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Overview

FELASA-AALAS Recommendations for Monitoring and Reporting of Laboratory Fish Diseases and Health Status, with an Emphasis on Zebrafish (*Danio rerio*)

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The exchange of fish for research may expose an aquatic laboratory to pathogen contamination as incoming fish can introduce bacteria, fungi, parasites, and viruses capable of affecting both experimental results and fish and personnel health and welfare. To develop risk mitigation strategies, FELASA and AALAS established a joint working group to recommend good practices for health monitoring of laboratory fish. The recommendations address all fish species used for research, with a particular focus on zebrafish (*Danio rerio)*. First, the background of the working group and key definitions are provided. Next, fish diseases of high impact are described. Third, recommendations are made for health monitoring of laboratory fishes. The recommendations emphasize the importance of daily observation of the fish and strategies to determine fish colony health status. Finally, report templates are proposed for historical screening data and aquatic facility description to facilitate biohazard risk assessment when exchanging fish.

Abbreviations and acronyms: BCS, body condition score; EU, epidemiologic unit; NNV, nervous necrosis virus; OIE, World Organisation for Animal Health – OIE; SLOM, screen less often microorganisms; SMOP, screen more often pathogens

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Introduction

Nonprotocol-induced variation in research can lead to misinterpretation of results and lack of reproducibility. Both clinical and subclinical infections of research animals can introduce such variation, resulting in negative effects on animal welfare and diminished translatability of the results to human medicine. Research using fish is no exception.[162,183,184,265,267] Variation in research using aquatic animals can result from noninfectious diseases, sometimes linked to water quality, husbandry, and care.[5,34,39,113,217,240,315] In addition, opportunistic and emerging agents may influence scientific data and/or fish welfare. With the increasing exchange of fish between and within institutions and even countries, the enactment of robust surveillance programs has become critical to document fish health status and reduce pathogen spread. Moreover, the definition of the microbiologic status of fish colonies is essential for assessing the risk of pathogen transmission from aquatic animals and fish water to personnel. Therefore, the goals of fish health monitoring programs in research should be to understand the current status of infectious and noninfectious diseases in the colony, inform and protect science, support the safe exchange of fish, and safeguard staff. These goals can only be achieved by implementing reliable health screening, robust biosecurity protocols, and sharing health-related data between collaborating groups. The development of a harmonized health monitoring program would help researchers and veterinarians at animal facilities worldwide.

Aims of the working group. FELASA and AALAS established a joint working group with 3 members from each continent to address this challenge. Fish veterinarians were asked to recommend how to monitor the health of research fish. This document is the fruit of their deliberation. The guidance proposes general information for all fish species and their main pathogens. However, the practical examples provided focus on zebrafish colonies because *Danio rerio* is the species most commonly used in biomedical research. The proposed health monitoring program relies on the following 6 recommendations: monitor fish performance, monitor fish morbidity and mortality, determine diseases of interest, establish a routine screening pattern, use both broad and specific diagnostic tools, and report results.

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We emphasize that effective health monitoring includes good communication among all stakeholders to set clear guidelines and expectations for the diverse domains that affect fish health: water quality, husbandry, physical and behavioral signs, diagnostic testing, and reporting. Our recommendations are informed by the current understanding of infectious and noninfectious diseases of research fishes, and we expect that the implementation of these recommendations will vary between facilities, depending on research and management goals, institutional capabilities, and personal experience and expertise available to the facility.

The working group reviewed current recommendations, practices in fish health monitoring, and data on pathogen prevalence.[22,39,54,126,134,137,158,176,188,203] A survey was used to collect data on current health monitoring practices, and results are detailed below. The survey revealed potentially deficient biosecurity processes in many facilities. Therefore, the working group decided to provide a second set of recommendations in another manuscript to emphasize the importance of proper biosecurity practices for the maintenance and introduction of fish and the protection of personnel from zoonosis. This second document also proposes scenarios illustrating how facilities of different sizes and biosecurity constraints can apply the health monitoring recommendations described here.[189]

Survey on fish health monitoring. An electronic survey was performed in spring 2018 to collect data on current health monitoring and biosecurity practices. Data were submitted by 145 respondents, including 111 from Europe and 24 from North America. A wide range of fish species was declared as used for biomedical research, and the survey did not allow the working group to reduce the spectrum of species to be addressed. Respondents showed a consensus on the main pathogens and noninfectious diseases that should be monitored in laboratory fish. About 3-quarters of respondents stated that they had a health monitoring system in place. The majority screened colony fish. Escapees, pre- or postfiltration sentinels, or environmental samples were tested by less than a third of the laboratories. Most facilities used PCR and histopathology, with a bias toward sampling fertile adults or older fish without considering fish sex.

Control fish health, exchange fish safely, protect science. Depending on the species, models, and facility characteristics, all stakeholders should evaluate the consequences of contamination by any bacteria, fungus, parasite, or virus (that is, all agents inclusively referred to below as microbes or microorganisms). These evaluations will determine which microorganisms should be excluded from a fish stock and how to organize a health monitoring program. In case of contamination, a contingency plan should be in place to allow prompt confirmation of the diagnosis and containment of outbreaks. Indeed, efficient health management requires thorough health monitoring, prevention of pathogen introduction, elimination of infected populations, eradication of pathogens, and prevention of dissemination of disease within the facility.[222] While the input of veterinarians or scientists experienced and knowledgeable in fish disease and health management of aquatic systems is essential, the health monitoring program must be designed and approved in a collegial manner to ensure compliance and buy-in from all stakeholders. Ensuring that all stakeholders are invested in the program is crucial to achieving the goals of controlling fish health and welfare, protecting animal models, and safeguarding personnel.

Key Definitions

Epidemiologic unit (also known as a biologic unit). An Epidemiologic Unit (EU) is defined as the complete set of tanks, racks, or systems likely to share the same water or for which water cross-contamination is unavoidable (for example, no reliable barrier between systems in the same room). All animals in an EU are susceptible to contamination with the same organisms.

A facility's overall health monitoring and biosecurity program may include several smaller programs, with one health monitoring and one biosecurity program dedicated to each EU. Rather than physical structures, EU barriers are determined by the work processes and the possibility of cross-contamination between rooms and systems. For example, a facility with several rooms and systems holding fish may consider all stock to be of the same health status and therefore have a single EU protected by a single set of biosecurity barriers.

Defining an EU is the first step in the establishment of health monitoring and biosecurity programs. This requires a robust understanding of biosecurity and sound knowledge of the flows and barriers in the facility, as well as the flaws of the biosecurity processes in place. To determine whether 2 fish are in the same EU or 2 separate EUs, one needs to assess whether reliable and robust barriers are present to prevent cross-contamination between the 2 fish. Water can be a vector of microbial transmission between fish in the same system and between systems. Thus, defining the EU must consider all items and surfaces that may contact system water and all processes in place to mitigate the ability of such equipment to be a vector of cross-contamination. In addition, aquatic pathogens can be transported by droplets of water and may sometimes linger in a dry environment.[147,178,236,322] Thereby, 2 fish in the same space or room are often deemed parts of the same EU, regardless of whether they are on the same system or not. When fish are held in 2 different rooms, internal biosecurity processes must be considered to determine whether each room constitutes an EU or whether the 2 rooms should be part of the same EU.

Health status. Health monitoring aims to establish the fish health status within an EU. The fish health status of an EU is determined by the combined impact of the following parameters on fish health, welfare, and performance: the prevalence of pathogens and noninfectious diseases, the prevalence of microorganisms relevant to the research programs, the biosecurity measures protecting the EU, and the husbandry conditions within the EU.

Diseases are monitored by a collection of observations and diagnostic tools used to identify and assess the occurrence of infectious and noninfectious disorders in a colony. Monitoring tools include fish performance evaluation, clinical or postmortem examination, morbidity and mortality records, histopathology, molecular biology, and other diagnostic assays.

