Facility Design and Health Management Program at the Sinnhuber Aquatic Research Laboratory

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Abstract

The number of researchers and institutions moving to the utilization of zebrafish for biomedical research continues to increase because of the recognized advantages of this model. Numerous factors should be considered before building a new or retooling an existing facility. Design decisions will directly impact the management and maintenance costs. We and others have advocated for more rigorous approaches to zebrafish health management to support and protect an increasingly diverse portfolio of important research. The Sinnhuber Aquatic Research Laboratory (SARL) is located ~3 miles from the main Oregon State University campus in Corvallis, Oregon. This facility supports several research programs that depend heavily on the use of adult, larval, and embryonic zebrafish. The new zebrafish facility of the SARL began operation in 2007 with a commitment to build and manage an efficient facility that diligently protects human and fish health. An important goal was to ensure that the facility was free of *Pseudoloma neurophilia* (Microsporidia), which is very common in zebrafish research facilities. We recognize that there are certain limitations in space, resources, and financial support that are institution dependent, but in this article, we describe the steps taken to build and manage an efficient specific pathogen-free facility.

Facility Overview

THE SINNHUBER AQUATIC RESEARCH LABORATORY (SARL) is a large off campus core facility that is heavily relied upon by multiple extramurally funded laboratories and research programs. It was designed to be the first Microsporidia-specific pathogen-free (SPF) facility.¹ A central design element is that fish are housed in distantly separated SPF and quarantine areas. The SPF husbandry spaces are adjacent to dedicated platforms for imaging, microinjection, molecular biology, and behavior assessments. The facility floor plan (Fig. 1) also integrates administrative and research staff offices and provides a modern conference space with a full suite of web-based communications to maximize personnel interactions. Below we detail the SARL design and workflow that support a strong track record of productivity and robust animal health management.

Design Philosophy

Keeping the water on

Water availability and quality are paramount, and thus, they impact nearly every aspect of husbandry facility design. For example, at the SARL, we have found that redundancy in every critical component of water purification and delivery, or at least

an onsite replacement, is an essential operating practice. Specialty, components that are difficult to quickly replace should be designed and built redundantly into the system, because they can and will fail at the worst possible time. Simple, but often overlooked considerations are can a component be bypassed for some period? If so, for how long? Is appropriate valving designed into the system to allow easy bypassing for replacement or maintenance? The apparent costs of system redundancy and careful design are high, but compared to the potential for negative impacts to colony health, lost or diminished productivity during the ensuing recovery period, and the enormous stress and cost of dealing with emergency heavy repairs, these costs are nearly insignificant.

Incoming water and processing systems

High-quality groundwater is the starting point for the SARL system water. Three groundwater pumps are available depending on need and serviceability. Dechlorination is not required for this source, as it would be with a municipal water supply, resulting in reduced water processing cost. Fish health protection is also enhanced by removing the potential for dechlorination equipment failure. Groundwater runs through an aeration/degassing column and is held in an outdoor cistern. Influent groundwater receives pretreatment before being delivered to the recirculating life support systems (RLSS).

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FIG. 1. Overall floor plan view of the entire Sinnhuber Aquatic Research Facility.

System pressure is boosted to 35 psi and particulate filtration ($100\ \mu\text{m}$) is employed using a self-cleaning screen filter. Afterward, the water undergoes ultraviolet sterilization to reduce threats from biological entities. This unit has been customized to include UV TOC (185 nm) bulbs to also degrade organic chemicals that might be present. This u.v. unit was repurposed from a system with higher throughput, delivering $\sim 480,000\ \mu\text{W}\cdot\text{s}/\text{cm}^2$. Pretreated water is then sent to the recirculating fish support systems.

