comprehensive welfare documentation as both an input and outcome-based WI. Furthermore, its applications are diverse: it can serve as a diagnostic tool for helping evaluate welfare state, as a prognostic tool, acting as a sentinel to predict the onset of undesirable events, and as a flexible biological matrix, allowing for targeted interventions to modify its structure and function as needed.

6 | Case Study: Utilizing WIs in Deciding Humane End-Points

Ensuring the prevention of unnecessary suffering is fundamental when animals are used for scientific purposes, and current legislation reflects that. The Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 states, 'The methods selected should avoid, as far as possible, death as an end-point due to the severe suffering experienced during the period before death. Where possible, it should be substituted by more humane end-points using clinical signs that determine the impending death, thereby allowing the animal to be killed without any further suffering'. Further, the Directive specifies, 'where death as the end-point is unavoidable, the procedure shall be designed so as to: (a) result in the deaths of as few animals as possible; and (b) reduce the duration and intensity of suffering to the animal to the minimum possible and, as far as possible, ensure a painless death' [15]. The latter refers to the numerous established methods and standard protocols required by legislation in areas like food safety and environmental toxicity, where mortality is a key evaluation parameter. Defining more sensitive end-points that improve animal welfare during experiments is a declared goal [821] and WIs play a key role in reaching it.

Humane end-points (HEPs) are used to determine when to intervene in an experiment to prevent unnecessary or alleviate necessary suffering [822, 823]. A HEP is aimed at preventing, minimising or ending discomfort and is, therefore, a means to comply with the refinement aspect of the 3Rs. HEPs can mean the animal is removed from the experiment (and euthanised or treated for recovery), or that interventions are made to reduce suffering within the experiment (provided there is no interference with experiment integrity), see [824]. Ellis and Katsiadaki [823] collate eight end-points that a stakeholder can consider. These include Experimental end-points (EEPs) which differ from HEPs as they are the point where the experiment has achieved its objectives and run its planned duration [822] and early end-points including Aim end-points; Biological error end-points; Technical error end-points; Mortality end-points; Moribundity end-points; Prognostic humane end-points; Non-prognostic humane end-points, see Ellis and Katsiadaki [823] and references therein. Prognostic HEPs are where the animal is '… removed at a pre-defined state, based upon clinical signs, predictive of a more severe end-point used historically' and Non-prognostic HEPs are where the animal is '… removed at a pre-defined state based upon clinical signs to limit suffering' [823]. Williams and Baneux [825] have also suggested the term Humane Experimental End-point (HEEP), stating the 'Animal reaches the end of the study in a humane manner and scientific goals are met for this animal' meaning HEPs and EEPs are both met. They have also built upon the term 'Humane Intervention Point' (HIP), which introduces 'non-euthanasia options for intervention' while acknowledging that the developing end-point terminology addresses this as well [825].

Irrespective of the applied end-point concept, these points should be defined before the trial starts and reflect developments in state-of-the-art knowledge, such as the recent recommendations on how to refine the TG203, including the proposal of a roadmap for refinement [821]. A key criterion for the application of a HEP is a score sheet [826], where the clinical state of experimental animals is recorded at predefined intervals, providing a health and welfare overview of the animal during the experiment. The practice has been adapted for fish research and is an ideal tool for incorporating WIs into HEPs, see [827]. Particularly, outcome-based WIs for individuals are excellent candidates to be included in score sheets during research trials. These trials often allow for individual surveillance of fish (due to e.g., clear water, small fish numbers or frequent planned handlings) and, therefore, facilitate the use of these WIs as presented in this review. Conversely, in applied aquaculture research, where individual scoring may not be possible, the outcome-based WIs for fish groups and a semiquantitative version of the individual-based WIs (e.g., the estimation of a symptomatic percentage of the group) can be applied. Either way, the key is to adapt the scoring so that values outside the WI tolerance range indicate current or certain future welfare issues that are not in line with the experimental goal and should be alleviated. However, developing score sheets for aquaculture research is not a straightforward matter. The outcome of a workshop entitled 'Establishing score sheets and defining end-points in fish experiments' that was held in 2020 reported a lack of consensus on score sheet use and which WIs to include in one [827]. It is crucial that harmonised score sheets, that include WIs and their corresponding criteria are developed for fish species that are being used in research settings, see [13, 29]. In addition, staff should be trained to recognise HEP in terms of species and life stage [827].

7 | Collecting Input- and Outcome-Based Welfare Data in an Appropriate and Representative Way

To be able to effectively use WIs in experimental settings, indicators should be measured accurately and precisely and at a frequency that can document the fulfilment of welfare needs or any deviations from this fulfilment. In the latter case, documenting that fish have *not* been exposed to welfare risks may require a higher frequency of measurements in space and time, as short-term drops or local deviations are likely to be missed with fewer measurements [4] or masked by use of averages [828]. While such requirements cause the optimal welfare assessment protocol to be very specific for each experiment, there is a justified trend in the opposite direction, toward standardisation of assessments during experiments. Standardized sampling methods make comparisons within and between experiments and facilities easier and more reliable. These standard methods should be appropriate, easy to carry out, and secure samples that are as representative as possible, that is, they are realistic to perform with sufficient precision within the limitations given by spatial and temporal variation. Further, sample sizes and frequencies must be realistic in terms of time spent on sampling and the number of individuals negatively affected by the sampling procedure [488]. At the same time, standardization and harmonisation of data collection will increase the requirements for consistency between sample sets. These strategies may lead to improved open-access data sources and allow for better comparison between data sets, but the quality and usability of the collected data will entirely depend on the data being collected using the standardised procedures [828]. Overall, such standards could be a minimal basis during experiments, while project- or trial-specific WIs can be added beyond the standards to ensure optimal data collection and fish welfare. However, both new standards and additional indicators mean more resources and, therefore, up-scaling, high-throughput, automatisation and digitalisation become more and more relevant.