Microbiologic status. The microbiologic status of an EU is the list of microbes detected in the EU by analyzing fish and/or environmental samples using techniques that include fresh mount microscopy, necropsy, histopathology, culture, PCR, and others. The microbiologic status will also inform risk assessment for personnel potentially exposed to zoonotic pathogens.

Exclusion list. Microbes that the monitoring and biosecurity programs intend to keep out of an EU constitute the exclusion list. The list of excluded pathogens should be approved by all stakeholders so that the long-term objectives of the laboratories are considered. The key questions relevant to establishing an exclusion list are: which microbes should be excluded from the EU because they may interfere with research or animal and personnel welfare; how can screening for the excluded microbes be performed; how EU contamination by these excluded microbes can be prevented; and what corrective measures should be followed when an excluded microbe is detected. Essential aspects

of a corrective plan are agreement among stakeholders on all actions necessary to provide an effective and prompt response to the outbreak and avoiding communication disruption during outbreak mitigation.

While fish that are potentially contaminated with microbes on the exclusion list ideally should not be accepted in quarantine, importation might be an option under some circumstances: when biosecurity barriers between quarantine and other EUs are robust and reliable, the surface sanitization of quarantined eggs will prevent contamination of other EUs, and a process to eradicate the pathogen in quarantine system and fish can be trusted.

Dynamic health screening. The health screening program should be dynamic. First, the list of screened microbes should include those on the exclusion list. The list should be expanded when biosecurity circumstances change and new risks are identified. Examples of this are when fish species are introduced in the facility, when fish are sourced from facilities deemed a risk for the EU, or when a biosecurity barrier breach is noticed and might compromise the EU health status.

Second, the list of screened microbes may also include non-excluded pathogens known to be present. This assessment is helpful for monitoring the effects of such pathogens, based on the occurrence and severity of clinical signs. An increase of morbidity or specific clinical signs may indicate suboptimal water and husbandry conditions and an increased infection pressure. Therefore, the routine monitoring of enzootic pathogens helps detect more general health issues and trigger further investigation.

Conversely, the list of screened microbes may be reduced if a specific microbe is demonstrated in the EU and follow-up screening is not deemed necessary (for example, asymptomatic picornavirus in zebrafish). Nonetheless, the health status report should indicate that the organism is known to be present.

More generally, more samples should be tested in instances of high morbidity or mortality until a diagnosis is reached. More samples are available when more sick fish are present, and samples can be divided for use in several different diagnostic tests (for example, inhouse necropsy, wet mount microscopy, culture, PCR, and histopathology). Also, an increased presence of clinical signs suggests an increased pathogen prevalence. In this situation identification of the responsible pathogen should be possible with a smaller number of samples than would be needed if morbidity and mortality are at background level. Therefore, sample numbers should be adapted to diagnostic needs. For example, when screening is performed to confirm that a given microbe is not present at a prevalence level that routine health screening would not detect, sample size must be increased.[254]

Management of Infection Pressure

Fish susceptibility to disease. Fish have a complex immune system with both innate and adaptive components. Susceptibility of a captive fish to disease is influenced by the normal development of physiologic immunity and by external factors including social stress (dominance), acute stress (capture), chronic stress (captivity, housing density), hormonal activity (reproduction), circadian rhythms, and water parameters.[71,80,126] Among these abiotic factors, temperature has the most significant impact on fish immunity, particularly when the temperature is outside of the fish species' suitable range or changes suddenly.[45] Some disease outbreaks occur during rapid seasonal changes in water temperature, as then strength of immune response lags behind growth of pathogen load (for example, *Flavobacterium psychrophilum, Ichthyobodo necator, Myxobolus cerebralis*, koi herpesvirus, spring viremia of carp, viral hemorrhagic septicemia virus).[3,13,100,318,321]

Many fish diseases are specific to cold or hot periods. Most pathogens thrive in a specific temperature range that is typically a narrow subset of the seasonal fluctuations a fish might experience. *Flavobacterium psychrophilum* disease, for example, is known as "cold water disease." Infectious Hematopoietic Necrosis Virus does not induce disease and is undetectable in salmonids at temperatures above 15 °C;[9] this temperature is an important factor to consider when screening. Furthermore, some treatments may include maintaining the fish outside of the pathogen's optimal temperature range (for example, cyprinid herpesvirus 3 (CyHV-3) is most virulent at 18 to 25 °C).[225]

Thus, infection is often the result of an interaction between the host's immune system (thermo-dependent activity and ability to adapt) and a pathogen's multiplication potential and infection pressure at a certain water temperature. The more pathogens are present to infect the fish, the higher the infection pressure will be, and the more infections will occur in the fish (that is, higher levels of infection in individual fish and/or more infected fish within a population). The dynamic relationship between fish immunity and the ecosystem can be an important driver of opportunistic infections.

Other factors like salinity (for example, increased salinity for freshwater fish, or reduced salinity for seawater fish within certain species limits) and diet (for example, provision of vitamins, immune stimulants, or pre- and probiotics) can be manipulated to help fish overcome infections.[211,263]

Husbandry and infection pressure. Husbandry and stock management are also key factors that affect fish immunity. For example, in aquaculture, suboptimal housing conditions (for example, high stocking density, or poor water quality) may lead to cutaneous lesions and infections.[169,211,263] During routine procedures, proper handling will reduce injuries to fish, and stress can be avoided by providing adequate acclimation times and feed availability.[169,211] Acclimation conditions can be refined by aiming to replicate the conditions at the facility of origin (for example, water parameters, light, noise, or vibration). Other essential variables include implementation of appropriate hygienic methods for cleaning tanks and sumps, water filtration, disposal of feed waste, prompt removal of sick fish and carcasses, and cleaning of handling equipment (for example, nets) to reduce biofilm and infection pressure.[44]

Containment of immune-deficient and older fish. Another way to control the number of organisms shed by fish into the system is to manage fish stock based on risk factors. Immune-suppressed fish (for example, due to phenotype, irradiation, or other treatment) are more likely to develop infections. Because of this, it could be tempting to use them as sentinels to detect pathogens. However, these fish could shed higher numbers of pathogens and contribute to an increased infection pressure in the system.[266] Therefore, immune-suppressed fish are not recommended for use as sentinels, considering the ethics of the practice and the difficulty in mitigating risk to the ecosystem.

On the contrary, whenever necessary, fish with higher sensitivity to infections should be housed in a biocontainment that will reduce their risk of infection and prevent potential contamination of other stock. This could be done, for example, by removing them from main recirculating water systems, and rearing them on a flow-through rack or a stand-alone (separate) system.

Infection pressure can be minimized by stock management aimed at reducing the presence of fish populations that are more

likely to shed pathogens. In the case of pathogens that induce chronic diseases, prevalence is expected to be higher in older animals,[38] and using older fish for breeding may increase the contamination of the new generations. For this purpose, some research establishments impose an age limit on fish that can be kept in the system. For example, to reduce *Mycobacterium* spp. or *Pseudoloma neurophilia* shedding, zebrafish should not be kept in a main system beyond 18 mo of age.[134] Histopathologic changes due to zebrafish natural aging also become significant from the age of 18 mo.[38] If aging studies are performed, the aged fish should be held under biocontainment (that is, on a separate rack that is not part of the main recirculation systems).

Diseases of Zebrafish

Although this manuscript is not intended to be a review of the literature on the diseases of zebrafish or other species, our recommendations are best based on published information identifying the microbes that are most prevalent or most often associated with morbidity, mortality, and variability of research results. These microbes are important targets for the testing described in these recommendations. Figure 1 indexes disease information that is provided as supplemental material and in dedicated sections of the text. The major diseases of laboratory zebrafish are summarized in supplemental material Table S1. Images of zebrafish pathologies are available in the cited literature.[39,136]

Infectious and noninfectious diseases of zebrafish. The infectious agents listed in the tables either induce high morbidity/mortality or are relatively common diagnoses. The most common pathogens of zebrafish are *Mycobacterium* spp. (bacteria) and *Pseudoloma neurophilia* (microsporidian fungal parasites).[137,158] Both are associated with a range of disease states from subclinical disease to acute mortality. Clinical presentation is determined by pathogen-specific factors (for example, *Mycobacterium* species variation), environmental factors (for example, water quality, diet, or husbandry), and host factors (for example, immune status). In addition, some bacterial and fungal organisms that are typically considered normal components of a fish's environment may induce disease due to suboptimal environmental and host factors. Noninfectious diseases attributable to husbandry and environment, genetics, toxins, and idiopathic causes are also briefly described in supplemental material Table S1.

