



Recommendations for control of pathogens and infectious diseases in fish research facilities [☆]

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ABSTRACT

Concerns about infectious diseases in fish used for research have risen along with the dramatic increase in the use of fish as models in biomedical research. In addition to acute diseases causing severe morbidity and mortality, underlying chronic conditions that cause low-grade or subclinical infections may confound research results. Here we present recommendations and strategies to avoid or minimize the impacts of infectious agents in fishes maintained in the research setting. There are distinct differences in strategies for control of pathogens in fish used for research compared to fishes reared as pets or in aquaculture. Also, much can be learned from strategies and protocols for control of diseases in rodents used in research, but there are differences. This is due, in part, the unique aquatic environment that is modified by the source and quality of the water provided and the design of facilities. The process of control of pathogens and infectious diseases in fish research facilities is relatively new, and will be an evolving process over time. Nevertheless, the goal of documenting, detecting, and excluding pathogens in fish is just as important as in mammalian research models.

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1. Introduction

The use of fish as models in biomedical research has dramatically increased in the last decade, largely led by the development of the zebrafish (*Danio rerio*) model (Zon, 1999; Dodd et al., 2000; Rubinstein, 2003; Wixon, 2000; Ackermann and Paw 2003). Indeed, by 2003 some 250 projects funded by NIH used zebrafish models alone (Henken et al., 2003; Rasooly et al., 2003). Japanese medaka (*Oryzias latipes*) is also widely used in biomedical research, particularly in toxicology (Law, 2001a; Hawkins et al., 2003; Hinton et al., 2005; Shima and Mitani, 2004) but this species is also being used for infectious diseases studies (Sarmasik et al., 2002; Broussard and Ennis

2007). Other fishes commonly used in biomedical research include goldfish (*Carassius auratus*) for infectious disease studies (Talaat et al., 1998), sticklebacks (*Gasterosteus* spp.) for behavioral and speciation research (Cresko et al., 2007), and rainbow trout (*Oncorhynchus mykiss*); mummichog (*Fundulus heteroclitus*), gulf killifish (*Fundulus grandis*), sheepshead minnow (*Cyprinodon variegatus*), fathead minnow (*Pimephales promelas*), guppies (*Poecilia reticulata*) and swordtails and platys (*Xiphophorus* spp.) for toxicology and oncology (Bailey et al., 1996; Hawkins et al., 2003; Law, 2001b; Walter and Kazianis, 2001; Winn, 2001).

Concern about infectious diseases in research fishes is increasing as fish resources in research institutions are becoming centralized, where breaches in proper biosecurity measures could lead to rapid spread of pathogens. In addition to acute diseases causing severe morbidity and mortality, underlying chronic conditions that cause low-grade or subclinical infections may confound research results (Baker, 2003). Whereas the occurrence of underlying diseases causing low mortalities may be acceptable for production aquaculture or with ornamental fish they should be avoided in fish (as with any laboratory animal) used in

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research. Fishes used for research also share the same domestication history that characterized the advent of laboratory mice in that evidence-based husbandry and preventive medicine came much later than research discoveries. Specifically, there are fewer peer-reviewed publications for fish in research today that describe optimal and experimentally validated nutrition, water quality, stocking density, veterinary care, etc. Consequently, laboratory fishes continue to be colonized with microorganisms that often cause disease, death or may affect research data. Because morbidity and mortality rates associated with these microbes are “acceptably” low, scientists have become accustomed to tolerating their presence even though reproductive capacity and research data may be adversely affected. In addition, the growing use of genetically engineered zebrafish is likely to modify current tenets of husbandry and disease due to novel and unpredicted interactions between host genes, pathogens, and environment.

Underlying, chronic infections in laboratory fishes has also become more important as the use of fish models (particularly zebrafish) expands to areas of research that require experimental animals to live in a healthy state for long periods, such as for research on infectious diseases, immune system function, and toxicology. Underlying chronic infections can enhance morbidity associated with toxicant exposure (Pascoe and Cram, 1977; Jacobson et al., 2003), and confound histological interpretations in chronic toxicant exposure laboratory experiments (Capps et al., 2004).

2. Infections and carcinogenicity studies

Underlying chronic infections may affect carcinogenicity studies. The inflammation and cell injury caused by infections can increase tumorigenesis, for example, by increasing the rate of cell turnover/proliferation (Ward et al., 1994; Hailey et al., 1998). Such unintended and often cryptic infections can have disastrous consequences in long term studies, at best resulting in a waste of time and money, and perhaps at worst leading to duplication of animal research and faulty conclusions concerning the safety of pharmaceuticals, industrial chemicals, or other compounds being tested.

Examples of this phenomenon are found in laboratory fishes; several parasites are recognized as promoters of chemically induced neoplasms in mammals. The capillarid nematode *Pseudocapillaria tomentosa* is a common intestinal infection in zebrafish, particularly those reared by ornamental fish dealers in ponds. An increase in intestinal neoplasms was observed in zebrafish exposed to dimethylbenzanthracene (DMBA) that were also infected with the nematode (Kent et al., 2002).

