

Zebrafish Housing Systems: A Review of Basic Operating Principles and Considerations for Design and Functionality

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Abstract

The strategies for housing zebrafish used in biomedical research have evolved considerably over the past three decades. To keep pace with the rapid expansion and development of the zebrafish model system, the field has generally moved from keeping fish at the level of aquarium hobbyist to that of industrialized, recirculating aquaculture. Numerous commercial system vendors now offer increasingly sophisticated housing systems based on design principles that maximize the number of animals that can be housed in a given space footprint, and they are thus able to support large and diverse research programs. This review is designed to provide managers, lab animal veterinarians, investigators, and other parties responsible for care and use of these animals with a comprehensive overview of the basic operating and design principles of zebrafish housing systems. This information can be used to help plan the construction of new facilities and/or the upgrade and maintenance of existing operations.

Key Words: aquatic life support; recirculating aquaculture; system design; zebrafish (*Danio rerio*) housing systems

Introduction

Dr. George Streisinger, considered by many to be the founder of modern zebrafish research, used glass aquariums purchased from a local pet shop to house zebrafish in his laboratory at the University of Oregon during the 1970s and 1980s. The systems were decidedly of the home-made variety: the tanks were covered with acrylic custom-cut lids, water exchanges were performed using a siphon system made from science catalogue parts, a household carbon filter was used to remove chlorine from tap water, and a thermostatic mixing valve that one might find in a photography studio darkroom mixed domestic hot and cold water to get the water to the “right” temperature for the fish. The “system,”

which operated as flow-through during the day, was turned off in the evenings so that the animals received no water flow overnight. This early precursor of the present-day fish room was more like an aquarium shop than a research laboratory.

Zebrafish housing systems have evolved considerably in the 30 years since Dr. Streisinger published his seminal work, “Production of Clones of Homozygous Diploid Zebra Fish (*Brachydanio rerio*),” in *Nature* (Streisinger et al. 1981). Although standard glass aquaria are still used in some settings, far more sophisticated life-support systems are now the rule. Zebrafish aquaculture housing systems combining design principles from industrial aquaculture, laboratory rodent housing, and research genetics are commercially available from a number of sources, and an increasing number of academic research institutions are constructing large centralized facilities to support their growing zebrafish research programs. The technology is growing by leaps and bounds; the newest systems now include such innovations as fully automated electronic water quality monitoring and control systems that can be accessed and operated remotely over the Internet, specialized spawning chambers, and robotic feeders (Harper and Lawrence 2010).

The use of the zebrafish model system is also rapidly expanding beyond its traditional applications in developmental biology and genetics to diverse applications in various fields, including ecotoxicology (Scholz et al. 2008), drug screening (Rubinstein 2006), cancer (White et al. 2011), xenotransplantation (White et al. 2008), and behavior (Cachat et al. 2010). In some instances, this may require the use of specialized housing equipment, especially in instances where study designs evaluate fish for weeks or months (Traver et al. 2004).

Given these increasing levels of complexity, both in housing systems and the experimental uses of the zebrafish, the task of choosing, designing, and planning for a new system or upgrading an existing one is critically important to the success of a research program. Above all, the fish housing systems that are ultimately selected should function to (1) provide a stable and favorable environment that produces and maintains healthy and productive fish and (2) support specific research goals of investigative staff. Importantly, the system should also be designed to facilitate adherence to regulatory requirements, which are also becoming more stringent as the use of fishes in biomedical research increases (Lawrence et al. 2009). Housing systems

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that do not facilitate the maintenance of stable environmental conditions can contribute to myriad problems in one or more of these areas. Although having a state-of-the-art fish housing system is important, its functionality is maximized by the quality of the people who manage it. With the proper personnel and strategy in place, a well-appointed fish housing system can greatly enhance the ability of those charged with the care of the fish to maximize the productivity and welfare of the animals living in the system while allowing researchers to concentrate on science instead of fish husbandry.

The goal of this review is to provide researchers, laboratory animal science professionals, architects, and others who may be involved in the planning of a new fish facility with a comprehensive overview of design concerns that should be considered when planning to purchase and install zebrafish housing and life-support equipment in biomedical research settings.

Life Support

The elemental operating goal of any zebrafish housing system is to provide a stable and favorable macroenvironment for the animals housed within it. This macroenvironment, for fishes at least, is synonymous with the concept of water quality, which incorporates the physical, chemical, and biological characteristics of water that the animals inhabit. The most important of these parameters are temperature, pH, salinity, alkalinity, hardness, dissolved oxygen, and nitrogenous wastes. All fishes have a preferred or optimum range of water quality parameters under which they perform best. Although these optima have not been formally determined for zebrafish, there are enough data, collected from both the natural habitats of the fish in Bangladesh (Spence et al. 2006)

and India (Engeszer et al. 2007) and various laboratory studies (Brand et al. 2002; Chen et al. 2003; Cortemeglia and Beitinger 2005; Sawant et al. 2001), to suggest the most favorable ranges for the fish in culture conditions (Harper and Lawrence 2010; Lawrence 2007) (Table 1). Housing systems must be designed in such a way that these conditions can be reliably maintained. The ability to achieve this goal is dependent upon both the preparation of source water before it gets into the housing system and the manner in which the water is treated once it is inside the system with the animals.