Viruses and other emerging diseases of laboratory zebrafish. Viruses are known causes of disease in many fish species,[60] and zebrafish have been experimentally infected with some World Organisation for Animal Health (OIE) notifiable viruses.[153,168,241,296] However, only in the last decade has the community identified naturally occurring viral diseases in laboratory zebrafish, and a few viruses are now considered as emerging pathogens in this species. Viral nervous necrosis (NNV) was identified in 2013 as the result of a betanodavirus infection in zebrafish and goldfish (*Carassius auratus*) purchased in India from an ornamental fish store.[20] In 2015, infectious spleen and kidney necrosis virus (ISKNV), an iridovirus infection, was documented in laboratory zebrafish in Spain.[19] More recently, zebrafish picornavirus 1 (ZfPV1) was identified as an asymptomatic infection of intestinal tissue in fish from 56% of 41 screened institutions.[6] Lately, natural infection with covert mortality nodavirus (CMNV) was reported in *Danio rerio*.[308]

Besides viruses, the zebrafish gut is home to several other emerging pathogens. In 2018, the transmission of intestinal neoplasms in cohabitated zebrafish was correlated with a *Mycoplasma* species.[32] A recent survey of zebrafish purchased from pet stores revealed an intestinal infection with 2 previously undescribed coccidia.[137] Descriptions of infections with *Edwardsiella ictaluri*[276] and the trematodes *Centrocestus formosanus*,[121] *Clinostomum* spp.,[258] and *Transversotrema patialense*[317] further highlight the risk of importing new pathogens with zebrafish from certain sources. In addition to the direct pathologic effect of these agents on target tissues, the associated microbiome should be considered. A recent study showed that infection with the intestinal nematode *Pseudocapillaria tomentosa* measurably disrupts composition of the intestinal microbiome.[199] Some research projects require the collection of fish from the wild; some facilities purchase fish from pet stores and fish farms; and some facilities house different fish species in close proximity. All of these are risk factors for the development of new and emerging diseases in laboratory fish populations.

Diseases of Other Fishes

In the supplemental material Table S1, which details zebrafish diseases, other fish species are also briefly addressed. References for pathogens of species other than zebrafish are summarized below and in supplemental material Table S2. Images of pathologies can be found in the cited literature.[112,117,211,263,315] Figure 1 indexes disease information provided as supplemental material and in dedicated sections of the text.

Bacteria. Most of the bacteria found in fish are Gram-negative and are considered normal flora of water and fish, potentially causing disease under poor husbandry conditions, secondary to other infections, or in immunocompromised fish. Contrary to these common secondary or opportunistic bacterial agents, primary or obligate pathogens are always associated with disease (for example, *Aeromonas salmonicida* causes furunculosis; *Renibacterium salmoninarum* causes bacterial kidney disease).

Disease category	Described in			
	Table S1	Table S2	Table S3	Dedicated sections in body text
Bacterial	A	A	C	4ac, 5aj
Fungal	C	A, B	B, D	4a, 5b
Parasitic	B, C, D	B	A, B	4ac, 5cdefg
Viral	E	C	D	4b, 5h
Noninfectious	F			4a, 5i
Neoplasia	G			

Figure 1. Index of disease descriptions in supplementary tables. This table helps readers to find information about diseases that are mentioned or described in Supplementary Tables S1 for zebrafish, S2 for other fish species, S3 in the context of a multispecies facility example, and in specific sections of the text. These supplementary materials are divided into smaller tables (A to G) referred to in the index. Although microsporidia are fungi or sister to fungi, they are commonly reported as parasites.[40] They are therefore classified as parasites and included here in the fungus and parasite categories.

In addition to the bacterial diseases of zebrafish summarized in the supplemental material Table S1, Gram-negative *Vibrio* infection can cause significant mortality and morbidity in other fish species, particularly when associated with chronic stress. Gram-positive bacteria have assumed an increasingly important role in aquaculture fish species; these bacteria include pathogens like the previously mentioned *Renibacterium salmoninarum* and Streptococcaceae representatives (for example, *Lactococcus garvieae* and *Streptococcus parauberis*).[13] Bacterial infections can induce high morbidity and mortality, and some bacteria, like *Mycobacterium marinum,* can affect both freshwater and saltwater fish. A few bacteria may be transmitted to offspring by vertical or gamete associated transmission, including *Flavobacterium psychrophilum*, *Mycobacterium* spp., *Piscirickettsia salmonis* and *R. salmoninarum*.[13,211,263] Vaccination, frequently used in aquaculture, is used less in fish research facilities. Fish vaccinated against a specific bacterium can still be asymptomatic carriers, which are an important hazard to consider when importing vaccinated fish. Extensive literature is available concerning fish bacterial infections (see references in supplemental material Table S2).

Fungi and fungal-like pathogens. Mycotic or fungal-like infections in fish are frequently caused by saprophytic, opportunist fungi or fungal-like pathogens that can affect injured, chronically stressed, or immunosuppressed fish. These fungal diseases can be secondary to bacterial, parasitic, or viral infections and are exacerbated by poor husbandry.

Fish mycosis refers to a varied group of parasitic agents previously attributed to fungal clades but recently excluded by DNA-based classification and comparisons with Oomycetes and Mesomycetozoea.[103] In consequence, fewer fish pathogens are still in the kingdom fungi, mainly represented by Exophiala and Microsporidia.[124] The latter are commonly referred to as parasites and are classified as such in the tables we present.

Among other fungal-like agents, oomycetes, or water molds, *Achlya* spp., *Aphanomyces* spp., *Branchiomyces* spp., and *Saprolegnia* spp. are common pathogens that can affect health and welfare of both freshwater and estuarine fish. Infection with *Aphanomyces invadans* causes epizootic ulcerative syndrome, the only fungal-like OIE notifiable disease.[296] Diagnostic and treatment measures are described in the literature.[87,211,263,318] Mesomycetozoea, a small group of Opisthokonta, include a few species and mainly fish parasites like Ichthyophonida (*Ichthyophonus hoferi*) and Dermocystida (*Dermocystidium* spp. and *Sphaerothecum destruens*). Ichthyophoniasis is usually diagnosed as a subclinical infection in marine, anadromous, and some freshwater fish. The typical disseminated pattern of lesions can compromise fish homeostasis and interfere with experimental outcomes.[210,211,263,318] *Sphaerothecum destruens* is thought to be a major freshwater fish parasite that causes high morbidity and mortality in salmonid and cyprinid species.[104] Other fungal-like pathogens and true fungi or eumycetes can also cause disease in fish.[76,182,211,263,318]

Importance of parasite life cycles. In closed systems, ectoparasites with a direct life cycle are the most problematic, as they can easily find suitable hosts. External parasites are often more contagious than internal parasites due to their ability to spread in the environment. Nevertheless, external parasites are typically easier to manage and can often be eradicated during quarantine by specific treatment or egg disinfection.

Parasites with complex life cycles may be of limited concern when obligate hosts are not present in the EU. Nonetheless, they can infest new hosts via cannibalism or predation (for example, infection of European sea bass [*Dicentrarchus labrax*] by metacercariae of the digenetic trematode *Bucephalus haimeanus* after predation of infected gobies).[321] Care should be taken not to introduce these parasites via food (for example, live or dead prey).

As compared with facultative parasites, obligate parasites are a major concern in research facilities. Among these are some protozoan and monogenean trematodes.