It is possible that some studies using fish may be confounded by unidentified, background oncogenic viruses. Beckwith et al. (2000) reported 100% incidence of cutaneous papillomas in Florida strain wild-type zebrafish following exposures to *N*-nitroso-*N*-ethylurea (ENU). Many zebrafish at the University of Oregon, Harvard University (Leonard Zon, personal communication), and Cornell University (Jim Casey, personal communication) have been mutagenized using ENU by protocols similar to those used by Beckwith et al. (2000), yet cutaneous papillomas have not been seen in those experiments. Likewise, we exposed 3-wk-old zebrafish fry (TL and KOLN lines) to ENU. While we observed a variety of neoplasms none developed epithelial skin tumors (Spitsbergen and Kent, 2002). One potential explanation for the differences in results in these ENU studies is that the population that developed papillomas was infected by an unrecognized oncogenic virus. Although oncogenic viruses have not been detected in zebrafish, there are several reports of these viruses associated with skin tumors in other fishes (Wolf, 1988; Bowser and Casey, 1993; Waltzek et al., 2005).

3. Present status of knowledge about infectious agents in research fishes

There are very few pathogens that have been recognized in fishes in the research setting, compared to the long list of those in fishes used in

aquaculture. Only a few viral diseases have been described from the many hundreds of aquarium fish species (Lewis and Leong, 2004; Hedrick et al., 2006; Whittington and Chong, 2007). This is likely not due to the absence of pathogenic viruses in medaka, zebrafish, etc., but rather reflects the lack of viral examinations that have been conducted on these species. Guppy reovirus (Choo, 2001) has been isolated from guppies in United States, but its pathogenesis is not fully understood. A nodavirus has also been isolated from moribund guppies (Hegde et al., 2003). Guppies (as well as zebrafish) are susceptible to spring viremia (Sanders et al., 2003), which is a common and lethal rhabdovirus of carp and other cyprinid fishes. Several iridoviruses have been reported from a variety of aquarium fishes. Most have not been cultured or characterized, but recently Jeong et al. (2008) showed that a group of similar iridoviruses infected many species of aquarium fishes, and were lethal to some of these.

Some of the fish species used in research are also popular aquarium fishes (e.g., zebrafish, swordtails), and thus information on their diseases and their control has been based largely on anecdotal observations and popular publications. However, as the importance of these models has increased in recent years, there are a few scientific reports documenting specific diseases afflicting them in a research setting (Matthews, 2004). A search of the literature reveals reports of infectious diseases in research fishes being caused by agents already recognized as pathogens in aquarium or food fishes, such as *Piscinoodinium pillulare* (Westerfield, 2007), capillarid nematodes in zebrafish (Kent et al., 2002), Gram-negative septicemias (Pullium et al., 1999) and mycobacteriosis (Abner et al., 1994; Teska et al., 1997; Sanders and Swaim, 2001; Astrofsky et al., 2000; Kent et al., 2004; Watral and Kent, 2007; Whipps et al., 2007b). A few other pathogens with narrower host specificity have caused problems in research settings, such as microsporidia in zebrafish (Matthews et al., 2001) and stickleback (Kent and Fournie, 2007).

Mycobacteriosis, caused by several species in the genus *Mycobacterium*, is the most common infectious diseases encountered in laboratory fishes reared in warm water (e.g., >20 °C). The following *Mycobacterium* species have been isolated from medaka or zebrafish: *M. abscessus*, *M. chelonae*, *M. fortuitum*, *M. haemophilum*, *M. marinum*, *M. peregrinum* (Astrofsky et al., 2000; Kent et al., 2004; Sanders and Swaim, 2001; Teska et al., 1997; Watral and Kent, 2007; Whipps et al., 2007a; Kent et al., 2008). Histological observation of acid fast bacilli in sections is reliable in routine surveillance for mycobacteria (Whipps et al., in press). Compared to Gram-negative bacteria, culture and conducting biochemical tests for species identification is usually more difficult for mycobacteria. Therefore, PCR and DNA sequencing or restriction fragment length polymorphism of the *hsp65* gene is the preferred method of identification for non-tuberculosis mycobacteria (Kim et al., 2005; Ringuet et al. 1999; Whipps et al., in press). This can be accomplished either directly from DNA extracted from infected tissues or from cultures. The ability to distinguish species and strains of mycobacteria cannot be underemphasized. With zebrafish, *M. chelonae* appears to be widely distributed but under normal environmental conditions is less likely to cause disease, while *M. marinum* and *M. haemophilum* are much more virulent but fortunately are less frequently encountered in zebrafish colonies (Astrofsky et al., 2002a, b; Kent et al., 2004; Watral and Kent, 2007; Whipps et al., 2007b; Ostland et al., 2007). Unique strains of species that infect zebrafish and medaka may be present in a single facility (Whipps et al., in press), and may exhibit dramatic differences in virulence (Ostland et al., 2007). The ability for at least some, if not all, of these species to persist in surface biofilms in aquatic systems (Beran et al., 2006) presents additional challenges for interrupting the cycle of infection once established. This highlights the role of a quarantine program to minimize the potential introduction of these bacteria, regular monitoring of populations to remove infected fish, and the routine cleaning and disinfection of impacted tanks and husbandry equipment.

4. Role of inflammatory mediators in development

Certainly, agents that evoke chronic inflammation, such as parasites and mycobacteria, may affect studies investigating inflammatory mediated pathways. Alterations in the balance of such inflammatory mediators due to infectious diseases can greatly disrupt normal tissue development and homeostasis, and may confound many of the parameters of interest to scientists studying zebrafish as models for human diseases tissues (Chaisson et al., 2004; Doherty, 2007; Hernandez-Gutierrez et al., 2006; Boersma and Meffert, 2008). TNF α is also a potent endogenous mutagen acting by production of reactive oxygen species (Yan et al., 2006).