While manual measurements do offer utility in certain situations or procedures, developing and refining this process using existing and emerging technologies should be a key objective of any WI documentation framework. However, this approach is not always simple and requires some guidelines to clarify if, when and how best to refine the documentation framework in relation to the latest technological developments in this field, irrespective of whether the framework is adapted by a consortium for legislation or by researchers for their experiment. Digitalisation offers many opportunities in this refinement process and has been the subject of many review articles over recent years, [829–834]. In particular, digital monitoring offers opportunities to take into account any indicator-specific aspects during the evaluation and development of the chosen technology. However, this simultaneously challenges developers and users to consider the indicator's peculiarities such as ranges or thresholds. It is not the aim of this paper to revisit or update some of the digital approaches, benefits or pitfalls outlined by other articles, but we hope to give the reader a brief introduction to the subject area, highlighting how digitalisation can be used to refine the monitoring of fish welfare in experimental settings.

7.1 | Sampling Input-Based Welfare Data

7.1.1 | Location and Frequency of Measuring Input-Based WIs

Many input-based WIs are water quality aspects, and while this clarifies most measuring technologies, it leaves the question of where and when to measure. As far as the authors are aware, there are no clear guidelines on where and how to document input-based WIs in aquaculture research according to the EU Directive 2010/63/EU [15]. However, its amendment, the Commission Delegated Directive (EU) 2024/1262 of 13 March 2024 states 'Water quality shall be monitored using a defined testing schedule at a sufficient frequency to detect changes in these critical parameters and action shall be taken to mitigate such changes' [16]. As those aspects of the defined WIs are key for measuring and documenting welfare in different rearing systems, we review a few characteristics of tanks, net pens and ponds.

In a tank environment, all water in the tank usually arrives from the same inflow(s), and physical properties that are not affected by the fish, like temperature and salinity, are mostly the same throughout the whole water volume, although they may vary with time. In contrast, properties that can be affected by the fish, in particular, oxygen but also metabolites like CO_2, may spatially vary in relation to fish distribution and water current conditions and are more frequently variable in time due to fish activity and metabolism, caused by, for example, feeding or stressors [276, 835]. Monitoring tank effluent water is a standardized way to measure water quality, and also ensures that measurements are made after the water has passed through and been affected by the fish. The Norwegian Standard 9417 'Salmon and Rainbow Trout – Terminology and Methods for Documentation of Production' [491] states that measurements in the effluent water should be done 5 cm outside the drain, while the point of measurements done inside the tank should be 1/3 into the tank at mid-depth. We propose that *as a minimum requirement*, measurements in scientific tank studies are done in the effluent water, that is, after the water has passed the fish. However, horizontal and vertical profiling might be needed for some water quality parameters that are not uniform throughout the entire volume or are related to system placement and the surrounding environment of the research tanks or net pens. Such profiling of water velocity, dissolved oxygen or CO_2 levels during different seasons or at different inlet pipe settings might be useful to reduce or account for variability in research data [344, 345].

When net pens are employed as the experimental rearing unit, water is supplied from natural currents and the physical properties of the water vary with depth and time, especially in stratified environments [84]. In addition to the natural levels in the inflowing water, oxygen is affected by, for example, current velocity, tides, local stocking density, planktonic activity and the biomass the water has passed through, as well as the fish's metabolic rate. Further, rapid local changes in oxygen levels can occur within a net pen, including variations across the horizontal plane [836–840]. It is important to consider where the sensors are placed within the farm environment in order to capture the circumstances that the fish are exposed to [839]. Environmental data in experimental net pens should ideally be measured at high frequency across the whole cage volume, but this is often not possible. As a minimum, measurements should be carried out daily and cover the main depth interval, for instance as a vertical profile, and to be measured at times where there is an expected minimum, that is, at the highest fish density and when the current speed is at its lowest [84, 488]. For salmonids, the NS 9417 [491] farming standard states that measures should be done at 3, 5, and 15 m and at the maximum depth of the cage. We propose to measure at least this detailed in scientific studies in marine net pens, and where feasible and possible, extended monitoring capabilities if it is relevant to the research question and the situation allows it.

Common carp farming mostly utilizes extensive or semi-intensive earthen ponds, providing unique challenges to measuring water quality for research purposes. For example, major sources of dissolved oxygen in ponds are either natural production from algae, leading to a circadian rhythm with the lowest oxygen concentrations before sunrise, or artificial enrichment through aeration equipment, causing a spatial gradient with the highest oxygen concentrations closest to the equipment [841, 842]. Additionally, organic buildup at the bottom of the pond may cause anaerobic zones due to decomposition leading to steeply decreasing oxygen concentrations in deeper areas [841]. These aspects of temporal and spatial oxygen gradients will be more pronounced the smaller the flow-through is, that is, the amount of fresh water per pond, and further depend on the size and shape of the pond [843]. The latter will further impact how WIs such as water temperature and light conditions fluctuate, with larger and deeper ponds showing less fluctuation potentially requiring less frequent measurements. Furthermore, indicators like nitrogen levels and pH will depend on feeding schedules and stocking densities influencing when and how much ammonium and carbon dioxide the bacteria and algae can convert to nitrate and oxygen [844]. It is therefore advisable to define the time and location of water quality measurement according to the specifics of each pond as well as the research question and, if needed, to test for temporal or spatial maxima, minima or gradients before a trial.