Protozoan ectoparasites. All obligate parasites, like *Ichthyophthirius multifiliis, Chilodonella ssp.,* and their marine counterparts, *Cryptocaryon irritans* and *Brooklynella hostilis*, should be viewed as major pathogens. They have low host specificity and can cause high morbidity and mortality.[206,211] Nonobligate parasites, such as Trichodinid species (for example, *Trichodina* spp. and *Trichodinella* spp.), affect skin and gills, and usually cause less morbidity and mortality. Infections commonly occur in stressed and debilitated fish and in systems affected by poor hygiene. Treatment is often effective. These trichodinids can be found in amphibians, which can thus be reservoirs.[211]

Protozoan ciliates of the order Scuticociliatida are facultative parasites. Genera like *Miamiensis, Philasterides,* and *Uronema* in salt water and *Tetrahymena corlissi* in fresh water can cause infections with high morbidity and mortality, often related to poor husbandry.[10,263,319] Corrective treatment and husbandry measures have been described and should be implemented upon diagnosis.[211,263]

Hematozoa, vascular parasites that can be external (for example, *Cryptobia* spp. in freshwater fish and *Haemogregarina* spp. in saltwater fish), can be transmitted via biotic vectors like leeches.[321]

Metazoan ectoparasites. Monogenetic trematode infections generally result in higher host morbidity and mortality than do the digeneans. This might be due to their direct life cycle and adhesion to the skin or gills. Almost all are ectoparasites that affect these 2 organs, and numerous species infect marine and freshwater fish. The genus *Gyrodactylus* encompasses over 20,000 species, including *Gyrodactylus salaris*, a notifiable disease in salmonids.[321] *Gyrodactylus* spp. are often specific to a fish species; however, some species, like *Neobenedenia melleni*, can infest multiple fish species. Treatments have been described.[211,263] Other relevant groups of metazoan parasites (for example, copepods, branchiurans, isopods, and hirudeans or leeches) should be detected in quarantine, where treatment can be initiated to prevent parasites from entering the main fish-holding rooms. Drug prophylaxis can sometimes be advisable if a particular parasite is suspected but not found in initial diagnostic testing.[81,211,263,315]

Trematode internal parasites. Trematodes (such as *Centrocestus formosanus*,[121,215,219] *Clinostomum* spp,[258] and *Transversotrema patialense*[317]), are metazoan parasites that are seldom described in zebrafish from biosecure sources[137] but are common in other fish species, frequently affecting their health status and research outcomes.[211,263] Fish are often intermediate hosts for digenetic trematodes; metacercariae, the larval stage of the parasite, can be found encysted in various locations. Brain infestation may cause behavior modifications, as described for *Euhaplorchis californiensis* in California killifish (*Fundulus parvipinnis*).[118] Digenetic larval stages can be found in the eyes or gills causing vision impairment (for example, *Diplostomum* spp. in several fish species) or gill lesions (for example, *Centrocestus formosanus* in *Cyprinus carpio*).[263,275] Frogs, toads, and snails can be intermediate hosts for some digenetic trematodes; this may represent a significant hazard for aquatic laboratories using both fish and such species.

Nematodes. Most nematodes have a complex life cycle. Intermediate and paratenic hosts can be crustaceans, snails, oligochaetes, tadpoles, and fishes. Host tissues can be affected by nematode larval stages.[321] In final hosts, adult forms of the parasites are usually located in the intestinal lumen with some exceptions, like *Anguillicola crassus* in the eel's swim bladder.[211] Like *Camallanus* spp. or *Capillaria* spp., some species may complete a direct life cycle within a recirculating system and parasitize the intestine of fish—the final host.[132,160,180,194] Some nematodes (for example, *Anisakis* and *Pseudoterranova)* are zoonotic parasites that can be introduced into the system via food (for example, live or dead prey). Freezing food before introduction can be used as a preventive measure (for example, −15 °C for 96 h, −20 °C for 24 h, or −35 °C for 15 h).[228]

Viruses. More than 50 viruses with veterinary importance have been described in fish, and within these, several are associated with notifiable aquatic animal diseases in the OIE Aquatic Manual.[142,296,318] Generally, viruses are host-specific or affect a closely related group of fish species. Their pathogenicity is often temperature-dependent and may depend on the target organ and the fish developmental stage (for example, fingerlings are more susceptible than adults and act as carriers).[211]

Particular attention should be paid to the viruses associated with notifiable diseases and their potential fish hosts and vectors. This is relevant both for regulatory compliance and because these diseases can be associated with significant morbidity and mortality. Vectors can act as carriers, infecting susceptible species. National or regional legislation may differ from the OIE list, and surveillance of these viruses should be carried out in accordance with local legislation. Besides these notifiable diseases, other viruses can be detrimental to wild species (for example, betanodavirus) or have a wild host specificity with variable pathogenicity, like some iridoviruses.[142] Lymphocystis, caused by an iridovirus, is one of these diseases. It affects more than 140 fish species, typically resulting in low mortality with self-limiting lesions that are white to greyish-pink, small, papilloma-like masses mainly in the skin and fins. Morbidity is sometimes associated with episodes of stress (for example, husbandry issues, mainly during quarantine or after transport).[142,211,263] Extensive literature concerning fish viral infections is available, and reports of emerging fish viruses are expected to expand (see references in supplemental material Table S2).

Noninfectious diseases. Noninfectious diseases are not linked with any pathogen, and can induce developmental disorders, morbidity, or mortality. They encompass intoxication, neoplasia, nutritional disorder, trauma, and hormonal disruption. Noninfectious diseases are common in fish and may be responsible for acute mortality (for example, supersaturation, ammonia or nitrite intoxication) or chronic subclinical impact (for example, hepatic megalocytosis). Some of these diseases are related to husbandry practices (for example, nephrocalcinosis, egg-associated inflammation).[39]

Fish depend on system water for the provision of heat, gas (for example, oxygen), and minerals (for example, calcium, magnesium, or iodine). Water temperature, pH, and salinity should be closely monitored. Temperature can influence gas solubility, salinity, and other chemical equilibrium (for example, pH and ammonia). Sudden variations of water parameters should be avoided. Nitrogen waste should be controlled in recirculating systems, considering fish susceptibility to ammonia and nitrite.[5,211,217,240] Inadequate water parameters can also lead to pseudo neoplasms (for example, goiter due to iodine deficiency).[16,63,204]

True fish neoplasia are described.[235,301] Some factors have been linked with an increased incidence. For example, melanoma in *Xiphophorus* spp. and thyroid neoplasm in *Oryzias latipes* are linked to sex,[31,257] while other neoplasia may be linked to age and strain.[39,130]

Fish nutrition remains underexplored for most species not used for aquaculture purposes. Research fish may therefore receive unbalanced diets. In general, protein and high unsaturated fatty acid content are key elements of fish feed. Vitamin C is an essential fish nutrient that is particularly relevant to storing diet in fish facilities since ascorbic acid is degraded in hot and humid environments. A wide range of clinical diseases have been linked with nutritional issues: cataract, exophthalmia, gill hyperplasia, anemia, skeletal deformity, convulsion, and anorexia.[156,214]

Emerging diseases of other fish species. Intracellular bacteria of the phyla Chlamydiae and Proteobacteria can induce epitheliocystis, characterized by mainly gill epithelium cysts. This emerging disease in aquaculture affects more than 90 marine and freshwater fish species, including elasmobranchs.[213,230] Although described as a usually benign infection, it can cause respiratory failure and death, mainly in juvenile fish.[74,128,186,187,213,248,249,318] The incidence and morbidity are associated with poor husbandry. Etiologically, this condition should be differentiated from pathogens like *Ichthyophthirius multifiliis*, *Dermocystidium* spp. and *Loma* spp. and from breeding tubercles on goldfish (C*arassius auratus*), carp (*Cyprinus carpio*), and common minnow (*Phoxynus phoxynus*). It should not be confused with lymphocystis, a previously mentioned viral infection.[13,21,263] Other emerging diseases such as erysipelothricosis, edwardsiellosis, and francisellosis can also be considered.[181]

Monitor Fish Performance

Fish husbandry is designed to optimize fish welfare and maintain expected performance standards. Several factors can be used to assess fish performance, including body condition score (BCS), fish length, weight, width-related ratio, egg production, and fecundity.[38,53,157,244] For a defined population, changes in average performance may result from disturbances of water quality or husbandry (for example, diet distribution, tank density) or contamination by a virulent pathogen. Expected ranges should be established in each facility to provide a reliable standard for comparisons. These ranges should represent fish of different ages, sexes, genotypes, and other relevant variables.