Water Production

The nature of the water source used to supply fish systems is critical and must be carefully considered prior to the establishment of the system. The objective is to provide a stable and clean “template” from which to operate. Use of a building potable water supply opens up the possibility that one or more critical water parameters may change without advance knowledge or planning. For example, in many instances, municipalities routinely treat water supplies with chemicals that may be toxic to fish, such as chlorines or chloramines (Kent et al. 2007). Depending on building infrastructure, contaminants, such as heavy metals, can leach from supply pipes and cause health problems or mortality in the fish (Kent et al. 2007). The chemistry of water supplies may also change with weather conditions. This variation may be problematic and is undesirable, especially if not anticipated. Given these realities, it is essential to have an in-depth knowledge of the water source and specifically the chemistry of the source water prior to installing a life-support system. Many private water testing laboratories are capable of carrying out the type of comprehensive analysis required to ensure the

Table 1 Recommended water quality parameters for zebrafish (adapted from Harper and Lawrence 2010)

| Parameter | Target range | Available testing methods | Testing frequency |
|------------------------------|------------------------|--|----------------------------|
| pH | Stable, within 6.8-8.5 | Colormetric kit, automated monitoring systems | Continuous-daily |
| Salinity | Stable up to 0-5 g/L | Refractometer, automated monitoring systems | Continuous-daily |
| Alkalinity | Stable, 50-150 mg/L | Colormetric kits | Monthly |
| Hardness, g/L | Stable, 75-200 mg/L | Colormetric kits | Monthly |
| Total ammonia nitrogen, mg/L | Zero | Colormetric kits | Daily ^a -weekly |
| Nitrite | Zero | Colormetric kits | Daily ^a -weekly |
| Nitrate | Up to 200 mg/L | Colormetric kits | Daily ^a -weekly |
| Dissolved oxygen | No less than 4 mg/L | Colormetric kits, automated monitoring systems | Continuous-monthly |
| Carbon dioxide | No more than 20 mg/L | Colormetric kits | Monthly |
| Temperature | Stable within 24-30°C | Handheld thermometer, automated monitoring systems | Continuous-daily |

^aAt system startup

water does not contain unwanted impurities and is stable in its chemical composition, and they should be consulted during the planning stages of a new or upgraded fish facility.

Once the qualities of the source water are determined, there are a number of ways to ensure it is suitable to use for fish. Although low-tech, inexpensive options exist (i.e., pre-treating water with off-the-shelf chemical additives, aerating supply to off-gas chlorine prior to use, or prefiltering with activated carbon), the more reliable approach is to purify the water using reverse osmosis, deionization, or a combined reverse osmosis/deionization system. Reverse osmosis water filters are actually several individual filters placed in series, with each filter type referenced as a “stage.” The two stages used to prefilter the water before it passes through the reverse osmosis filter membrane are a sediment removal stage (particulate filter) and, when using thin film composite membranes, a chlorine removal stage (activated carbon filter) (Evans 2005). Once prefiltered, the water is pushed through the reverse osmosis membrane in reverse of its natural osmotic flow, and the resulting filtered (“product”) water can be stored for use, whereas the waste (“reject”) water can be sent to a waste stream.

A higher level of source water control can be achieved by supplementing the reverse osmosis system with a deionization step, wherein the reverse osmosis product water is filtered through a deionization unit to remove unwanted ions, although it should be noted that the reverse osmosis itself removes some harmful ions. Although deionization filters can be used alone, the resin in them will tend to need more maintenance and the volume of water between resin replacement/recharge will be low compared with reverse osmosis/deionization filters (Evans 2005).

It is important to consider that the various treatment processes described above remove beneficial as well as potentially harmful substances from the water. For example, trace elements such as calcium and magnesium that are essential for fish metabolism and growth (Lawrence 2007) will be removed during treatment. Therefore, purified water must be conditioned by addition of synthetic sea salts before it is delivered to fish (Harper and Lawrence 2010). This can be done before the water enters housing systems in an off-system storage tank, or, more commonly, it takes place within the system itself. Indeed, most zebrafish housing systems are designed to condition pure water as it enters the system. This is achieved via automatic water quality monitoring and control equipment that continuously measures various parameters of the system water, including salinity/conductivity and pH. These parameters, often along with temperature, are affected when pure water is added to the system during water changes or replacement. Most monitoring systems are designed to maintain threshold setpoints of these parameters in the housing systems such that when probes detect changes, the monitoring and control system accordingly doses the water with saline and/or sodium bicarbonate solutions or, in the case of temperature, warms the water to bring the parameters back within the desired range.