5. Comparing aquatic and mammalian biomedical research

Approximately 80% of all animals used in research are rodents, compared to the 9% that are aquatics. Given the much longer use of rodents as laboratory animals we felt it was worthwhile to compare the two in regard to infectious diseases. It has been recognized over the years that doing research with pathogen free mice is important in order to minimize research impact and generate good research data. Although met with resistance by some researchers, this concept is gradually being accepted by laboratory aquatic biomedical researchers using zebrafish. The impact on research results, even for the most common diseases found in zebrafish housed in recirculating systems is not well understood or described. For example, one could speculate that *Pseudoloma neurophilia*, which infects the brain, spinal cord and muscle of zebrafish could potentially affect studies involving phenotype development, developmental neurotoxicity testing, brain-specific cell death, behavioral research and musculoskeletal development. Also, mycobacteriosis, which is known to cause nodules in various internal organs, can lead to granulomatous nephritis and hepatitis that could potentially affect kidney and liver function, the immune system and disease expression in genetically engineered fish such as the recombination activating gene 1 (rag1) mutant. The ability to reliably survey for and exclude murine pathogens from research colonies has escalated over the last several years. New murine pathogens continue to be recognized (e.g., Mouse Parvovirus, Mouse Norovirus, *Helicobacter*, etc.). Health surveillance is performed routinely as a combination of methodologies. With rodents, the methodologies frequently employed to detect pathogens include gross examination; cultural and *in vivo* isolation of microorganisms; serology for detection of microbial antibodies formed in response to infection; and infectious agent detection and identification by microscopic, biochemical, and molecular biology techniques. Thus, with some modifications, pathology, parasitology, and bacteriology can be performed equally well in fish and in rodents. Cultures are usually incubated aerobically at 35°–37 °C in rodent health screenings because the majority of clinically important bacteria are facultative anaerobes that will grow under these conditions. As fish are poikilothermic, culture of bacteria and viruses from them is usually done at lower temperatures. With regard to routine virus monitoring of laboratory mammals, these methods are generally performed as antibody assays with known antigens to delineate the strain specificity and thus the etiology of the viral antibody response. In addition to lack of information on potentially important viruses in most laboratory fishes, serological testing for viruses in fish in general is seldom used.

The availability of specific pathogen free (SPF) mice is a cornerstone for control of infectious diseases in laboratory research for this species. Whereas SPF salmonids are available, this concept has only recently been adopted for zebrafish (see below). Because many aquatic pathogens or opportunistic organisms colonize biofilms in biological filters, on tanks and pipes, the current state of knowledge regarding best husbandry practices and system designs may have to be reassessed to maintain SPF fish in recirculating systems. Confirmation that a fish is definitely SPF is predicated on the assumption that the diagnostic test is 100% sensitive. Also, as most diagnostic tests for

fish require lethal samples, designation of a population to be SPF is based on the number of fish sampled. Preventing the spread of diseases in fish facilities is minimized by the use of ultraviolet (UV) sterilization of water used in recirculating systems and avoiding cross contamination of tanks with nets, hands, and equipment.

Exclusion of murine pathogens is also enhanced by the broadening pool of individuals capable of embryo rederivation for the creation of “clean” stocks and cryopreservation for recovery in the face of a catastrophic outbreak. In salmonid aquaculture, sex products (e.g., ovarian fluid and milt) are screened for pathogens. This could also be applied to laboratory fishes, particularly as cryopreservation of gametes continues to expand.

We now face many of the same problems with fish species as we did with mice just a couple of decades ago. However, problems with fish are compounded by the diversity of species used. This diversity includes both a wide variety of species and a diversity of preferred habitat (e.g., marine, brackish, fresh-water). This diversity is further complicated by the source of the animal because many of the species used are not readily available from commercial vendors specializing in laboratory animals, as is the case with mice. Many times the researchers must rely on sources such as the ornamental fish trade, or farm-raised and wild-caught stocks. With the growth of zebrafish as a model we have seen a shift from pet store purchases to stocks that are available from the NIH Zebrafish International Resource Center (ZIRC) and collaborators at controlled research institutions. However, this is not necessarily the case with other species of fish.

6. Comparing laboratory fishes with aquaculture and ornamental fish

Zebrafish, swordtails, platys, and guppies are both important fishes for research and as popular ornamental fishes. However, the acceptability of underlying, chronic infections by infectious agents in research fishes is quite different compared to home aquaria. Fishes for the ornamental fish trade are usually reared in outdoor ponds, and often are infected with a variety of pathogens and parasites. While these often do not cause overt disease, as mentioned earlier, these infections should be avoided if fish are used for research. Therefore, quarantine strategies for fish destined for research are more complicated than what is recommended for a fish destined to a home or public aquarium (see below). The health status in food fish aquaculture also differs from that of research fishes. Subclinical infections that don't affect mortality and feed conversion may be acceptable when the endpoint is meat production, but these should be avoided, when possible, in research animals as they may confound experiments and results. For example, an important endpoint in zebrafish health is fecundity. A microorganism that caused reduction in fecundity (even if otherwise non-pathogenic) should be avoided.