7.1.2 | Digitalising the Measurement of Input-Based WIs

With regard to input-based WIs, many water quality parameters can be measured and processed automatically, but this is not the case for all indicators or all experimental facilities. Technological monitoring of input-based WIs can provide information about immediate changes in water quality parameters and can be actively used for welfare documentation and ensuring the objectives of EU Directive 2010/63/EU and its amendment Commission Delegated Directive (EU) 2024/1262 are met in all experimental settings. It is also important to note that some parameters are easier and more reliable to measure than others, but there are considerable investments in research and innovation, particularly in the salmon sector, to improve sensor technology to widen the scope and ease of measurement of the indicators, and this offers opportunities for aquaculture research. Improvements in the design and utility of measuring probes and signal transmission enable continuous measurement of basic water parameters and their logging or processing in real-time. However, problems can still occur, and there was an incident in Scotland where a subsea cable was damaged which led to a temporary loss in communications at aquaculture facilities, see [845]. There are various technologies that are available or emerging for monitoring input-based WIs at various points within and around a rearing system and also in areas in and around where the fish are. Sensors can be static and measure water quality at a single fixed point, or multiple fixed sensors can be placed at various horizontal and vertical locations to provide stakeholders with a broader picture of water quality within the extended rearing system. Several advanced measurement devices are also available that offer mobile water quality monitoring. These can be tools for the horizontal and vertical profiling of water quality parameters, for example, the WelfareMeter, outlined in [846]. Existing mobile camera-based technology for monitoring feeding can also have water quality sensors integrated into their hardware to help stakeholders gain an oversight of water quality parameters as these cameras are moved around the fish for feed monitoring purposes. Remotely operated vehicles (ROVs), Autonomous Surface Vehicles (ASVs) and Autonomous Underwater Vehicles (AUVs) can also be tasked to monitor water quality in and around a rearing system, see [847–849]. Small, wireless sensor arrays can also be used for documenting water quality during key operational and research routines that the fish are subjected to during fish transfer and crowding. The research animals themselves can also be used as sentinels for monitoring water quality if they are fitted with appropriate and relevant internal and external sensors, for example, electronic tags [850]. Earth observation (EO) and remote sensing can be used to monitor some water quality variables via satellites, planes, and aerial drones. EO is advantageous as it can be used in areas that might be difficult to access and can also cover relatively large areas, and as technology has improved, many aspects of EO have become capable of near real-time monitoring. For example, satellite data from the Copernicus Sentinel mission are freely available and have a spatial resolution of 10–60 m and a five-day revisit time [851]. Several parameters (such as sea surface temperature, chlorophyll A concentrations and harmful algal blooms) can be estimated using different satellite sensors (visible range, near-infrared range [852]) and these parameters can have high utility for marine-based open cage research infrastructures. However, for many important input indicators, EO is not a direct replacement for in situ water quality monitoring; for example satellites are unable to give a vertical profile of the water column, and there can be challenges with spatial resolution not being fine enough to capture the farm conditions. An emerging indicator of interest, both in experimental and applied studies is noise, and this is also addressed in Annex III of the Directive 2010/63/EU and its amendment by Commission Delegated Directive (EU) 2024/1262 [15, 16]. Passive acoustic monitoring (PAM) is an established method to record underwater sounds in natural environments [853]. In contrast to active acoustics, such as tags and echo sounders, PAM does not rely on sending out a signal and then receiving it. Instead, all sounds that are present are passively recorded. PAM could be utilized in aquaculture research to monitor background noise to make sure the fish are not subjected to unnecessarily high sound levels (an input-based WI) and to monitor sounds produced either actively or passively by the fish (an outcome-based WI). With regard to using PAM to monitor outcome-based WIs, the potential for monitoring feeding and feeding-related behaviour via PAM has received the most attention [854–856] and has been mentioned in recent reviews focusing on fish feeding behaviour and feeding control methods [831, 857]. Feeding systems that utilize PAM have been studied for turbot *Scophthalmus maximus* [858] and currently exist for shrimp [859], while emerging machine learning approaches are being tested for other species of fish [860, 861]. PAM is also showing promising results within stress monitoring, where an experiment involving the crowding of a mesoscale cage sparked a significant increase in a specific acoustic signature that was similar to known involuntary sounds produced by Atlantic salmon [862]. PAM is non-invasive, could provide real-time monitoring, and is not dependent upon visibility. However, sounds produced by fish are often species-specific and could easily be affected by noise [854], and their propagation will be affected by the environment [863], which could make it difficult to correctly infer behaviour from PAM.

7.2 | Sampling Outcome-Based Welfare Data

For the outcome-based WIs the where and when to sample are equally relevant as for the input-based ones, and the additional question of how many and which fish are subsampled adds further complexity in all phases of an experiment—the design, the execution and the analysis. During the design phase animal and sample numbers are key and should be estimated using power analysis frameworks [864]. These allow researchers to anticipate the costs associated with sampling and analysis, to comply with the 'Reduce' principle of the 3Rs and to ensure robust statistical results by accounting for anticipated mortality and dropout rates due to humane end-points. To maximise its effectiveness, a conservative power analysis incorporating as much reliable information as possible about treatment effects and data variability—which may be specific to different WIs—should be conducted during the design phase. Similarly, sample numbers during the execution phase matter, particularly as manual sampling of fish for the scoring of outcome-based indicators involves capturing and handling the fish either in or out of water, including the application of anaesthesia, which is laborious and can be detrimental to their welfare [631]. The EU Directive 2010/63/EU states that the handling of fish in experiments should be kept to a minimum [15], which may potentially restrict sample size and the frequency of sampling events. However, these considerations on the execution level can lead to issues during the analysis, as lower sampling numbers may lead to high uncertainty of the proportion of a given indicator score in the population. For instance, in a sample size of 20 fish, each individual constitutes 5% of the sample, and infrequent deviations easily become under- or overestimated. This leads to the risk of imprecise estimates of the welfare state due to low sample size and chance effects. One solution can be indicator-adapted sampling: using fewer but broader indicator categories during routine evaluations may be beneficial for both observation frequency and time spent per fish. Indicators that have high or rising frequencies may then be focused on for more detailed investigations, while indicators of less concern are not, as suggested by [8, 488].