Water Treatment

Once the source water is adequately prepared and delivered to fish in systems, a critical set of new challenges arises—that of how the water is treated once it is in the housing system with the fish. A central operating premise of aquaculture revolves around the fact that fishes excrete their metabolic wastes directly into their environment—the water. Some of these waste products are toxic (e.g., ammonia nitrogen; see “Biological Filtration”), so when they accumulate in closed systems as a result of fish metabolism and the breakdown of organic materials present in the system, they have the potential to kill or cause severe health problems for fish. Therefore, ammonia and other waste products must be removed from the water in order for the fish to be able to live in it. Hence, the life-support component of an aquaculture system must function to remove these metabolites from the system.

For the purposes of waste removal, there are two basic types of aquaculture systems that can be employed: flow-through and recirculating. The difference between these two systems has to do with the manner in which waste products are removed. In flow-through systems, clean water is pumped into tanks, fish excrete wastes into the water, and the effluent is flushed out. The flow is unidirectional—clean water in, effluent water out—and may be continuous or periodic. This type of system requires a large source of appropriately conditioned water. Recirculating systems operate differently. Clean water is pumped into tanks, fish excrete wastes into the water, and the effluent water is pumped into a “treatment” zone where wastes are removed before the water is returned “clean” to the fish. Hence, the water recirculates in the system. This mode of aquaculture greatly reduces water usage and space requirements. The great majority of zebrafish housing systems are recirculating, so this review will focus on the major treatment steps employed in this application: solids removal, biological filtration, chemical filtration, aeration, and disinfection.

Solids Removal

Solid wastes, which are produced by the accumulation of uneaten food and fish feces, can have a detrimental effect on the efficiency of biological filtration and can lower the overall oxygen level in the system because bacteria use oxygen to break down waste (Masser et al. 1999). They can also produce significant amounts of ammonia nitrogen. The accumulation of solids in a system also favors the growth of heterotrophic bacteria, which will compete for space on biofilters with the autotrophic species that are required to metabolize fish wastes (see “Biological Filtration”). The three types of waste solids found in a recirculating aquaculture system are settleable, suspended, and fine or dissolved solids (Losordo et al. 1999). Settleable solids are those that will drop out of the water column. Suspended solids are those that are carried by the water column and do not settle. It is critical

that both be removed from the water prior to the next treatment step—biological filtration.

The removal of these solids from the system water takes place at both the level of the tank and the system treatment zone. The various tank types available from commercial system vendors are all, to some extent, designed to facilitate the removal of solids from the tank to the system water treatment zone via flow of the water through the tanks. The precise mechanism by which the solids are removed from the tanks varies, but it often incorporates sloped tank bottoms and a tank baffle and/or siphoning mechanism. In some instances, solids will not be effectively removed at this step and will settle somewhere in the tanks, usually along the bottoms. This is undesirable, and steps should be taken to remove this manually, which can be labor intensive.

Once the solids are removed from the tanks, they are moved through the system to the treatment zone. It is crucial that the solids be removed before biological filtration. There are several different ways to achieve this. The most basic approach is to first pass all water through filter pads, usually made from polyester fiber. The goal of this step is to remove the largest particles, the settleable solids, from the water, and it is often done as wastewater is returned to collection sumps. This is generally effective, but it adds consumable items and can be labor intensive (pads must be washed and/or replaced on a regular basis). In addition, pads may not always remove all solids, so some will settle out in sumps or in other low-flow zones that may exist along the way to the next treatment step, where they will have to be manually removed by siphoning. After this step is completed, the water is usually pumped through a second set of mechanical filters designed to remove suspended solids. There are many different types of fine mechanical filters that can be used, but the most common include bag filters, screen filters, or cartridge filters. In all three cases, the filters must be regularly cleaned or replaced because they become saturated with collected solids. Again, although generally effective, this is labor intensive, uses consumable items, and has the potential to cause flow restriction problems if not regularly maintained.

More sophisticated means of solids removal are now a possibility in commercially available zebrafish systems. Rotating microscreen drum filters are one example. A typical rotating microscreen drum filter design consists of a cylindrical drum, the circumference of which is constructed with steel or nylon mesh with a pore diameter size of 25 to 100 μm . The drum sits inside a sump. Wastewater is pumped or flows by gravity into the drum and then flows out through the screen, leaving the solids trapped on the inside of the screen. Most drum filters are outfitted with some version of an automated high-pressure backwash system. Over time, the solids interfere with the flow of water through the screen, and the water level inside the drum rises, triggering a level switch that activates a rotary motor and high-pressure backwash sprayer that simultaneously rotates the drum and sprays the screen inside the drum with fresh water, washing solids into a collection trough that is connected to a drain. Once the screen is cleared, the drum stops rotating, the sprayer turns

off, and normal filtering operation continues until the process is triggered again by accumulation of solids. There are numerous advantages to this filtration option, including significant reductions in consumable materials use and maintenance required by filter pads, mechanical filter bags, or cartridge options described above.