Comprehensive reviews and books on diseases in captive fishes and reports on specific diseases and protocols for laboratory fishes can be found elsewhere (Woo, 2006; Stoskopf et al., 1993; Casebolt et al., 1998; Hoffman, 1999; Woo and Bruno, 1999; Noga, 2000; Ostrander, 2000; Dykstra et al., 2001; Astrofsky et al., 2002a,b; Lee and O'Bryen, 2003; Matthews, 2004; Nickum et al., 2004; Batt et al., 2005; Pimenta-Leibowitz et al., 2005; Scarfe et al., 2006; Ferguson, 2006; Kent and Fournie, 2007). Our intent here is to present general recommendations and strategies for researchers, fish technicians, and veterinarians for controlling pathogens of fishes in research situations.

7. Recommendations

7.1. Identification of important pathogens for aquatic species of interest

Knowledge of which pathogens are of concern for each species is a corner stone for developing control strategies — i.e., one must know which pathogens must be avoided and which might be mere

opportunists or cause little disease. Even for the latter, researchers should be aware of their presence when they occur in their experimental fish. Information on the following would be very useful for implementation of a control plan. 1) The ability of an agent to cause mortality, morbidity or cause other problems (e.g., reduce fecundity). For example, a pathogen may cause only subclinical infections in one species, while it may induce severe disease in another. 2) Modes of transmission – can the pathogens be transmitted between fish (horizontally), within eggs (vertically), and can parasites with direct life cycles rapidly proliferate in confined research systems? 3) Are there effective drugs or disinfectants? For example, it has been discovered that chlorine utilized at levels commonly recommended in zebrafish rearing are not effective for killing spores of the microsporidium *Pseudoloma neurophilia* (Ferguson et al., 2007). 4) Host specificity and geographic distribution – for example, some species of *Mycobacterium* are ubiquitous in fish systems that house various fish species.

7.2. Strategies for avoiding introduction of pathogens

Water provides an excellent vehicle to transmit pathogens, and is the source of many important diseases in captive fishes. However, research laboratories have an advantage compared to many large aquaculture operations, as the volume of incoming water is relatively small, allowing for thorough filtration and disinfection before exposure to fish.

The best way to control infectious diseases is to avoid their introduction, regardless if the facility is new or existing. In general, avoidance of introduction of fish pathogens is accomplished by 1) using fishes from pathogen free suppliers, 2) holding fishes in quarantine for several weeks before introduction into the research facility, 3) prophylactic treatment with therapeutic agents via static baths, parental drug administration, or orally via medicated feed to remove external parasites, treat a diagnosed disease, or reduce shedding of pathogenic agents, 4) avoiding introduction of contaminated water with new fishes into the main systems, 5) use foods free of pathogens (live foods, particularly tubificid worms, can be vectors for fish pathogens), 6) filtration and disinfection of incoming water to the main system by either ultraviolet light sterilization or ozonation, and 7) using only eggs for new stock whenever possible. Eggs should be surface disinfected with either iodophors (for salmonid eggs) or free chlorine (i.e. from sodium hypochlorite as used for zebrafish eggs) to remove external pathogens. Reviews on quarantine and prevention procedures are provided in several sources (e.g., Stoskopf et al., 1993; Gratzek, 1993; Ostrander, 2000; Noga, 2000; Wedemeyer, 2001; Wildgoose, 2001; Astrofsky et al., 2002a,b; Matthews et al., 2002; Kent and Kieser, 2003; Harms, 2003). One down side of excessive time in quarantine is that fish may be more crowded than usual, which may induce other health problems. Prophylactic treatments in quarantine may induce changes in the immune response, such as reducing the time of allograft rejection.

7.2.1. Source history

Avoidance of pathogens begins with obtaining research fishes from reputable suppliers that can provide a general disease history of their stocks. It is generally better to obtain fishes that have been reared in enclosed systems, versus pond-reared or wild-caught fishes. Aside from certain stocks of salmonids, most fish suppliers do not provide SPF fish.

Many apparently healthy fish, particularly those from the wild or ponds sources, arrive with varying degrees of infestations by external parasites (monogeneans, ciliates, and flagellates). The most common method to remove these parasites is to immerse the fish in short term chemical baths like formalin (Noga, 2000; Wildgoose, 2001; Harms, 2003) or prolonged immersion in lower doses of chemicals like copper sulfate (Wildgoose, 2001; Harms, 2003). The toxicity of formalin or copper sulfate baths varies considerably among fish species and with water quality parameters, so it is important to test your water

quality parameters, adjust your dosage accordingly, and treat a few individuals before applying any treatment to large numbers of fish.

7.2.2. Quarantine

Chemical baths will not eliminate internal pathogens. Thus careful observation of fish in separate quarantine systems is advised. In addition complete necropsy and disease evaluations on a subset of representative specimens of new stocks of fish before introduction should be considered, particularly if these fish are introduced directly to the main system, rather than using the next generation. Regardless of the length of quarantine, subclinical infection may be missed. Pathogens can also reside in aquarium biofilms and detritus. Therefore, quarantine tanks, filters, etc. should be thoroughly cleaned and disinfected following removal of each fish population (Wedemeyer, 2001; Wildgoose, 2001; Harms, 2003; Danner and Merrill, 2006).