Recirculating systems water

Water additions to the RLSS are further filtered utilizing a reverse osmosis filtration system followed by activated carbon filtration and a $1\text{-}\mu\text{m}$ particulate filter, before delivery to the supply water holding tanks in each of the two RLSS units. The components of RLSS units are familiar to the aquatic research community, but a brief overview is provided. An overview of the SARL water flow system is included as Supplementary Figure S1 (Supplementary Data are available online at www.liebertpub.com/zeb). Both of our RLSS units were provided by Aquaneering, Inc. (San Diego, CA).

Each RLSS unit has a clean and dirty water treatment loop facilitated by a semisegregated sump. The clean loop water is drawn from the dirty loop discharge side of the sump, particulate filtered to $50\ \mu\text{m}$ using a multiple bag filter manifold, and UV filtered ($40,000\ \mu\text{W}\cdot\text{s}/\text{cm}^2$ uvc irradiance older system, $80,000\ \mu\text{W}\cdot\text{s}/\text{cm}^2$ uvc irradiance newer system) before delivery to fish tanks. The dirty loop water is drawn from the tank drain return side of the sump, particulate filtered using a propeller bead filter, and biologically filtered using a fluidized bed filter.

We have implemented an additional side-stream activated carbon filtration loop in our RLSS units. This includes a small pump and granular activated carbon filter with its own plumbing. This filtration loop removes organic chemicals introduced by the mechanical system, plumbing, the fish, or the diet. The RLSS unit water parameters are computer monitored and logged. Programmable logic controllers are used to drive chemical pumps to maintain conductivity and pH levels. The monitoring system sends an alarm if parameters exceed limits and immediately contacts personnel through phone, text, and e-mail.

Iterative system for water supply to quarantine

SARL has three walk-in environmental chambers with quarantine racks. Because there can be no water return from quarantine to the SPF system, quarantine water is single pass and iterative to eliminate pathogens or research chemicals into the facility waste stream. Quarantine supply water comes from the filtered water pressure line from one of the RLSS utilizing an electric switch timer and an electronic ball valve. An iterative water delivery schedule is developed to maintain at least three water changes per day divided evenly over a 24-h period. Influent water is delivered to the three quarantine rooms in parallel, with no water being shared from one quarantine room to another, before being disposed. When necessary, effluent water can be filtered using particulate and chemical means for hazardous waste disposal.

Quarantine design and biosecurity

A well-designed quarantine facility is vital to the success of SPF maintenance, regardless of species, and for the SARL

is a critical line of defense against disease infiltration. Complete isolation, spatially and through workflow, of quarantine and the SPF colony is essential. A dedicated staff member is charged with daily husbandry and line propagation responsibilities. Any person entering and interacting with quarantine water or fish is not allowed into the SPF space for the remainder of the day. Entry into the quarantine space requires the use of gloves, shoe covers, and full-length gowns to minimize potential spread of organisms into the common areas of the facility. Fish food for each isolation chamber is dispensed into separate disposable containers.

Additional separation levels within quarantine can also be a valuable tool to achieve a more thorough level of testing to prevent disease transmission when importing fish. For example, the SARL quarantine space consists of three fully self-contained isolation chambers, (1) RED—new acquisition, unknown status but assumed Microsporidia carrier, (2) YELLOW—Microsporidia detectable, status known, (3) GREEN—final round of screening indicates no Microsporidia, fish can transition to SPF facility. Each chamber has a fish rack, benchtop, sink, and climate control and ventilation and dedicated supplies. These levels within quarantine, combined with a countercurrent workflow, reduce recontamination of fish, speeding up the overall progress of new acquisitions to SPF status.

SPF design and biosecurity

We have invested substantially to establish and maintain the SPF status of the SARL, and aggressive facility access controls are used to protect it. Many facilities allow full researcher access to the main fish rooms to help increase research productivity and to allow researchers to gain experience in husbandry and fish handling. We have taken the opposite approach and have implemented a closed core facility. Entry into the fish rooms is limited to only facility staff and select researchers who have undergone extensive training on the methods needed to adhere to all aspects of the SPF biosecurity plan.