Another more advanced type of solids removal is the employment of expandable granular media filters (Losordo et al. 1999). In this design, solids are removed by passing water through a bed of granular media, usually sand or plastic beads. Solids in the water become trapped in the media, and water flows out clean. The solids eventually clog the media, so it needs to be periodically backwashed with fresh water, with the solids flushed to a drain. Again, this is a process that can be easily automated. As with the rotating microscreen drum filter, this approach can result in significant cost and labor savings when compared with more traditional pads, filter bags, and cartridge combinations.

Biological Filtration

After solid wastes are filtered from the water, the next step in the treatment process is the removal of total ammonia nitrogen produced during fish metabolism and the catabolism of uneaten feed and other solid wastes. Total ammonia nitrogen consists of un-ionized ammonia and ionized ammonium, the relative fractions of which are dependent on pH, temperature, and salinity (Timmons et al. 2002). Un-ionized ammonia is highly toxic to fish and must be removed from the system (Masser et al. 1999; Noga 2010). This is achieved via oxidization of ammonia to nitrite and then nitrate by nitrifying bacteria in a process referred to as biological filtration or biofiltration. These microbes, which are ubiquitous in air, soil, and water, colonize and grow on the surface of all substrates in the fish system. The biological filter or biofilter is a specially designed substrate in the treatment zone of the system with a high surface area on which these nitrifying bacteria attach and grow. All effluent water in a recirculating system is passed through the biofilter. As this happens, certain species of bacteria living on the filter oxidize the ammonia (e.g., *Nitrosomonas* sp.) in the water to nitrite, and then others (e.g., *Nitrobacter* sp., *Nitrospira* sp.) oxidize nitrite to nitrate. Nitrates, which are toxic to fish in only very high concentrations (Hrubec et al. 1996), can be controlled through removal by regular water changes (5-10% of system volume). Nitrates may also be reduced in zebrafish housing systems as a result of passive denitrification (conversion of nitrate to nitrogen gas) that naturally occurs in anoxic zones of recirculating systems (Masser et al. 1999; van Rijn et al. 2006). The efficiency with which the nitrifying bacteria operate is highly dependent on a number of chemical and physical parameters, especially alkalinity and dissolved oxygen, and to a lesser extent on salinity and temperature. Thus, biofiltration components of water treatment zones are designed to be highly oxygenated in order to maximally support the health of the bacteria. Alkalinity is maintained in the

system by addition of sodium bicarbonate or calcium carbonate. Reductions in either of these parameters will negatively impact the biofilter and compromise the system's ability to support fish.

Although there are numerous biofilter designs available, all options strive to maximize three variables: (1) surface area of the media, (2) water contact, and (3) oxygenation. The surface area is critical because of the positive relationship it has with microbial population size: the higher the surface area, the greater number of bacteria it can support in a given space or footprint. The design must also maximize water contact with the media where the bacteria are; if the water doesn't come in contact with the substrate, nitrification won't occur. Further, areas that don't come in adequate contact with the water will be subject to clogging. Finally, because nitrification is an aerobic process, the filter must be designed in such a way that it maintains dissolved oxygen at adequate levels. Nitrifying bacteria become inefficient at dissolved oxygen levels less than 2 mg/L (Masser et al. 1999).

The most commonly encountered biofilter designs in commercially available zebrafish housing systems are trickling media, moving bed bioreactors, and fluidized beds. Each of these types varies qualitatively with respect to its performance in the above-mentioned operating areas and has inherent advantages and disadvantages that should be considered during the selection and design stage of facility planning (Table 2).

Chemical Filtration

Once toxic nitrogenous waste products have been removed by the biofilter, the water in most, but not all, commercially available zebrafish housing systems is moved through chemical filters. The most commonly employed chemical filtration types include activated carbon (charcoal), ion exchange resins, and ammonia binders. This process is not considered to be essential, but it can be important. Activated carbon, in particular, is often used because it binds to and removes organic compounds that contribute to color and odors in effluent water that are not eliminated in the first two water

treatment steps—solids removal and biofiltration. It will also remove other chemicals from the water, most notably chlorine, which is sometimes present in municipal water supplies and is toxic to fish. It should be noted that in some instances, the introduction of new activated carbon to a system may result in pH spikes, so only pretreated, acid-washed forms should be used. Ammonia binders detoxify the ammonia that may be present in the water but perform no removal of it (Holmes-Farley 2003). Ion exchange resins bind to and remove positively charged ions, such as ammonium. Ammonia binders and ion exchange resins will work to save fish from the harmful effects of ammonia poisoning, but in most cases, use of either is a precautionary step to prevent total ammonia nitrogen that has not, but should have been, removed during the biological filtration step of treatment. Thus, a well-designed and properly functioning system should not require these latter two chemical filtration types. Each of these chemical filtration methods adheres to basic principles of surface area and contact time to maximize efficiency and requires regular maintenance and replacement because the filter media will load over time and become less effective or inoperable. Importantly, all of these chemical media types must be thoroughly rinsed before addition to the system.