The duration of quarantine can be anywhere from two weeks to as long as ninety days depending upon the pathogens of concern and their rate of detection, the purpose for the animals, and the established biosecurity practices at the facility. During quarantine fish should be monitored for signs of sickness (e.g., anorexia, lethargy, respiratory rate changes, equilibrium disturbances, skin lesions, changes in skin color or body conformation, and behavioral abnormalities). When seen, sick animals should be isolated in separate tanks until diagnostic evaluation by veterinary services or fish health professional. During this time the remainder of the population should be monitored for the development of similar or different clinical signs. Treatment of fish during quarantine should be limited, unless the animals will be eventually introduced into a captive population. All treatment should be based upon appropriate diagnosis and selection of efficacious agents used for the appropriate period of time. Prophylactic treatments can inhibit the development of clinical signs and inappropriate use of antibiotics will lead to the development of antibacterial resistance (Wildgoose, 2001; Harms, 2003).

7.2.3. Eggs only

Transport of fish into new facilities using only surface disinfected eggs, the “eggs only policy”, is a cornerstone of many regulatory agencies overseeing salmonid health (Wedemeyer, 2001; Kent and Kieser, 2003). There should also be strict adherence to bringing in only surface disinfected eggs into the facility. With salmonids, this is facilitated by the long time hatching time (many weeks). Other fishes used in research have much shorter hatching periods, and thus it may be more practical to ship adults, hold the fish in quarantine, spawn fish, surface disinfect eggs, and then use only resulting progeny in the main facility. With live-bearers (e.g. viviparous fishes in the family Poeciliidae), it is essentially impossible to avoid transmission exposure from adults to progeny. Therefore, very thorough inspections of fish in quarantine with perhaps prophylactic treatments is the most practical alternative. In addition, live-bearers should be housed on separate systems from egg-layers (oviparous).

7.2.4. Specific pathogen free (SPF) fish

Aside from certain stocks of salmonids, most fish suppliers do not provide SPF fish. If incoming fish are infected with pathogens capable of vertical transmission, then quarantine and eggs only methods may not prevent introduction of these pathogens. In this situation, the only way to prevent introduction of the pathogen would be to assure that the fish are free of the specific pathogen. A strategy to have SPF zebrafish (*Pseudoloma*-free) at a new, centralized zebrafish facility at the Sinnhuber Aquatic Research Laboratory (SARL) at Oregon State University (OSU) has been implemented. The following summarizes the approach: 1) spawn fish as individual pairs or small groups, 2) rear larvae in isolation, 3) spawn the same pair or small groups multiple times if needed until 1000 larvae are obtained. This group of 1000 fish will represent 1 family. Using terminal procedures, 258 fry from the family at 10 days post fertilization and the brains of the parents are

screened for *Pseudoloma* infection using the PCR test developed by Whipps and Kent (2006). This allows for detection of infection at 1% prevalence or greater with a 95% confidence (AFS-FHS, 2007). If all samples are negative, then the remaining fry are transferred to the SARL. To date 11 families of 8 lines have been screened and transferred to this new facility. This approach could be implemented for other pathogens that may be vertically transmitted.

7.3. Controlling spread of pathogens within facilities

Even with strict entry measures, most facilities will eventually encounter some problems with infectious diseases. These are often opportunistic pathogens that are common in aquarium water and do not require fish hosts to proliferate. An example is *Mycobacterium chelonae*. There are many reports of infections in fish by this species, but it might be better considered an ubiquitous opportunist as it is often found in aquarium water, biofilms and in fish in the absence of disease (Schulze-Röbbecke et al., 1992; Beran et al., 2006) or in water in the absence of fish (Primm et al., 2004).

Moribund and dead fish should be removed at the first sign of detection. Often the very first sign of morbidity in infectious diseases is anorexia. Therefore, staff responsible for feeding should be trained to carefully observe feeding and other behaviors of fish. Culling of older fish (e.g., zebrafish older than 1.5 y) will often reduce the prevalence of chronic diseases, such as neoplasms and mycobacteriosis. In addition to avoidance and culling strategies, we recommend the implementation of surveillance programs so that the identity, prevalence and distribution of pathogens and their associated diseases are known. Two programs facilitate this: sentinel programs and routine monitoring of fish (particularly moribund fish) from the main research facility.

7.3.1. Routine monitoring

We recommend at least the opportunistic sampling and histological analysis of moribund or dead fish be apart of every fish health program. In some cases, when no signs of disease are present in a colony, it is desirable to know whether or not a pathogen is present in the population. This may be particularly important for distributors that wish to declare their fish free of specific pathogen, or for screening the founder population when a new colony is established. A realistic sampling regime is one that will detect at least one infected animal with 95% confidence (AFS-FHS, 2007; Office International des Epizooties OIE, 2006). To determine sample size from a given population, the equation below can be used (Thrusfield, 2005). The calculation requires a presumed prevalence of the pathogen which is ideally based on empirical data from previous surveys. In all likelihood, one must estimate this value from studies at other facilities or extrapolate from small pilot surveys or data collected from histological evaluation of moribund fish. Another assumption of this estimation is that the diagnostic method has 100% sensitivity and specificity. AusVet's EpiTools are an excellent resource (<http://www.ausvet.com.au/content.php?page=epitools>) to determine sample sizes that incorporate known sensitivity and specificity values, or for other epidemiological calculators,

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

where:

- n required sample size
- N population size
- p probability of finding one infected fish, usually set at 95% ($p=0.95$)
- d minimum number of infected fish expected given a presumed prevalence (P) so that $d=P \times N$.