Body condition score (BCS). Scoring body condition is a noninvasive method to describe the relative weight or fat cover of an animal. It is routinely used in mammals, and some fish can similarly be scored easily and quickly without anesthesia.[53] BCS is useful to monitor the appropriate feed provision by assessment of whether the average fish is too thin or too fat. Other practical applications include the establishment of an accepted BCS range according to life stage. Thus, by training staff to estimate and record BCS, fish falling outside of the accepted BCS range can be identified, and corrective actions initiated (for example, morbidity investigation, treatment, or euthanasia). Ideally, a group of fish of the same age and genetic background should be relatively homogenous (score within a small BCS range). Heterogeneity may signal a husbandry issue and/or a microbial contamination.[244]

Fish length and weight. Fish grow based on their genetic background, husbandry conditions (for example, tank density and temperature), and diet (quality and quantity). Consistent applications should result in consistent outcomes. Thus, it is helpful to monitor the weight and length (with or without the

caudal fin, depending on species and strain) of the most common lines in a facility (for example, commonly shared wild-type lines) at specific ages across generations. Monitoring weight and length during the growth of an individual clutch also allows the identification of unexpected changes in growth that may warrant investigation. However, measuring fish should not interfere with their welfare, physiology, or growth. When appropriate, disturbance can be reduced by performing the procedure concomitantly to other interventions, and by minimizing time out of water. For example, many fish species, including zebrafish, can be weighed without sedation by weighing a container filled with system water to which the fish is then added. By subtracting the weight of the vessel of water from the weight of the same vessel after adding the fish, the weight of the fish can be obtained. For smaller life stages, a more practical approach may be to measure larval length rather than other parameters. This measurement can be made using a photo of a larva in a small amount of liquid with a ruler in the frame.

Monitor Fish Morbidity and Mortality

Pathogens and husbandry issues can increase morbidity or mortality in fish colonies. Morbidity refers to the rate of disease in a population and is defined here as the percentage of a population that shows a clinical sign or subclinical lesion(s) during a specific time period. Mortality rate refers to the proportion of deaths in a population during a given period of time. Because both measures may refer to events that reduce welfare, both can be used as welfare indicators and not only as health monitoring tools.[79] Mortality rates include the number of fish found dead and the number of fish euthanized on welfare grounds. Comparing the number of fish found dead to the number euthanized can be another welfare indicator, reflecting the ability of care staff to detect poor welfare and intervene, and potentially the local culture of care.[237]

Record morbidity and mortality. Most health surveillance programs incorporate a method for recording levels of morbidity and mortality on a timed basis (for example, daily or weekly).[79] Daily health and welfare checks of fish should be used for that purpose. By keeping records of morbidity and mortality, trends can be recognized in a population more quickly, and responded to with appropriate follow-up observations and testing. Genetic identity, age (date of fertilization), sex, study assignment, system, and even location on the system are useful factors to include in sick or dead animal reports.

Deaths should be reported to the research personnel as well, so that researchers can collect postmortem samples. The researcher may also provide genetic or treatment history that could indicate the cause of death. The tank containing the dead fish can be marked with a mortality sticker for future reference. Fish not needed for laboratory sampling can be submitted for diagnostic testing. Fish exhibiting any kind of clinical abnormality may be documented by the use of a sick animal report, including general factors and a description of the abnormality noticed. The tank can be marked with a sick animal sticker for future reference. When sick fish reach clinical endpoints, or sooner, they can either be isolated for treatment under veterinary guidance and with permission from the study leader, or euthanized.

Analyze trends in morbidity and mortality rates. The levels of morbidity and mortality deemed acceptable in an individual facility may vary based on the species, phenotype, and immune status of the fish present, the types of work occurring with the fish (that is, infectious disease, toxicology, or oncology), fish age and developmental stage, and known pathogens in the facility. Genetically manipulated fish may be more susceptible due to their phenotype, and morbidity and mortality records for individual lines may reveal the severity of genetic alterations. For comparison and for monitoring of the general fish population, an important goal is to define baseline morbidity and mortality levels that correspond to expected life events of large wild-type populations under good care. When levels exceed baseline consistently and significantly, facility personnel must immediately begin to investigate possible causes, including water quality issues, improper feeding, temperature control, congenital defects, and toxin or pathogen introduction. To that end, recording the locations of tanks housing fish with higher mortality rates is essential to focusing investigation on racks or systems that may present adverse conditions. In general, an increase over 2 sequential observation periods would trigger veterinary investigation.[188] Each facility should determine its baseline rate and time frame threshold specific to the length of the monitoring period and the population's developmental stage. For example, mortality rates between fertilization and the age of independent feeding may be higher than those of adults.[79] Similarly, older fish may have high morbidity and mortality rates due to chronic infectious processes (for example, mycobacteriosis)[134] or natural aging. We recommend regular recording and assessment of morbidity and mortality rates, daily if possible. This information will alert staff to problems and will be used to direct diagnostic analysis and develop solutions to reduce colony losses.[79]

Determine Diseases of Interest

For the purpose of disease monitoring, microorganisms can be categorized into those more likely to cause impactful diseases and therefore tested for at greater frequency (SMOP for Screen More Often Pathogens), and those less likely to be present and cause impactful disease and therefore tested for less often (SLOM for Screen Less Often Microorganisms). We give examples here of selections of SMOP and SLOM; these should be adapted to each EU's context.

Zebrafish SMOP and SLOM. In this example for *Danio rerio*, SMOP include *Mycobacterium* spp., *P. neurophilia*, and *P. tomentosa*, all 3 of which are relatively common diagnoses in zebrafish facilities and are associated with disease.[137,158,326] SLOM include microorganisms that are infrequently diagnosed but still sometimes cause disease (that is, *Edwardsiella ictaluri*, *Flavobacterium columnare*, *Ichthyophthirius multifiliis*, *Piscinoodinium pillulare*, and *Pleistophora hyphessobryconis*), and those that are more common but not usually associated with disease (that is, *Myxidium streisingeri*). Viruses are also included in the zebrafish SLOM category; testing for these is at the facility's discretion. The science on zebrafish viruses is emerging.[137] Researchers should decide whether their models may be affected by nonpathogenic viruses[6] or if their fish are at risk of contamination by viral pathogens,[19,20] which may depend on the sources of the fish or concomitant housing with other susceptible species. We thereby recommend that each facility make an informed decision to include or exclude from the SLOM each virus that has been described in the relevant fish species.

SMOP and SLOM for multispecies facilities. The source of the fish (that is, laboratory-reared, aquaculture, pet shop, or wild) is a key question in determining which pathogens to monitor. Consulting the literature may identify potential pathogens, although publications may be limited for unusual or less studied species. Therefore, determining prevalence and impact on fish welfare and research can be challenging. Emphasizing regular observation of behavior, evaluation for external lesions, and

broad diagnostic strategies can be used to make a general assessment of fish health.

The SMOP and SLOM lists should be revised in accordance with the infectious risks posed by other species that are present in the facility, though not necessarily in the same EUs. Indeed, even with additional biosecurity measures, the risk of cross-contamination between EUs is present, and even pathogens adapted to a particular species may ultimately cross over to different species (see supplemental material Table S1, Table S2, and Table S3, which assess pathogen cross-contamination risk in a multispecies facility scenario and suggests SLOM/SMOP designation for common pathogens of each species).[189] Therefore, these lists depend on the overall epidemiologic context, namely previous screening results from the other fish species.