Aeration/Degassing

Recirculating aquaculture systems are also typically engineered to maintain adequate levels of dissolved gases in system water. The most important of these dissolved gases for aquaculture are oxygen, which should be kept as close to saturation as possible (no lower than 4 mg/L), and carbon dioxide, which should be kept at levels less than 20 mg/L (Losordo et al. 1999). The exchange of these and other gases in a system is achieved by moving the surface of the water into contact with the atmosphere through various means, most typically air diffusers or air stones, surface agitators, and pressurized or nonpressurized packed columns (Losordo et al. 1998). When this occurs, oxygen is added to the water, and carbon dioxide is removed (off-gassed). The gas removal

Table 2 Features of commonly used biofilter types in zebrafish systems

| Biofilter type | Substrates used | Advantages | Disadvantages |
|------------------------|--|---|--|
| Trickling media | Various plastic materials (e.g., rings, blocks), sponges | Good oxygenation, simplest design, no mechanical or electrical requirements | Prone to fouling, high maintenance, low load capacity |
| Moving bed bioreactors | Bioballs, siporax, kaldness | Simple design, low maintenance, moderate to high load capacities, can use neutrally buoyant or positively buoyant media | Reduced load capacities, requires additional pumps, aeration, increased electrical requirements |
| Fluidized bed | Sand, silicon (glass) beads | Highest load capacities | More complicated design, requires additional pumps, aeration, increased electrical requirements, media is negatively buoyant |

functionality of such equipment may also provide some measure of protection against problems arising from supersaturation of the water with dissolved nitrogen or oxygen (Hargreaves and Tucker 1998).

Ideally, the aerating equipment should be sited at the point in the system where dissolved oxygen levels are at their lowest and carbon dioxide levels are at their highest (Losordo et al. 1998). In most zebrafish housing systems, this location is typically just prior to where recycled water enters the housing tanks (usually after ultraviolet sanitization).

Disinfection

All recirculating aquaculture systems contain and support populations of numerous microbes, including bacteria, viruses, protozoa, and fungi. Although many of these organisms are benign, or even beneficial, for the fish (e.g., nitrifying bacteria), some may also be pathogenic, especially when populations are high. By design, recirculating systems conserve water and exchange only a small percentage of overall system volume each day to facilitate the removal of nitrates. One of the consequences of this conservation strategy is that many of these co-occurring organisms in a system will accumulate over time. To keep the populations of these organisms in check, aquaculture systems utilize a disinfection step, wherein the system water flows through a disinfection unit of some type subsequent to solids removal, biological filtration, and chemical filtration (if used). Ultraviolet disinfection is the most frequently applied method of water disinfection in zebrafish housing systems (Harper and Lawrence 2010).

Ultraviolet disinfection units, also called ultraviolet sterilizers, use light in the ultraviolet range to damage DNA of organisms, thereby killing them or rendering them inactive. Typically ultraviolet lamps are placed in a quartz sleeve, and water is passed around the sleeve, thereby exposing the water to the ultraviolet range light. Important factors in the effectiveness of ultraviolet sterilizers are the wattage of the lamp (intensity), the ability of the ultraviolet light to penetrate the water (transmittance), the length of time the water is exposed to the light (contact time), and the size and biological complexity of the organism targeted (Yanong 2003). There is no published standard for the level of irradiation (or fluence) required for disinfection in zebrafish facilities, although most commercial systems offer units that operate at a dose rate of 110,000 $\mu\text{Ws}/\text{cm}^2$ at end of lamp life (100% of bulb life).

The main advantages of ultraviolet sterilization are that it is relatively safe to operate and it is not harmful to the cultured species (Losordo 1999). It is critical that these units be serviced regularly (e.g., bulb changes, quartz sleeve cleaning and replacement) to ensure they are operating at maximal efficiency.

Water Quality Control and Monitoring

Water quality in a recirculating system is extremely dynamic. The complex interactions between the fish, bacteria,

and other microorganisms in the system both affect and are affected by chemical and physical parameters in the environment. The list of these parameters is expansive, and a complete description of their complex relationship with the organisms in the system is well beyond the scope of this review. However, there are a number of factors that are of particular importance to the function of life support, and they will be treated here, relative to the effects they have on the fish and the biological filter as well as the manner in which they must be monitored and controlled.