Table 1

Sample sizes required for a population (N) to detect at least one infected animal assuming a prevalence (P), with 95% confidence

N	P												
	0.5	0.4	0.3	0.25	0.2	0.15	0.1	0.05	0.02	0.01	0.005	0.001	
10	4	5	6	7	8	9	10	10	10	10	10	10	
20	5	6	8	9	10	13	16	20	20	20	20	20	
50	5	7	9	10	13	16	22	35	48	50	50	50	
100	5	7	9	11	14	18	26	45	78	96	100	100	
200	5	7	9	11	14	19	27	51	105	156	191	200	
300	5	7	9	11	14	19	28	54	118	190	260	300	
400	5	7	9	11	14	19	28	55	125	211	311	400	
500	5	7	9	11	14	19	29	56	129	225	349	500	
600	5	7	9	11	14	19	29	57	132	236	379	597	
700	5	7	9	11	14	19	29	57	134	243	403	691	
800	5	7	9	11	14	19	29	57	136	250	422	782	
900	5	7	9	11	14	19	29	58	138	255	437	868	
1000	5	7	9	11	14	19	29	58	139	259	451	951	

There are very few non-lethal diagnostic tests for fish. Therefore, conducting a statistically relevant survey for pathogens in small, valuable populations is often not possible. For example in a population of 20 fish and presumed prevalence of 10%, all 20 fish would need to be examined (Table 1). Therefore, the best alternative with small populations is to focus on examination of moribund fish. Many aquaculturists and aquarium fish hobbyists don't pursue the cause of mortality when mortality rates are minimal – i.e., they consider this “normal mortality”. However, there is great value in routine evaluation of the cause of morbidity, even if only a few fish are affected. These examinations not only provide a background of pathogens and pathological changes in the population, but also allow for early detection and implementation of control measures to head off an outbreak.

7.3.2. Sentinel programs

As stated above, it is quite often impractical to examine sufficient numbers of fishes from a sufficient number of distinct populations to have adequate knowledge on the pathogen status within a facility. An effective solution is the use of a sentinel program, as frequently used in murine research laboratories. The recirculating systems used in most fish facilities are well suited for sentinel programs. The concept here is to expose sentinel fish to effluent water from each system before the water is filtered or treated with UV. SPF fish are placed in a sentinel tank, continually exposed to effluent, and then all the sentinel fish are examined at a specific date. For zebrafish, we recommend that they be held at least 3 months to detect chronic infections such as mycobacteriosis and *Pseudoloma*. For zebrafish and medaka, fish are examined by histology. With other fishes such as carp and trout, the sentinel fish could also be screened for specific bacteria and viruses using culture.

Detection of a specific disease or pathogen in the sentinel fish demonstrates that this is occurring in at least one tank animals on the main system. Various actions would be taken depending on the severity of the disease and the needs of the researchers. Options include screening individual populations to determine which stocks are afflicted and quarantining the system to avoid transmission to other systems. If a new or highly virulent pathogen that is untreatable is found investigators and veterinarians may decide to euthanize the population and clean and disinfect the system (Danner and Merrill, 2006).

8. Systems design

The design of fish holding facilities for toxicological, biomedical and behavioral studies is often based upon the welfare requirements for maintenance of the fish in terms of water source and volume, flow rates, temperature and environmental enrichment requirements. However, consideration also needs to be given to the prevention and control of infectious disease.

Fish housing systems are typically configured in one of three main designs; static, flow-through, or recirculating. Systems can also be configured using a combination of these designs. Recirculating and static configurations are most commonly used for small tropical fish like zebrafish and medaka, whereas flow-through is most commonly used for larger more traditional aquaculture species like salmon and trout. (Ostrander, 2000; Wedemeyer, 2001; Astrofsky et al., 2002a,b; Courtland, 2002; Stoskopf, 2002) The design of fish holding facilities, including the rooms, corridors and tanks is crucial for effective bio-containment, prevention and control of infectious disease.

Water sources for these systems can come from municipal sources (tap water), unprotected sources (surface water from lakes or rivers), protected sources (wells, aquifers, or springs), or from artificial sources (reverse osmosis or distillation). The latter source is used most commonly in zebrafish and medaka facilities, whereas municipal or protected sources are used most commonly with larger aquatic research facilities and aquaculture facilities. Regardless of the source selected, the water must be adequately pre-treated (particulate filtration, degassing, activated carbon filtration, sand filtration, and UV sterilized) to remove unwanted agents (e.g., chlorine, chloramine, heavy metals, excess dissolved gasses, etc.) before it should come in contact with the fish species to be housed (Harms, 2003; Wedemeyer, 2001; Courtland, 2002). If a process like reverse osmosis or distillation to pre-treat water is used, then salts, minerals, and electrolytes will have to be added after these filtration processes. There should be enough on-site holding capacity of pre-treated water or the ability to provide a constant supply, depending upon your systems configurations, especially in the event of emergencies.