Equally relevant to how many fish are sampled is which fish are subsampled from the experimental population. Sampling can easily become biased due to individual characteristics of the fish or the fish group that can influence the likelihood of them being caught. Firstly, individual variation in spatial distribution is common [85], and can be affected by a number of variables such as hunger level, environmental preferences, territorial behaviour, parasite load, health status, sexual maturation level and size [4, 289, 865–867]. For instance, larger pen-held salmon have been found to spend more time in deeper waters than smaller conspecifics [868] leading to a greater mean weight of individuals that position themselves further down in the net pen [865]. Secondly, small or weak individuals may isolate themselves from the rest of the fish group or be less likely to escape during a sampling attempt, especially if they lack the ability to respond, for example, loser fish [869, 870] or are visually impaired. An example of this challenge was observed in a tank experiment on Atlantic salmon, where 378 fish were sampled and examined for a range of WIs including eye injuries (data from [482]). In the study, 28% of the first 50 fish sampled had severe eye injuries, where fish were classified as being blind in at least one eye, while the true proportion in the entire tank group was 8.2% (data from [482]). A common method to reduce biased sampling is crowding parts of, or the whole fish group to reduce their ability to flee [871]. Crowding is however stressful, and the physical contact between fish, the crowder or the rearing system may lead to physical injuries [4, 571, 872, 873]. Furthermore, sampling may still be biased even if a stakeholder crowds the entire population, in both tanks as well as in net pens [874]. Therefore, the choice of sampling method and number of individuals sampled must depend on the type of experiment and data collected and be based upon, among others, group size, the rearing unit size, the level of stress the fish will be subjected to, requirements for precision of the sample estimate, and so forth. Avoiding sampling bias is difficult, and its potential impacts should always be considered in all experimental phases—design, execution and analysis. Further considerations should also be paid to the choice of sampling equipment. For example, if a dip net is used to take the fish out of the rearing unit, it may induce further stress and fin and skin injuries that may, in addition to the welfare load on the affected individuals, influence the results of indicator scoring [631]. Dip nets made of appropriate netting material like rubber coating [875] and with fine mesh [876] are less likely to injure the fish. Using dip nets that have the least possible welfare impact and ensuring that the operator captures a low number/density of fish in the dip net has been recommended when sampling for welfare evaluation [488].

With regard to the monitoring of outcome-based WIs, existing and emerging technologies hold substantial potential for advancing the assessment of many indicators of interest for aquaculture research. When considering group-level outcome-based indicators, camera-based technologies can be used to monitor behaviour [877, 878], appetite [879, 880] and mortalities [881–883] in real-time. Further, costs for acquiring and deploying the technology are decreasing, in tandem with improvements in video quality and processing capabilities [834, 857, 877]. This data can then be analysed using machine learning and artificial intelligence [832, 884], streamlining and optimising the analytical processes to offer real- or close to real-time data on both the status of the indicator and any potential changes in it. Camera-based technologies, however, may potentially suffer from many of the sampling biases experienced with manual sampling and should be considered a valuable contribution to, but not a replacement for, a comprehensive welfare monitoring system.

7.3 | Examples of Technologies and Approaches for Documenting Fish Behaviour, Physiology, Injury Status and the Microbiome

When one considers fish behaviour, it can be documented at either the individual or group level, using camera-based technologies, passive or active acoustic technologies or tag-based technologies [85, 885–890]. If the whole group of fish cannot be monitored due to limitations in the field of view (camera and active acoustics) or due to challenges in tagging all the fish, the data from the fish that are sampled can be used to infer the welfare state of the rest of the group. Below we elaborate on a few concrete examples of using different technologies to assess behavioural WIs.

Fish behaviour can be audited using existing camera infrastructure such as those used for monitoring feeding in commercial aquaculture facilities [857, 877, 890, 891], or research-specific cameras can be deployed [857, 892]. For example, camera technologies that utilize the visible spectrum can be used in tanks [866, 893] and net pens [894–896], and 3D cameras utilizing the stereo vision principle are increasingly used for 3D fish position monitoring in tanks [897] and net pens [898]. Meanwhile, omnidirectional cameras can be combined to gather more information, although computational complexity is increased [848]. Such camera-based technologies offer many advantages, such as providing low-cost data and allowing simple manual monitoring of the fish. However, numerous cameras are dependent upon good water visibility in terms of light penetration, which can limit their utility at night (if the nocturnal period is not illuminated) [899] or in turbid production systems such as RAS [900]. Here, near-infrared light can be used to extend the monitoring period outside daylight hours, but some fish species are sensitive to infra-red spectra [404] and certain infrared wavelengths are more quickly absorbed in water than most visible light [901]. With all camera technologies, the field of view can be limited particularly if there are high numbers of fish in the rearing system. Additionally, manually analysing camera footage can be laborious and time-consuming [848, 902].

Echosounders are a completely non-invasive technology that allows for online (real-time) data measurement. They actively emit acoustic signals and then listen to the echoes produced by objects in their beam. In the simplest case, the location of an object is based on the round-trip time of the echo, and thanks to the Doppler effect, the object's radial velocity—the component of movement along the beam—induces a measurable shift in the echo's frequency. The echogram hence contains information on the densities of objects and their velocities. However, echoes from different objects tend to interfere with each other and this complicates the interpretation of these echograms. Some echosounders (multi-beam) can discriminate between directions within the beam, so each echogram is a cross-sectional image, not a one-dimensional profile. This may help ‘resolve’ different objects and hence reduce the interference between objects.