Automation of Water Quality Monitoring, Control, and Alarming

The central operating goal of a zebrafish housing system is to maintain a favorable and stable environment (water quality) that supports the health and function of both fish and the nitrifying bacteria that drive the life support within the system. Therefore, water quality in the system must be both managed and monitored to ensure that it remains within target ranges (Table 1). This can always be achieved manually by regular testing of the water with colorimetric or titrimetric water chemistry kits and physical or chemical manipulation of the system based on these findings. However, as the scale of zebrafish housing systems increases, so too does the need for automation of these processes. Automation facilitates a more continuous level of monitoring and control not otherwise possible in systems of any size. It also gives a manager a tighter degree of control over what is happening in the system, which is important as the margin for error becomes smaller in closed, intensive housing systems.

Virtually all commercially available options for zebrafish housing systems are now designed to allow for some degree of automated monitoring, alarming, and control of water quality. There are various permutations of design, but most setups allow users to continuously monitor, record, and view water quality parameters in the system and control various parameters and system components; they also provide a measure of security by sending alarms and/or automatically shutting components down when selected parameters move above or below threshold setpoints.

By and large, these monitoring systems all involve the use of probes or sensors that are either placed in contact with the sample flow stream or, in the case of physical parameters, are in an appropriate location in the system. These instruments interface with software programs, some of which allow for access of the system over the Internet, and in some cases, it is possible to view and control the system using a handheld device or cell phone.

Monitoring and Control of pH

The relative concentration of acids and bases in solution determines its pH. A major challenge of water quality

management in a zebrafish housing system is to maintain water within a pH range that supports both fish health and biological filtration. This is a constant balancing act because a number of processes in the system, including fish metabolism, the catabolism of organic wastes, and especially the oxidization of total ammonia nitrogen by the nitrifying bacteria in the biofilter, produce acids and continuously decrease the pH of the water. If the water is not buffered, the pH will typically first drop below the threshold required for biological filtration, resulting in an ammonia spike that will have the potential to stress, injure, or even kill the fish. Therefore, bases must be added to the water accordingly to offset the production of acids. This can be achieved via manual addition of buffers, usually sodium bicarbonate, to the system. Systems with automated pH monitoring and control options measure the pH continuously via in-line probes and can be programmed to automatically dose the system with sodium bicarbonate solution when the pH level drops below a predetermined setpoint. When this happens, the system is dosed until the pH moves back above this target. At that point, the dosing stops, and the system resumes normal operation until values drop below setpoints again.

Some systems also may be constructed to include a fluidized aragonite reactor, which is an alternative to the standard sodium bicarbonate dosing method. Aragonite is a form of calcium carbonate that dissolves into its constituent parts, calcium and carbonate, when in contact with water with a pH less than 7.8. The carbonate helps to maintain pH, whereas the calcium contributes to hardness values, which are important for maintaining fish health (Harper and Lawrence 2010). A fluidized aragonite chamber is designed to upwell filtered system water through a bed of aragonite, thereby continuously maintaining pH values within target ranges. Aragonite can also be used in conjunction with a standard pH sodium bicarbonate dosing system to provide an extra measure of pH stability while simultaneously contributing to hardness values. When adding aragonite or any other substrate to systems, it is important to ensure the product being added does not pose a biosecurity risk; substrates should be autoclaved or irradiated prior to their introduction to a system.

Monitoring and Control of Temperature

Temperature exerts a significant influence on all biological and chemical processes that take place in the microenvironment of a fish system. Zebrafish are tolerant of a broad range of temperatures but appear to perform best at stable values in the range of 25–30°C (Lawrence 2007). Temperature also affects the efficiency of the nitrifying bacteria in a recirculating water system, although warm-water systems should see no problem in this regard (Masser et al. 1999). However, given the profound influence temperature can have on the system, it is imperative that the housing system give users the ability to tightly regulate this parameter.

Temperature is typically regulated by heaters or heat pumps that are incorporated into the housing system design, but temperature can also be controlled by ambient room temperature as controlled by a building heating, ventilating, and air conditioning system. The use of both approaches in concert can help to reduce the workload of heating elements while providing some measure of redundancy.

Although temperature can be measured by daily checks using traditional thermometers or handheld infrared thermometers, it is preferable to record this parameter continuously. Again, most commercially available housing systems facilitate this by including temperature probes that are part of the overall monitoring and control system. This provides users with the added benefit of being able not only to remotely check and adjust temperature in the system in real time but also to track the temperature of the system over time. This especially valuable tool is helpful when troubleshooting problems in the system.