For example, in a multispecies facility, NNV can be classified as SLOM in a zebrafish EU and SMOP in a European seabass (*Dicentrarchus labrax*) EU, despite biosecurity barriers between the 2 EUs. This distinction is made in view of the high NNV prevalence in European seabass and reports of NNV infections in zebrafish.[20,261] If the 2 species were part of the same EU, NNV would be a SMOP for both species.

Also, some species may potentially act as vectors. For example, *Dicentrarchus labrax* is not sensitive to viral hemorrhagic septicemia virus disease but may carry and transmit the virus if originating from a trout farm. In this case, including the virus in SMOP or SLOM panels of the seabass importing facility may be relevant.

Glugea spp. would be considered a SMOP for African turquoise killifish (*Nothobranchius furzeri*) due to reported high prevalence. Zebrafish sharing a quarantine room with the species should be screened for this parasite, at least as a SLOM. When the risk of contamination is higher (for example, when zebrafish and African turquoise killifish share the same EU), *Glugea* spp. would be a SMOP for both species, despite the lack of reported infestation in zebrafish.

Eimeria funduli infections are reported in several species of the genus *Fundulus* belonging to the family Fundulidae. In the absence of reported cases for a species of this family, *Eimeria funduli* should still be considered at least a SLOM.[264] For African turquoise killifish, which belong to a different suborder of the order Cyprinodontiformes, sharing the same SLOM classification may not be necessary, but the diagnosis of *Eimeria* infections should be a concern since species from the same suborder (Aplocheiloidei) can be infected with coccidian.[91] These examples illustrate some aspects of risk assessment necessary to determine SMOP and SLOM status for the facility species.

Establish a Routine Screening Pattern

Number of animals to sample. One can use a described formula to calculate the number of fish to sample from a population to detect one or more infected fish.

$$n = \left[1-\left(1-p\right)^{\frac{1}{d}}\right]\left[\frac{N-2}{d}\right]+1$$

With test sensitivity and specificity set at 100%, 3 parameters must be defined to determine the required sample size (n): the population size (N), the desired degree of confidence that one or more infected fish will be detected if the pathogen is present (p; usually $p = 0.95$), and the minimal number of expected infected fish given a presumed prevalence (P) (that is, $d = P \times N$).[134,254]

Considering the large size of fish populations, the key parameter is the pathogen prevalence. For a population of 1,000 fish, 29 fish should be sampled to detect a pathogen with an assumed prevalence of 10%, and 259 fish sampled to detect a pathogen with an assumed prevalence of 1%, both with 95% confidence and a test with 100% specificity and sensitivity. Achieving these sample sizes quarterly is not realistic for many facilities. If screening for pathogens with a 20% prevalence, the required sample size can be reduced to 15 fish. This seems a more achievable number for routine health monitoring, although it would only detect pathogens afflicting at least 20% of the EU. To some degree, the statistical disadvantage of sampling fewer fish can be addressed by taking a biased sample of fish more likely to be infected, like sick fish or prefiltration sentinels.

We propose screening 15 fish quarterly. When the population is of sufficient size (> 1,000 fish) and the test specificity and sensitivity are set to 100%, a sample size of 15 colony fish would detect, with a confidence of 95%, pathogens infecting at least 1 out of 5 fish (that is, prevalence threshold of 20%). We chose a sample size of 15 fish for practical and financial reasons. This sample size does not demonstrate an absence of pathogens. We propose to increase the probability of pathogen detection by testing prefiltration sentinel fish and environmental samples. Quarterly repeats of the screening also help to improve detection. However, the limitations of the proposed sample size must be understood by stakeholders. Facilities that want to detect less prevalent pathogens must use the formula to estimate the number of samples required for each EU population size and pathogen prevalence threshold. Screening for pathogens in smaller populations may require a smaller sample size to achieve the same confidence in detection.

Sample colony fish. Fish that are euthanized due to illness or that are recently found dead in the colony should be tested regularly to monitor for pathogens and associated disease severity.[22,54,185,203] However, such samples only provide information about pathogen prevalence among sick fish, not the population as a whole. Nonetheless, these are particularly important samples in EUs with low fish numbers. When euthanasia for pathogen screening is not an option, due to low animal numbers or the high value of available specimens (for example, rare fish or brood stock such as meagre *Argyrosomus regius*), screening in quarantine and holding rooms is based on clinical evaluation, testing of anesthetized fish,[177] and environmental screening (at least for zoonotic mycobacteriosis). An alternative is to import a surplus of fish to allow a small number to undergo lethal screening tests at the beginning, during, and end of quarantine, and eventually during their stay in the main EU.

Set-up sentinels. In a recirculating aquaculture system, water exits fish tanks to a sump before it is filtered. The sump water can be used to expose sentinel fish to pathogens drained from the fish tanks. Several techniques are used to place prefiltration sentinels. For example, sentinels can receive sump water by insertion of a water pump in the sump to deliver sump water to one or more sentinel tanks. Alternatively, but not preferably, escapees can be removed from the sump, or sentinel fish can be placed out of the recirculation loop(s) and held in sump water renewed manually on a regular schedule.[191] The latter option has the advantage of preventing contaminated sentinels from shedding pathogens back into the recirculating system. Finally, so-called postfiltration sentinels are fish exposed just as colony animals and are not a recommended option because they do not present the advantage of a prefiltration exposure and are therefore not complementary to normal colony fish sampling.

The origin and exposure time of sentinels must also be considered. One practical option is to set up a pool of sentinels originating from the EU and to sample them regularly for a

prolonged period of time, until the pool is extinct or fish have reached an age limit. Because exposure of these prefiltration sentinels to effluent water is cumulative, this sentinel strategy would not be appropriate to gauge the success of a program to limit or eliminate a pathogen. Similarly, the detection of a pathogen at a later sampling point could indicate the presence of a new pathogen, a pathogen with a long incubation time, or the accumulation of an infective dose of an existing pathogen over time. Other sentinel strategies are also appropriate, including setting up new sentinel tanks every quarter, or varying the amount of time that sentinel tanks are exposed on the system.[191] When monitoring for a specific pathogen, sentinels from a source deemed free of the pathogen can be used, as long as they are not vaccinated against the pathogen.[192,200] Preferentially, sentinels would be used at the most sensitive life stages. In the case of rare colony specimens, other species sensitive to the monitored pathogens can be used as sentinels.

Environmental samples. Pathogens may grow in the environment, for example in biofilm, live feed cultures, or within an intermediate host.[308] Fish may shed waste that reveals the presence of a pathogen, for example, feces containing parasite eggs.[202] Thus, a combination of fish and environmental sample types can be used to detect infectious agents.[12,172,185,273,276,299] The detection of some pathogens in the environment is sufficient for establishing the microbiologic status of the EU. However, the lack of environmental detection is not informative, since some fish pathogens may not be reliably discernable by environmental screening.[62,185] Moreover, organisms detected in the environment may not always cause disease in the fish. Further analysis is required to determine the effect of the detected organisms on fish health status (for example, morbidity, mortality, interference with rearing and breeding, and experimental bias). More specifically, *Mycobacterium* spp. will commonly be present in the environment, sometimes without significant impact on fish health.[313,324] The same happens with several *Vibrio* species, frequently detected in the water and not necessarily linked to disease. This is true in freshwater fish, which are less affected by vibriosis, and in marine and estuarine fish.[22,211,263]

Sludge analysis consists of collecting fish and food waste, detritus, and biofilm at the bottom of sumps, tanks, or breeding devices. It can be used to detect some bacteria (for example, *Mycobacterium* spp.) and parasites (for example, *Pseudocapillaria tomentosa*). This technique can also be used in quarantine to screen imported fish.[191]

The sump wall surface can be swabbed at the air/water interface to identify the presence of some bacterial species by culture and PCR (for example, *Mycobacterium* spp.). Multiple samples should be tested, as not all system samples will always yield mycobacteria.[324] Attempts to detect parasites with this technique have not been successful.[191]

Tank water can be filtered, and the filter screened for pathogens. *Mycobacterium* spp. were identified with this process, but detection of parasites was less reliable.[62,97,185]

Monitoring the bacterial load of the water systems can be used to assess the efficacy of UV disinfection or to detect other circumstances that can lead to excessive bacterial load (for example, high fish density with deficient system cleaning).[188]

Live feed cultures should be monitored regularly to assess their quality. These can be screened for unwelcome commensal organisms by microscopy, culture, or PCR.[42,299] Test results for continuous live cultures of feed (for example, paramecia and rotifers) can indicate whether a culture is safe to propagate for feeding to fish. Brine shrimp are usually fed out the day a sample is taken (noncontinuous cultures). However, results can still be useful for documenting efficiency of cleaning and disinfection of the culture equipment and to potentially account for above normal mortality of larval fish. Dry feed can be screened for pathogens, though interpretation of positive PCR results in that situation may require further investigation to differentiate between the presence of a viable pathogen and the detection of residual inactivated DNA because the survivability of aquatic pathogens in dry feed is less likely than in a wet environment.