Further, sonar technology can provide information on, for example, circadian horizontal and vertical fish distribution, helping decipher the influence of abiotic factors such as light, waves and water current on fish swimming preferences within the rearing system [885]. Additionally, echo strength can be used to evaluate the swim bladder fullness in physostomous fish [903]. The application of echosounders in aquaculture settings presents unique challenges [904]. Firstly, the quality of the determined parameters can be influenced by the fish distribution in the sea cage because the high biomass blocks/shadows the signal [905]. Secondly, the reverberation of acoustical signals from the cage boundaries must be taken into account. Addressing the issue of removing the cage signal from the received echoes is a crucial consideration. Lastly, ensuring the stability of the transducer is essential, as transducer orientation influences the signal quality.

Biosensors, such as tags have been designed for monitoring the physiological parameters of fish. Electronic tags are miniature devices containing sensors that may be implanted into or attached to individual fish to measure physiological, behavioural or environmental parameters [906]. They can deliver systematic and detailed data on the welfare state of individual fish, and these fish can then be used as sentinels to infer the welfare state of the rest of the group [907–909]. A comprehensive overview of different biosensors eligible for electronic tags is available in [906, 908]. The most relevant sensors used in electronic tags for fish welfare documentation may broadly be divided into two groups: biosensors designed for monitoring the physiological parameters of fish, and biosensors exploiting the molecular elements of biological processes.

In the first category, electrocardiogram (ECG) based heart rate sensors have become increasingly popular as a research tool due to their commercial availability and ease of use. The most common tool using this sensor is the DST milli-HRT from STAR-ODDI which has been tested in several studies on Atlantic salmon and rainbow trout [910–913]. Some studies have used such devices for several weeks to gauge the long-term variations in heart rate during production [909]. While the aim is to use heart rate as a proxy for the stress level of monitored animals, further work may be needed to confirm this correlation and define the factors that may influence it [914]. In addition, the potential of using electrocardiography in fish remains largely untapped. Comprehensive ECG analysis—studying, for example, normal and abnormal waves, rhythms, intervals, and so forth—is often neglected when employing ECG measurements in fish and can be used as a powerful tool for diagnostic decision-making in aquaculture and aquaculture research [915].

The second category of sensors observes biological processes via enzyme and antibody levels, to identify target substances [908]. Some sensors measure glucose levels in fish blood, which will typically rise in response to stressful events and can remain high for more than 24 h [677]. Wu et al. [916] created a device containing such sensors transmitting real-time data. This 1.5 $\times$1.5 $\times$0.6 cm biosensor (3 g without battery) was implanted in the interstitial glass of the eyeball because the glucose levels at this site are highly correlated with blood glucose. Relatedly, AEFishBIT is an example of a device that contains elements from both physiological and behavioural biosensors. This miniaturised device is a tri-axial accelerometer that is externally tagged to the fish operculum. It provides proxy measurements of physical activity (using the x and y axes of the accelerometer) and ventilatory frequency (an indirect indicator of basal metabolism [407]) through opercular movements measured along the z axis [917]. AEFishBIT has been validated by means of exercise tests in swim tunnel respirometers, video recording and differential operculum and body tail movements across fish species with different swimming capabilities [917–919]. The suitability of this device for the welfare monitoring of free-swimming fish has been shown with gilthead seabream under numerous biotic and abiotic stressors in tanks [920]. AEFishBIT has also been employed to assess behavioural habituation to repeated stressors in gilthead seabream reared at high stocking densities. In this context, fish exposed to confinement stress exhibited faster recovery times in physical activity and respiration after repeated exposures, indicating a degree of habituation [921]. Further, it has been successfully tested in Atlantic salmon, rainbow trout, gilthead seabream and European seabass, though the tagging procedure must be adapted to each species depending on the ossification level of the operculum. This is due to the operculum being more fragile in salmonids than in other species, but it is considered minimally invasive as fish recover their basal activity and behaviour in a range of 1.5 (gilthead seabream) to 8 h (Atlantic salmon) after tagging [407]. When these factors are taken into consideration, this device can be considered a reliable tool for accurate behavioural profiling and welfare monitoring of farmed fish at a laboratory scale. However, tag use can negatively impact upon the fish both acutely and chronically, which can be either due to the procedures needed to deploy the tag (capturing, handling, anaesthetising and subjecting the fish to surgical procedures) or the size and placement of the tag, that may place additional burdens upon the fish, see [922, 923]. Attaching electronic tags to the fish usually involves (i) the surgical implantation of the tag into the body cavity, (ii) gastric insertion of the tag via the mouth or (iii) external attachment [407]. Internal implantation by surgery usually requires a higher level of expertise than what is needed for the other attachment procedures, and 3–10 days or even longer, are required for recovery from surgery and stabilisation of the basal levels of stress indicators and other monitored parameters [923–925]. This recovery time also depends on the welfare state of the fish, as chronic stress negatively impacts wound healing in internally tagged fish [926]. It is generally accepted that the ratio of tag to fish body mass should be lower than 2%, see Jepsen et al. [927] and references therein, to prevent detrimental effects of the tags on fish welfare and performance, but this practical rule is not applicable in all cases. For instance, Toomey et al. [928] reported that surgically implanting an accelerometer tag (2.9 g in water) in rainbow trout of ca. 260 g led to a significant reduction of specific growth rate (SGR) even 8 weeks after recovery. This case could be species-specific, as an SGR reduction was not observed in 320 g gilthead seabream or European seabass implanted with a similar device [929]. Gastric insertion of a tag may be potentially less invasive, though it is mainly limited to when fish are undergoing fasting [407]. Conversely, external tagging can be convenient for rapid application in fish that have a body shape that is not appropriate for surgical implantation [407], but some potential issues depending on the attachment procedure can lead to tissue damage, risk of tag loss or reduced swimming capacity [927]. In recent times, experimental trials have explored the potential of using devices from human medicine for pulse oximetry in salmon

[930], however, these devices are still in early developmental stages.