Tank rooms should be located away from laboratory areas that may be used for disease diagnostic purposes and should have controlled access for permitted laboratory staff only. Standard procedures for entering the facility (logging in and donning protective clothing) need to be established before entering specific rooms. Careful logging of materials entering (and leaving) the facility should also be maintained. Individual tank rooms may be used for different purposes, based on the size and shape of tanks within the rooms or the nature of the work to be undertaken. For efficient cleaning and disinfection, the floors, walls and even ceiling finishes should be non-porous, impervious, non-corrosive, and resistant to disinfectants. Light fixtures should be sealed and be of waterproof construction. If natural light is not provided from windows, then lights should be on programmable timers in order to provide adequate photoperiods for the fish species housed. Light timers must not be routinely overridden and if access to the animal housing area is required during the dark portion of the light cycle red lights can be used to help minimize interruptions in established photoperiods. Electrical outlets should be individually ground fault interrupted (GFI), be connected to specific GFI circuits and located in areas in which their exposure to water is minimized. In areas where this is not possible, outlets should have appropriate protective covers (plastic bubble type or self closing type). For each room, there should be a hand-washing sink or hand sanitizers for staff entering and leaving the room. If necessary there should be facilities for disinfecting footwear before entering and leaving. This additional measure depends upon the type of work done within the facility and the biosecurity measures required. Within each tank room, the positioning of each tank is usually determined by the space available and the original designed layout of the room, particularly for insulated tanks containing larger volumes of water. Care must be taken to avoid cross contamination by splashing. Moreover, fish pathogens can also be spread by aerosols created by aeration (Wooster and Bowser, 1996; Roberts-Thomson et al., 2006). Adjacent tank lids should be kept down and if practical, physical barriers be installed between tanks. Each tank room needs an area of benching for manipulation of aquaria, etc. Because of the liberal use of disinfectants and possible need to conduct experiments with marine fishes, fittings should be stainless steel or plastic wherever possible. To facilitate

cleaning and disinfection of the room, storage of consumables and equipment within tank rooms should be minimized. A dedicated feed storage area outside the tank rooms but within the bio-secure area should be provided. For large species, a post mortem room or area should be similarly provided with easily disinfected surfaces and stainless steel benches.

The size and shape of tanks can be extremely variable and custom designed systems are often built. In general, tank systems for larger fish often utilize round opaque and insulated tanks between 50 and 1000 l capacity with continuous flow-through, whereas toxicological or behavioral studies frequently use small fish species in glass or plastic aquaria maintained on rack systems in temperature-controlled facilities with limited water exchange depending on the experiment. Glass should be minimized due to its extra weight and the issues and dangers associated with breakage. Zebrafish and other small tropical species racks, with self contained recirculating systems have become popular. Large facilities have begun incorporating large recirculating systems with sometimes redundant capacity in which the same water system may be used for hundreds of tanks. These are equipped with industrial grade biofiltration systems (like fluidized sand filters) and multi-lamp UV sterilizers. Regardless of the size or scope of the systems, it is critical to perform and record routine scheduled maintenance on each systems associated components at least as per manufactures recommendations. It is very important to adequately pre-filter the water to remove particulate waste, maintain the clarity of the quartz sleeves and to change the UV bulbs at the recommended schedules to optimize the effectiveness of UV sterilizers. UV dose is defined as intensity \times time and is expressed in units of microwatts per square centimeter per second (mW/cm²/s). The required dose is related to the size and transparency of the organism to UV radiation (Wedemeyer, 1996, 2001). Recommended UV dosages needed to inactivate common fish pathogens are as follows. For bacteria like *Aeromonas* spp., *Vibrio* spp., and *Pseudomonas* spp. between 4000 and 5000 is required. As low as 2000 is required for viruses like Infectious Hematopoietic Necrosis Virus (IHNV) and Channel Catfish Virus (CCV), but agents like Infectious Pancreatic Necrosis Virus (IPNV) 150,000 is required. For myxozoans (e.g., *Myxobolus cerebralis*) 27,600 is required. To inhibit the growth of fungal hyphae from *Saprolegnia* spp. requires at least 230,000 (Wedemeyer, 1996, 2001). Microsporidia (now also considered relatives of fungi) are more fragile. A three log reduction in viability of *Encephalitozoon* spp. spores requires between 6 and 19,000 (Huffman et al., 2002; Marshall et al., 2003). Indeed, our observations from one large zebrafish facility suggest that 30–50,000 reliably kills spores of *Pseudoloma neurophilia*.

A principle design feature should be that cleaning and maintenance can be easily carried out. Invariably, this means that adequate space is provided between tanks or runs of tanks. There are compromises to be made regarding maintenance of pipe work, valves, etc. and the need for disinfection and cleaning. Each tank or water system must have its own net(s) and other equipment as required with facilities within the room for initial disinfection of these.

8.1. Quarantine

All facilities should have a dedicated quarantine room, or at least a separated quarantine area. As the name implies, strict separation of fish, water, and equipment must be maintained between the quarantine area and the main facility. This is accomplished by having dedicated equipment and only staff trained in sterile or aseptic techniques and other policies for avoiding pathogen transmission that are allowed in the area. Rigid policies should be in place to ensure staff working inside the quarantine area does not transfer potential pathogens outside of the area or into the main facility. Policies should include having designated protective clothing, dedicated husbandry and care equipment for use in the quarantine facility, prohibiting staff from moving directly from quarantine to the

main facility, hand washing after leaving the facility, and thorough disinfection of equipment and wastes required to leave quarantine. For the former chemical disinfection may be adequate but for the latter, autoclave disinfection may be advised (Wedemeyer, 2001; Harms, 2003).