Dedicated shoes for all long-term staff and shoe covers for all visitors are required for entry into the fish rooms and SPF space. Sticky mats are at the main entry where shoe changes occur. All persons entering the facility must immediately wash their hands with Sporidicin Antimicrobial Lotion Soap (Contec, Inc., Spartanburg, SC) at a dedicated hands free wash station. Only new and unopened disposables may be brought into the facility. Any equipment or supplies that have come in contact with other aquatic species are not allowed in the SPF fish facility. All persons having come into casual contact with another aquatic colony (aquarium, tour, etc.) are not allowed in the fish rooms for at least 24 h, and anyone coming in contact with the fish or water systems is not allowed regular contact with other aquatic colonies.

Because of the closed core status, facility staff are responsible for all husbandry, line maintenance, spawning, and genotyping needs within the facility. Researchers submit requests for embryos, adult fish, or other special services such as fin clipping. Embryos, fish, or samples are transferred to an incubator in a neutral area for pick up and use. All fish or embryos leaving the SPF area must be classified as having a terminal endpoint, as fish are never allowed back into the SPF facility after leaving. All experiments that must remain within the SPF space are executed in a collaborative manner

between the researcher and facility staff to ensure that biosecurity is maintained. Placing limitations on who comes into contact with the colony allows for substantially better control over biosecurity and ultimately creates a more stable SPF research environment.

Husbandry

Quarantine testing and import procedures

The multistage quarantine scheme at the SARL isolates animals by import status and generation, and provides dedicated husbandry workspace for health assessment and decontamination before introduction into the SPF facility. As mentioned, newly imported lines are housed in the RED quarantine chamber and visually monitored for signs of illness or parasitic infection before spawning. The majority of imported lines arrive as embryos; therefore, they are visually monitored until they reach sexual maturity at ~3 months of age. Imported adults would be monitored for ~4 weeks before spawning.

Once spawned, embryos are surface sanitized using methods previously described² and moved to the YELLOW quarantine chamber 2. All contributing parents are kept in RED for secondary testing while the offspring are raised until 15 days postfertilization (dpf) and a portion of the population¹ is sacrificed for analysis in-house through polymerase chain reaction (PCR) for the presence of *Pseudoloma neurophilia*. Whole larval fish are pooled in groups ($n=10$ per sample), and DNA is extracted as templates for PCR. If larval fish are negative for the pathogens, the remainder of that population is isolated until all contributing parents are tested. Contributing parents are euthanized, frozen, and shipped overnight for diagnostic testing (IDEXX Bioresearch, Columbia, MO). All animals are tested through PCR for *P. neurophilia* as well as *Mycobacterium* species: *Mycobacterium abscessus*, *Mycobacterium chelonae*, *Mycobacterium fortuitum*, *Mycobacterium haemophilum*, *Mycobacterium marinum*, and *Mycobacterium peregrinum*.

If all parent animals are negative for pathogens, the remaining larval animals in isolation can be transferred to SPF rooms within the facility. In the event that the offspring test positive for a pathogen of concern, the founding animals are spawned until clean offspring are obtained, utilizing the GREEN isolation chamber as needed. If clean offspring or parents cannot be obtained, either a secondary source of the line is found or alternatively, all research using this line must stay within the confines of quarantine. Only lines that produce two clean subsequent generations of animals may be introduced into the SPF fish room.

Elimination of live feed

All live feed sources have been removed from our feeding regimen in an effort to reduce labor cost and to eliminate variations introduced by inconsistent live food quality. Adult fish are fed size-appropriate GEMMA Micro (Skretting, Inc., Fontaine Les Vervins, France) twice a day. Larval and juvenile fish also receive age-appropriate GEMMA Micro feed but are fed thrice a day.