Another approach which can be used is the fish microbiome. The change in microbial populations in relation to differing stimuli represents a hybrid WI and one can find several communities of microorganisms strictly associated with different districts of the host and environment, which when considered altogether, define the holobiont system. Microbial communities in the water can be investigated as an input-based WI, defining, from a biological perspective, the quality of the aquatic medium used for fish rearing, which is particularly important for RAS systems. The skin and intestinal microbiome, in addition to having possible direct and indirect relationships with the environment, have an important influence on physiological features of the holobiont and offer utility as an outcome-based WI. These connections and causal dependencies do not only have an individual focus on the animal, as previously discussed [766–768, 772], they can also influence group level indicators, such as behaviour [408, 813]. Therefore, the identification and use of functional microbial clusters and specific taxa within the microbiome community structure represent a multi-level welfare audit, as it allows a diagnostic and prognostic evaluation of a phenomenon, as well as a possible route of intervention to modulate the holobiont, considering the experimental and farming needs.

Finally, as stated at the start of this section, injury-based indicators are traditionally evaluated via manual handling, anaesthetising and physically examining fish, and can be undertaken both in or out of water. If the assessment is conducted out of water, potential air exposure times (and air temperatures) must also be considered [4]. Manual scoring can also be done on high-quality underwater images to avoid negative interactions with the fish and make scoring at different depths possible [931]. Beyond that, there are a number of technological solutions available at the research scale that can be used to document, for example deformities [932] or diseases [933, 934] using various advanced systems. However, these have not yet been commercially developed to assess injury levels in fish. Further, other camera-based technologies can be introduced to the rearing unit to document different injury types [935], and severities in real-time and these are being increasingly developed by various commercial companies for deployment in aquaculture. Furthermore, hyperspectral imaging can be used as part of a manual handling assessment in order to streamline and introduce more precision and accuracy to the injury monitoring process [936]. It combines digital imaging with spectroscopy to capture detailed spectral information across a wide range of wavelengths, allowing for the quantification of the chemical compositions of materials through the analysis of light interactions in the visible and near-infrared spectrum (400–2500 nm). This technology can be employed to monitor parameters such as fin erosion or haemorrhaging [936] and, in comparison with the standard imaging cameras, it can obtain much more information about the appearance of the fish. Additionally, Pettersen et al. [937] demonstrated that underwater hyperspectral imaging can efficiently detect and distinguish between different stages of sea lice on Atlantic Salmon in laboratory conditions. The possibilities of hyperspectral imaging for aquaculture were further validated by Svendsen et al. [938], which showed its effectiveness in determining the smoltification status of juvenile Atlantic salmon, potentially reducing risks associated with transferring poorly smolted fish to the sea. Therefore, hyperspectral imaging is a powerful technology that, like in terrestrial livestock, is expected to have increased utility for welfare documentation in the future. In addition, a recent study by Juhasz-Dora et al. [939] demonstrated that hyperspectral imaging can non-invasively analyse biofluorescence in lumpfish to detect stress, suggesting its potential for measuring and monitoring welfare. Further work is required, however, to determine which welfare traits are best diagnosed with these imaging systems, and how to develop standards of use such that results can be compared between studies and fish populations.

7.3.1 | Streamlining Data Collection and Processing Using AI and Machine Learning

The utilization of artificial intelligence (AI) in monitoring input- and outcome-based OWIs holds immense potential for the monitoring and documentation of animal health and welfare in aquaculture and aquaculture research. For example, AI-driven computer vision systems can process and analyse high-resolution images or videos of fish, extracting valuable insights into the presence and severity of different types of injuries [935], swimming behaviour [895, 896, 940, 941], ventilation rate [942] or the number of emaciated loser fish with poor health within a population [870]. This approach allows for continuous, non-invasive monitoring of key WIs, offering a more comprehensive understanding of their dynamics in relation to fish welfare. It also circumvents the time-consuming need for manual extraction of the information from the footage [870, 893]. Furthermore, AI algorithms can provide real-time alerts with regard to potential welfare risks, enabling prompt interventions and the application of mitigating actions, if these are available. However, the application of AI and machine learning does not come without its challenges. For example, solutions are often designed for specific conditions and specific fish species and can sometimes be difficult to adapt to other species and conditions. However, this can be overcome via recent AI developments (e.g., via the use of convolutional neural networks, CNN) that can easily be adapted to new arenas as they do not require deep programming knowledge, and developers can re-train the CNN by manually annotating data. However, in all approaches, enough data needs to be collected to adequately train the AI models.