Embryos and larvae can be affected by predators like *Coleps* spp. and *Tetrahymena* spp. These are not transmitted by other fish and are a water quality problem. They can be detected by regular observation of embryo media and clutch water.[10,179,278]

Water can also be screened for chemicals. The screening technique is not designed to detect pathogens but instead monitors the adsorptive efficiency of the carbon filtration by measuring the concentration of key molecules before and after filtration. The molecules that are monitored should be adapted to the local water supply, bearing in mind that some may not be filtered by reverse osmosis (RO) membranes (for example, toluene). Monitoring can be done for byproducts of the chlorination process (for example, chlorodibromomethane, bromodichloromethane, or chloroform), volatile organic compounds (for example, toluene), and some heavy metals (for example, arsenic, copper, or uranium). Several studies have demonstrated the effects of such pollutants on the zebrafish digestive tract and microbiota.[11,46,47]

Routine health screening. An example of a routine screening pattern is summarized in Figure 2. It relies on analyzing a set of 15 fish quarterly per EU using at least 10 prefiltration sentinels and 5 colony fish. The screening pattern also includes 2 environmental samples and 1 live feed sample (see Figure 2). The number and type of these nonfish samples should be adapted to the facility. For example, when propagating large numbers of paramecia cultures, samples can be taken from a subset of representative cultures from which all future cultures would be split.

Each sample type is screened quarterly, and the testing can be staggered so that at least one sample type from the EU is tested per month. Leaving too long a period of time between screening events may delay the detection of a contaminant or of a new problem and thus delay its mitigation. Facilities with higher biosecurity risks (for example, frequent fish imports) may opt for more frequent testing. Alternatively, closed facilities with static fish populations and limited staff may find biannual sampling acceptable. Whatever the routine sampling frequency, monitoring for clinical signs of disease and changes in environmental parameters is incorporated into daily staff tasks, and abnormal findings should trigger additional sampling as needed.

Considering that pathogens may affect one sex more than the other,[50,279] or that clinical conditions may vary in relation to fish sex,[66,244] age,[24,38,134] or genotype,[119,280,313] the fish included in the samples over a 1-y period should represent all categories of the population: age, sex, and genotypes as appropriate. This can be difficult to achieve in a large facility with hundreds of lines, in which case the screening may be weighted more toward testing sick fish.

One option is to annually establish a prefiltration sentinel tank with 25 female and 25 male wild-type fish, all over 3 to 6 mo of age after fertilization, representing the most common genetic background in the EU. Ten sentinel fish are then sampled quarterly, after a minimal exposure duration of 3 mo. Any sentinels that become sick or are found dead are screened as soon as possible. After 12 mo of exposure, at the fourth sampling time

Origin of sample	Over a 3-mo period	Histopathology, microscopy or the following PCR panel
Colony fish	Screen at least 5 colony fish per quarter (young or sick fish)	Quarterly: SMOP Every other quarter: SMOP+SLOM
Prefiltration sentinels	Screen 10 prefiltration sentinel fish quarterly	
Sludge analysis	1 quarterly sample for PCR	*Mycobacterium* spp., *P. tomentosa*
Sump surface swab	1 quarterly sample for PCR	*Mycobacterium* spp.
Feed	1 quarterly sample for PCR	*Mycobacterium* spp.
Quarantine	Screen imports, sick fish, escapees, subset of retired fish, sump sludge	SMOP+SLOM
Mortality	Monitor and increase number of fish samples if mortality increases	Adapt to investigation

Figure 2. Quarterly routine screening pattern for an epidemiologic unit. Histopathology can be performed on all euthanized and promptly fixed fish. Note that quarantine should not be part of the main epidemiologic unit, it is included here for convenience. In absence of fish that are imported or otherwise screened, sample the quarantine quarterly as appropriate (for example, sludge, sump surface swab; at least one sample type per quarter and per system).

point, all remaining fish in the sentinel tank are euthanized and available for screening. The 5 colony fish sampled every quarter would then be less than 6 mo of age after fertilization and/or any fish euthanized due to illness. Colony fish should also be selected based on their genetic background, using genetically altered fish or wild-type lines that differ from the sentinels. This approach has the benefit of requiring selection and tank placement of prefiltration sentinel fish only once a year, while colony fish of different ages can be selected throughout the year.

Use both Broad and Specific Diagnostic Tools

Testing at least some young and some aged fish by histopathology is recommended. Infectious and noninfectious diseases, including those likely related to husbandry, can be identified in histologic sections, whereas techniques like PCR and bacterial cultures only allow the identification of specific pathogens. Screening fish that are less than 6 mo of age by histopathology may augment monitoring of fish growth performance. Monitoring young and old fish by histopathology may be key for identifying effects of husbandry practices by allowing assessment of lesion and tumor prevalence or reproductive organ development and health. Fish found dead should not be screened by histopathology due to the promptness of postmortem autolysis. Dead fish may be useful for PCR, but postmortem bacterial overgrowth should be considered as a possibility when interpreting results.

Limitations of PCR. PCR can be performed on a large variety of samples (for example, fish, live feed, and environmental samples). However, the assay must be validated for use on pooled samples of the size submitted and on materials not previously tested with the assay. For example, algae and yeast found in raw materials (feed, filters, etc.) or yolk reserves may produce PCR inhibitors that can cause false negatives.[4,212,251] Some agents, like mycobacteria, may be hard to distinguish by simple PCR due to lack of specificity and may require sequencing.[90] Direct PCR screening of fish samples may lack sensitivity and yield false-negative results. For example, Infectious Hematopoietic Necrosis Virus surveillance may require a cell culture isolation step before molecular identification.[283] A diagnostic lab can advise on appropriate samples to submit for specific pathogen detection.

On the other hand, PCR can detect small quantities of inert DNA, which may be interpreted as a false positive. Any unexpected positive result should therefore be confirmed by a secondary method whenever possible (for example, visualization of a parasite or their eggs by microscopic examination, histology, or confirmation of the presence of bacteria or virus by culture or serology).

Nevertheless, PCR remains a master tool in the diagnostic toolbox. Amplification of target DNA extracted from fixed tissue in paraffin blocks is even possible. Considering that histopathology does not allow identification of *Mycobacterium* species, acid-fast positive histopathology lesions can be investigated further by PCR to determine the species of *Mycobacterium* present (for example, *M. haemophilum* or *M. marinum*) and to differentiate these pathogens from less pathogenic mycobacteria in a lesion. Still, identification of mycobacteria by PCR from formalin-fixed tissues is not always possible, and inconclusive PCR results may occur due to poor quality of the extracted DNA. The need to test such lesions can be mitigated by environmental screening and data representing morbidity, mortality, and lesion prevalence; a small number of localized acid-fast positive lesions (for example, due to opportunistic infection with environmental mycobacteria) may be expected when a large number of fish is screened.