The SARL has been free of early-stage live feed such as paramecia or rotifers since 2009. Brine shrimp (*Artemia nauplii*) was removed from the SARL feeding regimen after a multiyear study in which both larval survival and lifetime

embryo production were assessed utilizing only GEMMA Micro without supplementation of any live feed, both at the larval and adult life stages. This trial clearly indicated that we could maintain high-level embryo production without the use of any live feed. In addition to equivalent fish performance and reduced risk of introducing contaminants through live feed, a labor assessment showed that the overall cost of our feeding program was reduced considerably.

Tank housing

A large portion of the fish at SARL are housed in 50 or 100 gallon brood stock tanks. All additional fish are housed in small polycarbonate enclosures at ~6 fish per liter. SARL houses ~50,000 adult fish within the SPF fish rooms. Although stocking densities as high as 12 fish per liter have been reported as appropriate for research zebrafish,³ SARL utilizes the lower density to allow better fish observation during daily health surveillance screens. Dedicated outcross tanks are utilized to reduce potential for biological or genetic contamination back to our main wild-type stocks. All fish in the outcross tanks are euthanized after they are no longer needed and are not returned to the main wild-type population. In an effort to reduce handling of wild-type spawning populations, they are housed in large brood stock tanks and spawned in place using an internal collection apparatus that is plumbed to an external collection spigot.

Tank cleaning

Tank cleaning is regularly scheduled and the procedures are tailored to the type of enclosure. Large brood stock tanks are cleaned in place by manual scrubbing and flushing of debris. The large brood stock tanks are designed for zero light ingress for use as light cycle tanks, and each has its own timed overhead LED light source. Sidewall growth has been significantly minimized to a point that they only require quarterly cleaning. When fish stocks are removed, the tanks are emptied, isolated from the system, and sanitized in place using sodium hypochlorite. All other tanks and tank supplies are changed out every 2–4 weeks and are washed in a Tecniplast 650a Aquatic Cage Washer, which utilizes a three-stage chemical sanitization and neutralization process to eliminate biologicals. The cage washer is tested quarterly using adenosine triphosphate (ATP) detection probes to ensure proper function.⁴

Health monitoring and testing

The health monitoring program within the SPF portion of the facility has four main components: sentinels, wild-type stocks at both 15 dpf and retirement, select retiring generations, moribund and mortality.

Sentinels. The large volume of fish sampled from the room at retirement allows us to have a sentinel program mainly composed of prefiltration exposed animals. Fish are introduced to the sentinel program at approximately 4 months of age. SARL houses prefiltration sentinels in the fish room, rather than in the return water sump, to ensure they receive the same level of care as the fish in the main facility. Fish are placed on a bottom shelf within the fish room that has been modified to pump water directly from the dirty water collection sump. Potential stress induced by vibrations from the life support system, disruptions in the circadian rhythm due to

afterhours entry into the CLS room, or potential differences in temperature are eliminated by housing all sentinels within the fish room.

A total of 80 fish are used for each sentinel group and remain in the program for 6 months postexposure or until 10 months of age. At the specified sample date, 60 fish are euthanized and dissected. Brain and spinal column material are removed for analysis through PCR for *P. neurophilia*.¹ The remainder of the carcasses are frozen and submitted overnight to IDEXX for additional PCR testing for Mycobacterium species: *M. abscessus*, *M. chelonae*, *M. fortuitum*, *M. haemophilum*, *M. marinum*, and *M. peregrinum*. The remaining 20 fish are preserved in fixative and sent to the Oregon State University Veterinary Diagnostic Laboratory for sectioning and histopathology assessments.

Supplemental testing. In addition to extensive sentinel screening, a large number of nonsentinel animals are tested to gain a greater understanding of the health status of the colony. Wild-type stocks are tested at several points throughout life and from different housing conditions. At 15 dpf, 60 fish from all wild-type stocks are sampled for analysis through PCR for *P. neurophilia*. Given that the majority of our wild-type fish are housed in 50 or 100 gallon tanks, we also sample a portion of each of these stocks. When a stock reaches retirement age, 10 fish are removed and submitted for histological analysis.