In addition to its use with OWIs, AI-based histopathological systems are being developed to improve the comparison and quantification of LABWIs, such as tissue structure or development and responses in different tissues. One model that has already been published uses the Aiforia platform to evaluate the skin of the Atlantic salmon [943]. The evaluation of skin development throughout a commercial production cycle, following major farming operations, for example, delousing, showed skin tissue changes in relation to how long the fish had been in the sea and also around differing farming operations [943]. This work illustrated how AI-based histopathology and automated tissue analyses can improve our understanding of Atlantic salmon skin health and physiology and how it relates to life in intensive production systems. However, AI-based histology results must be handled with care and precaution since results may be misinterpreted and biased by poor samples. Similarly, numerous approaches have been employed to model and characterise microbiota communities. Among many, SAMBA is an aquaculture-dedicated machine learning tool that aims to identify causal relationships between operational taxonomic units (OTUs) and biotic and abiotic variables, such as environmental factors, experimental conditions or host genetic background, by means of a Bayesian network [944]. The advantage of this bioinformatic platform is based on the identification of strictly correlated bacterial clusters, which can be used as substrates for functional enrichment. The use of this information, together with the combination of multi-omics data layers and the prediction power of the membrane computing algorithm, constitutes the base for the better comprehension of the cross-talk between microbes and host, and the functional plasticity of the holobiont as the environment changes [781].

Despite the advancements in technology, many methods for measuring input-based and outcome-based WIs in aquaculture are still used in isolation. This fragmented approach limits the potential for comprehensive monitoring and documentation of fish welfare. There is a significant gap in developing integrated tools that can replace manual sampling methods with advanced sensors, in addition to artificial intelligence (AI) and machine learning (ML). By leveraging these technologies, we can achieve more accurate and real-time data collection, and we need catalysts for synergizing research efforts toward this goal. A unified approach would not only enhance the precision of welfare documentation but also streamline the process, making it more efficient.

8 | Summary and Conclusions

The utilization of fish in aquaculture research across Europe is protected under Directive 2010/63/EU and its amendment Commission Delegated Directive (EU) 2024/1262 [15, 16]. Fish can be subjected to procedures or experimental objectives that are stressful and can lead to varying levels of suffering; WIs can be used to help quantify the effects of this exposure in addition to help protect and optimise welfare where it is possible. An aim of this article has been to collate and propose a WI toolbox that presents the stakeholder with an updated state of the art regarding the measurement, monitoring, auditing, assessment and safeguarding of fish welfare when fish are used for scientific purposes [15], for the top five European aquaculture fish species in terms of production volume: Atlantic salmon, rainbow trout, European seabass, gilthead seabream and common carp [31]. The indicator toolbox is summarised in Table 7 at the end of this section.

Directive 2010/63/EU (and its amendment Commission Delegated Directive (EU) 2024/1262) has an expansive structure for addressing the welfare of various animal species used in scientific procedures, considered under a detailed list of recitals, articles and annexes [15, 16]. The needs of fish are specifically addressed under Annex III of the Directive, and this section provides information on a broad range of mostly input-based WIs. However, specific details regarding the application of these indicators are somewhat lacking, especially with regard to the fulfilment of some species- and life-stage-specific welfare needs. Outcome- (animal-) based WIs are only briefly addressed in general terms regarding the behaviour of fish in experimental settings. In this current review we have extended the range and scope of indicators that can be considered when documenting the welfare of fish used in scientific procedures and also outlined cases of how to measure these indicators in both small- and large-scale experimental settings.

While our proposed toolbox is harmonised in terms of its framework and function, it also provides information that allows stakeholders to consider species- and life-stage-specific welfare needs. While it is comprehensive, it is not all-encompassing. There are a large number of indicators that are not considered in this review, such as various input-based indicators including hydrogen sulphide, various metals and wave action, in addition to numerous outcome-based indicators related to the condition of an extended range of internal organs such as the spleen or kidney. Although these indicators have marked utility in assessing fish health and welfare, information on them is either limited or addressed elsewhere.

We also consider our proposed toolbox as a way to introduce relevant stakeholders to a range of possible indicators that have utility in an extended welfare monitoring and documentation programme for the fish. Nevertheless, when tools are selected from toolboxes they must always be adapted and appropriate to the objectives of each scientific procedure, rearing system and husbandry practice the fish are subjected to. However, as a precautionary measure, if little is known about the effect of a procedure or practice upon a particular species or life stage, or if it is entirely novel, it would be prudent to undertake a comprehensive investigation of potential welfare risks. Such an evaluation not only safeguards the animals involved but also provides clear and transparent information on the potential effects of adopting and implementing the practice in the wider aquaculture industry. This requirement is as equally appropriate in aquaculture research as it is in aquaculture, where an increasing number of WI toolboxes are being developed and refined for differing species and also production systems and operations [4, 34, 56, 331, 333, 334]. The updated state-of-the-art outlined in this review can also be used as a further channel for promoting, revising and refining the use of WIs in the wider aquaculture industry and its associated stakeholders. For example, the extended section on the challenges of applying thresholds in differing research and farming situations can help stakeholders formulate and update their own assessment protocols and monitoring frameworks accordingly, underlining the unequivocal need to consider numerous input-based WIs in tandem with others.

WIs are central to the delivery of refinement goals of the 3Rs [945], and have utility in the 3S and harm-benefit analyses; see earlier sections of this review. Further, WIs can help define the severity of scientific procedures [13, 25] and also identify where the severity of procedures can be minimised or reduced [946]. Additionally, they are central to the application of humane end-points and humane intervention points in scientific procedures involving fish; see [823, 825, 827]. For applying humane end-points in fish research it is key that harmonised score sheets or auditing frameworks that include various types of WIs are developed, [29]. In addition to this, appropriate staff training to aid the recognition of humane end-points for a given species and life stage is needed [827]. These benefits do not only have an ethical

TABLE 7 | Summarising the welfare indicators covered in our proposed WI toolboxes, broken down into Input-based and Outcome-based indicators (at either the individual or group level).