Other investigative techniques. Clinical examination and diagnostic techniques in live fish include external gross examination, visual observation of oral and opercular cavities, and microscopic observation of cutaneous mucus, gill, and fin biopsies.[49,52,59,75,78,161,167,211,263] Blood collection for serology or molecular biology testing and percutaneous or laparoscopic biopsies of organs like the kidney and liver are other possible diagnostic techniques.[1,8,89,95,164,193] Serology is not used frequently in fish health surveillance. ELISA is the most common serological assay, mainly used for vaccine-related screening and epidemiologic survey.[48,125]

Figure 3 lists the fish pathogens that can be detected by nonlethal diagnostic examinations performed mainly by microscope (fresh smear with or without staining) and stereomicroscope.[12,73,164,211,263] Inhouse microscopic examination of wet mounts or stained preparations of mucus, gill, and fin biopsies can often identify parasites to the family or genus level. Alternatively, parasites can be sent to a parasitological

Pathogens	External observation	Cutaneous mucous	Gill biopsy	Fecal observation	Blood sampling	Coelomic fluid	Laparoscopy
Monogenetic trematode	Y	Y	Y	—	—	—	—
Digenetic trematode	Y	Y	Y	Y	—	—	Y
Nematode	Y	Y	Y	Y	—	Y	Y
Myxozoa	Y	Y	Y	—	Y		Y
Microsporidia	Y	Y	Y	Y	—	Y	Y
Protozoa	Y	Y	Y	Y	Y	Y	Y
Apicomplexa	—	—	Y	Y	—	Y	Y
Crustacea	Y	Y	Y	—	—	—	—
Bacteria	—	Y(1)	Y(1)	Y(1)	Y(1)	Y(1)	Y(1)
Saprolegnia spp./ Other fungi	Y	Y	Y	—	—	—	Y
Viruses	Y(2)	Y(2)	—	—	Y	—	—

Figure 3. Use of nonlethal samples for pathogen detection. Nonlethal diagnostic examinations are performed mainly by microscopy (that is, fresh smear with or without staining) and stereomicroscopy. Some procedures obviously depend on fish dimensions. Y indicates that pathogens of the listed category are detectable in fresh smear, biopsies, or serology. Lesion biopsies can be used for histology, microbiologic cultures, and molecular biology tests for speciation. (1) indicates that only a few bacteria can be identified to their genus level in a fresh smear due to their unique characteristics (*Flavobacteria* spp., *Tenacibaculum* spp., *Epitheliocytosis*, *Candidatus arthromitus*) or after specific staining (for example, acid fast bacteria). Most bacteria must be identified by culture, bearing in mind the difficulties of aseptic sampling and bacteria isolation from skin and gills. (2) indicates that only lymphocystis can be diagnosed on fresh smear.

laboratory for identification. Bacteria (for example, *Tenacibaculum* spp. or *Flavobacterium* spp.) can also be identified in wet mounts or stained smears observed under a microscope. Cutaneous mucus can be observed in wet mounts, stained in smears, or sent for microbiologic culture or PCR. Cutaneous mucus smears from ulcers stained with Ziehl-Neelsen are used to identify acid-fast bacteria such as *Mycobacterium* spp. and, more rarely, *Nocardia* spp., which can eventually be differentiated from mycobacteria by a branching presentation.[211]

When investigating sick, retired, or even recently dead fish, many techniques can be applied: macroscopic necropsy, serology, fresh mount microscopy, microbiologic culture, and histologic assessment. However, histology should only be used in freshly euthanized fish, ensuring that tissue fixation occurs before the onset of autolysis.[39,235,252,303,327] Both molecular biology and bacterial cultures are commonly used as complementary testing after necropsy.

Report Results

A complete reporting of health monitoring data must be accompanied by a clear description of the facility and its husbandry and biosecurity procedures to present an accurate picture of the microbiologic status and biosecurity risks of an EU. Moreover, this information may help explain nonprotocol variation when trying to reproduce experimental data in an aquatic animal model in a different facility. We propose reporting this information in 2 key documents per EU. Templates are provided online as supplemental materials Table S4 and Table S5. The documents are designed to be completed electronically using dropdown menus and automatic coloring.

Description of the EU. The first document (supplemental material Table S4) details the setting and management of the EU and includes sections detailing facility organization, water supply and quality, fish performance and husbandry, morbidity and mortality numbers, internal biosecurity procedures, and importation processes and quarantine procedures. The document should initially be completed when establishing a health monitoring program and updated when significant changes occur. This document is mainly designed to provide information for the purpose of exchanging fish. The template is designed for all fish species in an individual or multispecies EU and should be modified as necessary based on the facility.

Historical screening data. The second document (supplemental material Table S5) provides the health data derived from screening the EU. The provided template is an example of zebrafish data only and should be adapted as needed to include data from other fish species. It contains sections for recording test results for SMOP (*M. haemophilum*, *M. marinum*,

P. tomentosa, *P. neurophilia*), SLOM, other microorganisms, and other *Mycobacterium* spp. Another section lists and describes lesions identified by histopathology that are not attributed to a pathogen. This template is based on other templates reported in the laboratory animal literature,[54,232] with the goal of conveying the microbiologic status and the prevalence of infectious and noninfectious diseases in the EU. The reports should be updated as new results become available.

When this document is shared prior to shipping fish, the importing facility can use it to make decisions to protect the biosecurity of the resident fish and optimize the welfare of the imported fish. For example, the presence of relevant *Mycobacterium* species is recorded in different tables. The most pathogenic mycobacteria, *M. marinum* and *M. haemophilum*, are reported in the SMOP part of the first table. The presence of such microbes in an exporting facility may trigger a decision by the importing facility to decline the import or to prepare for strict quarantining and rederivation with diagnostic screening.[189] Conversely, detection of other mycobacteria (for example, *M. chelonae*, *M. fortuitum* and *M. abscessus*) is expected in aquatic facilities, and documentation of the presence of these agents may not impair exchange of fish between laboratories. Even so, a sudden increase in these organisms as a trend should trigger attention in a facility. That may include histopathologic evaluation of morbidities and corrective husbandry measures, especially when linked with increased morbidity or mortality, and also bearing in mind the zoonotic risk.[90]

Conclusion

As the use of fish as model organisms in biomedical research has expanded, so has our knowledge of the infectious and noninfectious diseases affecting these animals. The recommendations for health monitoring and reporting described here are based on our knowledge of diseases to date. We regularly add new diseases, pathogens, and clinical presentations to the list of factors that afflict laboratory fish and anticipate that the list will only grow as fish research expands. This is particularly true in the case of viruses, which are emerging potential pathogens for zebrafish. Health monitoring will continue to evolve to meet the needs of researchers grappling with reproducibility problems, identify new diseases, and enhance animal welfare. However, 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.

As discovered in the health monitoring survey, significant work is needed to improve biosecurity measures in some facilities. Given the importance of these measures, the working group developed a second manuscript to expand upon these concepts.[189] Further, to help facilities implement the above recommendations, scenarios have been developed using the documents presented in this publication.[189]

Laboratory fish husbandry is likely to change over the years to come. For example, the aquaculture industry is actively working on new feed options, new technologies for equipment disinfection are being developed,[190] and personnel working patterns include contingency plans. All of these parameters will affect biosecurity risks. Moreover, the widespread development of health monitoring and the availability of health reports and facility descriptive documents may help establish better epidemiologic sources of information and identify the main risks to manage in aquatic laboratories. To that effect, we would like to encourage establishments to make their health monitoring reports available publicly as that would open communication about disease and pathology between fish users.[7,154,247,326] Finally, we suggest that these recommendations and templates be reviewed in a few years to incorporate new data on biosecurity risks, emergent pathogens, research impacts of known pathogens and contaminants, and microbe prevalence.

Supplementary Materials

Table S1. Zebrafish diseases

Table S2. References on relevant pathogens of fish species other than zebrafish

Table S3. Multispecies facility context and assessment of pathogen contamination risk

Table S4. Fish health monitoring description

Table S5. Zebrafish Health Monitoring Report

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