In an effort to ensure that testing done in quarantine is effective, follow-up testing occurs with lines recently transitioned to the SPF space. At the specified sample date, 60 fish from the most recently transitioned population are euthanized and dissected and undergo the same rigorous testing described earlier. In addition to the transitioned animals, any offspring produced by the line within the first year post-transition are also tested at retirement using the same parameters. In addition to routine screening of wild types, newly transitioned lines, sentinels, and all moribund fish exhibiting symptoms of concern are fixed and sent for histological analysis.

Sentinel program results. To date, 1519 sentinel fish have been sampled at the SARL. Of this number, 1025 fish have been analyzed through PCR for the presence of *P. neurophilia* and all results have been negative. Four hundred ninety-four fish have undergone histological analysis with the same results. All fish were negative for signs of *P. neurophilia*. Of these fish, 18 were positive for Mycobacterium, which has been cultured and identified as *chelonae*. More recently, the SARL added multiplex PCR testing for multiple species of Mycobacterium. Since adding this additional level of surveillance, 140 sentinel fish have been tested. Of 35 pooled samples (3–5 fish per pool), 4 pools have tested positive for *Mycobacterium chelonae*, with the remaining samples (127 fish represented) testing negative for the Mycobacterium species.

Summary

SPF is a relatively new concept for zebrafish research facilities. Commercial diagnostic tests aimed at the zebrafish market have only recently become available, and access to veterinary staff trained in aquatic diseases can still be a limitation for those interested in improving their facility's overall health status. Maintaining an SPF colony requires a

strong commitment to both facility design or retooling, special husbandry approaches, and the realization that operational costs will be higher. Costs increase because of the resources needed for quarantine, diagnostic testing and for the additional personnel needs to provide a full-service closed facility. Some of these costs can be offset by continually streamlining operations, such as increasing the efficiency of feeding, spawning, and tank washing.

Although maintaining an SPF colony comes at a higher overall cost, there are numerous benefits. If pathogens can be eliminated at the door, the colony will be healthier and more productive, reducing the number of fish needed to accomplish the research. The presence of pathogens also introduces an element of variability that can affect research outcomes.^{5,6} For example, a recent study indicated that background infections could impact zebrafish behavior.⁷ One of the most tangible benefits is increased insurance against a catastrophic colony loss due to a virulent pathogenic outbreak. Although the number of serious pathogens affecting zebrafish continues to increase, diagnostic technology has advanced to the point that there is no longer an excuse to not stay on top of it. Careful infrastructure design and rigorous adherence to protocol have allowed SARL to maintain an SPF colony status for over 8 years and provide a path for other facilities to achieve an improved health management program.

Disclosure Statement

No competing financial interests exist.

References

1. Kent ML, *et al.* Development and maintenance of a specific pathogen-free (SPF) zebrafish research facility for *Pseudoloma neurophilia*. *Dis Aquat Organ* 2011;95:73–79.
2. Kent ML, *et al.* Toxicity of chlorine to zebrafish embryos. *Dis Aquat Organ* 2014;107:235–240.
3. Castranova D, *et al.* The effect of stocking densities on reproductive performance in laboratory zebrafish (*Danio rerio*). *Zebrafish* 2011;8:141–146.
4. Guide for the Care and Use of Laboratory Animals. 8th ed. National Academies Press, Washington, DC, 2011.
5. Ramsay JM, *et al.* *Pseudoloma neurophilia* infections in zebrafish *Danio rerio*: effects of stress on survival, growth, and reproduction. *Dis Aquat Organ* 2009;88:69–84.
6. Kent ML, Harper C, Wolf JC. Documented and potential research impacts of subclinical diseases in zebrafish. *ILAR J* 2012;53:126–134.
7. Spagnoli S, Xue L, Kent ML. The common neural parasite *Pseudoloma neurophilia* is associated with altered startle response habituation in adult zebrafish (*Danio rerio*): Implications for the zebrafish as a model organism. *Behav Brain Res* 2015;291:351–360.

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