Monitoring and Control of Dissolved Oxygen

Dissolved oxygen is a very important water quality parameter. Low levels of dissolved oxygen are responsible for more mortalities than any other parameter in aquaculture, although this rarely is a limiting factor in zebrafish systems. Typical stocking densities and associated feed rates in most zebrafish colonies dictate a target dissolved oxygen level near saturation, for example, 7.75 mg/L at sea level atmospheric pressure and in water warmed to 28.0°C (Lawrence 2007; Masser et al. 1999). In any case, a dissolved oxygen level less than 5.0 mg/L is a cause for concern in warm-water systems (Masser et al. 1999), and normal zebrafish system operation should keep the dissolved oxygen level above this minimum.

Dissolved oxygen can be measured by chemical test kits, by handheld meters, or by in-line probes that are part of an automated monitoring system. Many system-level devices have displays that indicate current dissolved oxygen levels and include outputs for external monitors that will facilitate dial-out notification and data logging. Handheld meter/probe devices can be used where infrequent checks are desirable or for verifying system-level readings. Oxygen enters the water through surface exchange, and disturbing the water surface by means of influent water flow (drip or stream) or by means of in-tank aeration (bubbles) will ensure no oils from foods or other obstructions reduce surface oxygen exchange. In extreme cases when large numbers of fish per water volume and associated oxygen use by fish and bacteria exceed available dissolved oxygen, surface oxygen exchange can be supplemented with injections of pure oxygen, although this type of extreme case is not recommended in zebrafish systems.

Monitoring and Control of Salinity/Conductivity

Zebrafish are freshwater fish, although they can tolerate water approaching brackish levels of salinity (Best et al. 2010;

Lawrence 2007). For all freshwater fish, there is an energy expenditure that takes place as internal body salts and water are balanced against the salinity of the surrounding water (osmoregulation) (Lawrence 2007). To maximize energy use for growth and reproductive purposes, zebrafish systems monitor and regulate salts, principally sodium chloride, in the fish water to try to minimize the cost of osmoregulation to the fish. Salinity levels frequently vary in zebrafish systems whenever fresh water is added to the system or when water is lost to evaporation.

Salinity is measured in parts per thousand or as the water's ability to conduct electricity (conductivity) expressed in microSiemens per centimeter. Again, although salinity can be measured using a refractometer, in-line probes and meters that are part of automated monitoring systems can measure conductivity (or salinity) continuously. In a scenario similar to that of the above-described sodium bicarbonate method of autos dosing to maintain pH values, these systems can be employed in concert with controllers and dosing pumps that automatically add salt solution to the system water when probes detect values below established setpoints.

Monitoring and Control of Total Dissolved Gas Pressure

If the total gas pressure in fish water is not allowed to equilibrate with atmospheric gases, supersaturation results and excess gas, mostly nitrogen, may enter the bloodstream of the fish and result in illness or death (Noga 2010). Gas bubble disease is caused by gas supersaturation. Gas supersaturation is the result of mechanical failure or poor system design, and known causes include cavitating pumps, Venturi injectors, and rapidly heated water (Noga 2010).

Total gas pressure is commonly expressed as a percentage of total barometric pressure. In general, saturation levels of 110% or greater are dangerous to fish (Noga 2010). Zebrafish systems can monitor total dissolved gas pressure using logic controllers with meters and probes connected to dial-out notification. The total gas pressure monitor can be connected to water distribution pumps, so in the event of a mechanical failure that causes total gas pressure to exceed 100% saturation, the pumps can be shut down, thereby saving the fish from supersaturated water, and at the same time system managers can be notified by means of an email, telephone call, or text message. Additionally, local alarms or horns can be used to notify people in the immediate vicinity of the system when such an event occurs. In addition to total gas pressure monitors, saturoimeters can be purchased to test water for gas supersaturation (Noga 2010). Whether flow-through system or recirculating system, with proper design providing degassing/gas stripping areas, only mechanical failure will cause gas supersaturation, and the proper use of monitoring and control equipment can reduce or eliminate fish illness or death resulting from such an event.

Despite the clear advantages offered by automated monitoring system technology detailed above, confidence in such a system, no matter how sophisticated, must be paired with regular checks, calibrations, and maintenance on the system itself. For this reason, it is critical that the system and its components (e.g., probes, sensors) be cleaned and calibrated regularly to ensure they are functioning properly. A probe that is not measuring properly is not controlling properly, and such an event could have disastrous consequences for the fish. Therefore, in addition to routine maintenance, it is strongly recommended that the system parameters be routinely cross-checked with other reliable testing means, including colorimetric or titrimetric water chemistry kits. Simple water testing strips are also available for most parameters and can be used for this purpose as well. These particular testing tools are especially useful to deliver a quick preliminary readout of a water quality situation before more sophisticated and accurate methods of measurement are employed.