8.2. Staff

Appropriate training of husbandry and investigative staff in understanding the importance of control of infectious diseases and procedures to avoid pathogen exposures is critical. Prior work experience of fish husbandry staff in many research facilities comes from caring for pet or ornamental fish, which can be entirely different than caring for large numbers of valuable research animals. At least one staff member should have some training in fish health, and this person could be the key point to interact with the institution's laboratory animal veterinarian and outside diagnostic laboratories. Several short courses on fish health are available and many are geared towards aquarists and aquaculturists, fish health professionals and veterinarians. The staff should follow written standard operating procedures (SOPs). These documents are considered "living documents" in that they should be modified or updated as frequently as necessary by the manager or supervisor such that these SOPs reflect what is actually being done by personnel within the laboratory and animal facilities.

9. Biocontainment designation and fish pathogens

The principles of biosafety incorporate strict adherence to standard microbiological practices and techniques, the use of appropriate primary barriers and personal protective equipment within a secondary barrier designed and constructed with the primary objective of the containment of potentially harmful biological agents (Biosafety in Microbiological and Biomedical Laboratories, 2007). As with mammalian biomedical research, normal biosafety and animal biosafety levels (BSL and ABSL) are applicable. The BS and ABS levels typically used for piscine pathogen research, biomedical research, and diagnostic endeavors include levels 1 and 2. *Mycobacterium* species, including those found in fish, are BSL-2. BSL-3 and ABSL-3 work with piscine or other aquatic animal pathogens is further clarified as BSL/ABSL-3-Agricultural (Ag) by the United States Department of Agriculture (USDA). BSL-3-Ag is unique to agricultural because of the necessity to protect the environment from economic, high risk pathogen in situations where studies are conducted in which the facility barriers now serve as primary containment (BMBL 5th Ed., 2007). Examples of piscine pathogens that would require handling and housing at BSL-3-Ag include Spring Viremia of Carp Virus (SVCV) and Infectious Salmon Anemia Virus (ISAV). Currently there are no known piscine BSL/ABSL-4 pathogens.

As the specific description of the recommended design of BSL/ABSL-1 to BSL/ABSL-3-Ag facilities for aquatic animals is beyond the scope of this paper, please refer to the BMBL 5th Ed. 2007 for more information and consult with your local laboratory animal veterinarian and environmental safety officer.

10. Some recommendations if disease and pathogens are detected

The following is an outline that one might follow regarding occurrences of mortality and disease in a facility.

- I) Low mortality
 - A) Determine cause by conducting diagnostics and evaluating water quality parameters.
 - 1) Infectious disease. Document macroscopic changes, isolate tanks, examine all moribund fish and a subset of healthy fish to determine prevalence of condition. Routine disin-

fection of equipment that comes into contact with water or tanks housing morbidities of mortalities.

- a) High prevalence: consider euthanizing population
 - b) Low prevalence: monitor, treat if option is available, consider isolation of population
- 2) Non-infectious cause. Investigate potential toxicity problems related with water source or feed. Evaluate water quality records for parameter perturbations.
 - 3) Unknown cause. Isolate fish and monitor mortality. Routine disinfection of equipment that comes into contact with water or tanks housing morbidities of mortalities.
- II) Significant mortality
- A) Determine cause by conducting diagnostics and evaluating water quality parameters.
 - 1) Infectious. Isolate fish, ensure adequate disinfection of all equipment that has had contact with water or tanks housing morbidities of mortalities. Most likely euthanize remaining population. Consider potential treatments if stocks are very valuable.
 - 2) Non-infectious. Investigate potential toxicity problems related with water source or feed. Evaluate water quality record for parameter perturbations.
 - 3) Unknown cause. Isolate fish, ensure adequate disinfection of all equipment that has come in contact with water or tanks housing morbidities or mortalities. Most likely euthanize remaining population — go to III).
- III) Further investigations on diseases of unknown etiology.

It is very possible that the cause of disease is from a previously unknown agent. Thorough investigation (actually a small research project) is often done in collaboration with a laboratory conducting fish disease research. For a thorough examination, multiple samples should be taken; frozen at -80°C (for DNA and toxicology), preserved in formalin-based fixative for thorough histological investigations and preserved in other fixatives for electron microscopy (e.g., visualization of viruses). Fresh samples are provided for bacteriology and virology. Conducting *in vivo* exposure experiments have been very useful for demonstrating an infectious etiology, even if a specific agent is not found (Kent and Dawe, 1993; Kongtorp et al., 2004; Verner-Jeffreys et al., 2008).

11. Conclusion

Although similar for the most part, differences in strategies for control of both pathogenic agents and infectious diseases in fish used for research can be compared to terrestrial animals. This is due, in part, their unique aquatic environment that is modified by the source and quality of the water provided, the species of fish to be housed and the configurations and components of the housing systems. We have provided a diverse compilation of recommendations that are currently employed at various fish research facilities. The process of control of pathogens and infectious diseases in fish research facilities will be an evolving process over time. Nevertheless, the goal of documenting, detecting, and excluding pathogens in fish is just as important as in mammalian research models.

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