Indicator type	Welfare indicator	Addressed in the directive and its amendment	Species specific information addressed in this article?				
			S. salar	*O. mykiss*	*D. labrax*	*S. aurata*	Common carp
Input-based	Temperature	Yes	Y	Y	Y	Y	Y
	Oxygen	Yes	Y	Y	Y	Y	Y
	Nitrogen compounds (ammonia, nitrate, nitrite)	Yes	Y	Y	Y	Y	Y
	pH	Yes	Y	Y	Y	Y	Y
	CO_2	Yes	Y	Y	Y	Y	Y
	Salinity	Yes	Y	Y	Y	Y	Y
	Lighting	Yes	Y	Y	Y	Y	Y
	Noise	Yes	N	N	N	N	N
	Stocking density	Yes	Addressed in other articles aside from this one				
	Water volume	Yes	Y	Y	Y	Y	Y
	Water velocity	Yes	Y	Y	Y	Y	Y
	Water exchange rate	Yes	Y	Y	Y	N	N
	Turbidity	No	N	N	N	N	N
	Total suspended solids	No	N	Y	N	Y	N
Outcome group-based	Behaviour	Yes	Applicable to all species, see Table 2 for ethogram in Section 4.1				
	Hunger and appetite	No	Indicator applicable to all species, see Section 4.2				
	Scales or blood in the water	No	Indicator applicable to all species, see Section 4.3				
	Health	No	Indicator applicable to all species, see Section 4.4				
	Mortality and survival	No	Indicator applicable to all species, see Section 4.5				

(Continues)

TABLE 7 | (Continued)

Indicator type	Welfare indicator	Addressed in the directive and its amendment	Species specific information addressed in this article?				
			S. salar	*O. mykiss*	*D. labrax*	*S. aurata*	Common carp
Outcome individual-based	Gill status	No	Indicator applicable to all species, see Section 5.2				
	Opercular deformities	No	Indicator applicable to all species, see Section 5.3				
	Vertebral deformities	No	Indicator applicable to all species, see Section 5.4				
	Skin damage	No	Indicator applicable to all species, see Section 5.5				
	Fin damage	No	Indicator applicable to all species, see Section 5.6				
	Snout/jaw damage	No	Indicator applicable to all species, see Section 5.7				
	Eye damage	No	Indicator applicable to all species, see Section 5.8				
	Condition factor	No	Y	Y	Y	Y	N
	Maturation state	No	Indicator applicable to all species, see Section 5.10				
	Integrative stress responses	No	Indicator applicable to all species, see Section 5.11				
	Internal organs	No	Indicator applicable to all species, see tables in Section 5.12				
	Faecal consistency	No	Indicator applicable to all species, see Section 5.13				
	Microbiome	No	Indicator applicable to all species, see Section 5.14				

Note: Some group-based indicators can be utilized at the individual level, for example, appetite, behaviour or health. Y = species-specific information on the indicator is addressed in our article; *N* = species-specific information on the indicator is not addressed in our article.

and legislator perspective, but it is in the interests of conducting better science, as discomfort in an animal may give unreliable results. Lastly, improving the welfare of animals used for scientific purposes is central to the 'Culture of Care' concept that, for example, 'provides support to all staff to strive for continuous improvement in: animal care and welfare; care and welfare of staff involved in the animal care and use programme; scientific quality; and openness and transparency' [947]. While this is not directly addressed in Directive 2010/63/EU per se, it is referred to in other guidance materials [946, 947].

A better overview of the WI toolboxes that are available and emerging for a range of top European farmed fish species also has utility beyond delivering the objectives of European Directive 2010/63/EU in aquaculture research. Other European Directives, such as those addressing food chain security and safety, which are closely linked to the welfare of animals in food production because of the connections between animal health, welfare and food-borne diseases (see e.g., https://www.efsa.europa.eu/en/topics/topic/animal-welfare), can also benefit from improved animal welfare via better welfare documentation. These include Regulation (EU) 2017/625 of the European Parliament and of the Council of 15 March 2017, which addresses official controls and other official activities performed to ensure the application of laws concerning food and feed, animal health and welfare, plant health and plant protection products [948]. Pavlidis et al. [949] also outline further European regulations that also affect fish welfare, such as Council Regulation (EC) No 1/2005 of 22 December 2004 on the protection of animals during transport and related operations [950]. The upcoming European Commission legislative framework for sustainable food systems (FSFS), under the Farm to Fork Strategy [951, 952], will also benefit from an improved overview of fit-for-purpose WIs that can be applicable to farming and auditing whether and how sustainable farming can improve animal welfare.

Finally, functional and sensitive WIs are a prerequisite for the development of novel monitoring routines in the face of environmental challenges that threaten finfish farming, such as harmful algae blooms or cnidarian attacks. Here, WI-based observations could be one example of an early warning tool for threats that are invisible to many other sensors [953, 954].

Overall, a WI toolbox that has the scope to consider both the species- and life-stage-specific welfare needs of the fish is key to the effective delivery of the goals of the EU Directive 2010/63/EU and its amendment Commission Delegated Directive (EU) 2024/1262. An improved welfare knowledge base will help shape other key parameters that can impact fish welfare, such as the design of the rearing system, the decisions and practices that animal care staff and researchers impose upon the fish, in addition to the interpretation and application of relevant regulatory frameworks by regulatory bodies and policymakers. Further, where the objective of an experiment is to subject the fish to conditions that challenge their health and welfare, a toolbox can help document how severe this challenge is, in relation to one or more welfare needs.

Author Contributions

Chris Noble: project administration, visualization. **Gunhild Seljehaug Johansson:** visualization. **Lars Helge Stien:** visualization. **Linda Tschirren:** visualization. All authors: conceptualization, writing – original draft, writing – review and editing.

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Conflicts of Interest

Some of the authors were involved in the development of the AEFishBIT device and the SAMBA tool, which are referenced in this article. Although they hold no direct financial interests, this involvement is disclosed as a potential conflicts of interest.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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