Racks and Tanks

Zebrafish housing in the research laboratory is often referred to as "racks and tanks." In truth, the zebrafish research drives the need for the animal housing and that need can be satisfied by anything from a single glass aquarium on a lab bench to a large housing room with rows of racks and thousands of small plastic tanks. With three decades of exponential growth in zebrafish research (Lieschke and Currie 2007) and its associated need for animal housing, commercial companies have emerged to fill the zebrafish housing need and though some innovative ideas are present, the standard for zebrafish housing in the research lab is the rack and tank system that, from a distance, might resemble books on shelves in a library. Still, such systems represent an elegant mix of commercial aquaculture, laboratory animal housing, and research genetics, and can be tailored to meet the needs of multiple research programs in one facility.

Racks

In its most basic, generic form, a rack in the context of zebrafish housing is a structure that safely holds tanks. The size and shape of the tank will dictate the rack requirements. The housing needs of the zebrafish, usually kept in groups whose numbers are driven by the research, will dictate the requirements for the tank. In its most developed form, a rack supports its tanks not only by organizing them in a 3-dimensional grid but also by providing the infrastructure that helps each tank successfully house the fish. Examples of this infrastructure include influent and effluent water pipes, aeration, lights, integrated control and monitoring capability, and incorporated work surfaces, among others. Ergonomics is also an important part of rack design. Rack designs that include forethought to ergonomic issues benefit researchers, facility managers, and

animal care personnel. Commonplace tasks like feeding, removing and replacing tanks, and daily health checks can be aided by creative design elements in lid features and attention to height and accessibility, for example. In any case, racks can be designed with regard to human safety and usability concerns as well as concerns for proper fish housing.

Configurability

Because research moves in many directions, rack designs that allow for some customization by the researcher will likely serve the research better than static designs that cannot be modified. Racks can be constructed to allow for such variations and allow for changes required by the researcher. The ability for a rack to accommodate tanks of differing water volumes is an important aspect for many researchers. Considerations for the tank infrastructure and the ease of replacing one tank size with another can be incorporated into rack designs. Some research may require racks to accept influent lines from different water sources or require racks to empty into different waste streams. Racks can be designed with water system components built into the structure. These “stand-alone” designs use space under the lowest rack row to house water filters, pumps, ultraviolet sterilizers, and all other equipment necessary to provide water for the fish. Racks can be constructed with photoperiod capability for one or more rows, allowing for research that demands alternate photoperiods for the fish. Racks can be as small as a single table-top model or as large as room size, building codes, and ergonomics will allow.

Construction Materials

Successful zebrafish rack construction requires attention to the wet, humid, warm environment that results from zebrafish aquaculture and the use of materials that will withstand this environment. Although zebrafish are classified as a freshwater species, zebrafish system water contains some salts and minerals that can corrode many metals. Therefore, racks constructed of corrosion-resistant material that will endure in this type of environment are preferable.

Materials toxicity is a concern when introducing any new equipment, including racks, into the facility or fish room. Some standard plumbing fittings are known to be constructed with fungicides and other compounds toxic to aquatic life. Silicone sealant used for household kitchens and bathrooms is not appropriate for the zebrafish rack because of the additives that make it resistant to mold, for example. There is no requirement for commercial rack vendors to test materials for fish safety, and therefore the burden is on the researcher or facility systems manager to ensure all rack materials are safe for fish.

When choosing materials, the standards for sanitation and disinfection found in the *Guide for the Care and Use of Laboratory Animals* (NRC 2011) should be remembered, especially the standards for repeated use of disinfectants and

sanitizing agents like oxidizers. Although, because of their infrastructure components, aquatic racks are generally difficult to move, sanitizing parts of the rack through disassembly is an option, and some rack designs allow for easy disassembly and reassembly. In some instances, racks can be constructed to rest on casters, and can be disengaged from the plumbing to facilitate removal and disinfection if the need arises. Implementation of influent and effluent water pipe clean-outs and system-wide particulate flushing capabilities are also welcome additions to rack designs.

Additional Considerations

In some geographic regions, seismic activity necessitates safety considerations for bracing. Racks can be constructed with seismic concerns in mind, and attachments to accommodate bracing can be incorporated into rack designs, thereby lessening concerns that racks would topple during a seismic event. Vibration and noise may also negatively affect aquatic life (NRC 2011), and considerations for these factors should be a part of rack design and construction. Such measures may include dampeners on stands that support pumps or other equipment that create vibration during operation. Ideally, pumps and other water treatment equipment will be sited in a separate enclosed room, thereby implementing a facility design consisting of discrete zones for housing and water treatment that minimize vibration, heat, and other undesirable effects associated with having mechanical equipment and housing in the same space. This arrangement also facilitates routine maintenance and repairs. When this option is not available, however, appropriate measures to mitigate these issues must be taken.

Tanks

Tanks used in zebrafish housing systems may vary in shape, size, and materials with which they are constructed. They also vary in terms of how water is delivered and removed from them as well as the manner in which they contain fish (prevent fish from escaping into the system). These factors all impact the well-being of the fish, the functionality of the system (maintenance of stable and favorable water quality), and the pace and efficiency of research being conducted with the animals. Therefore, the choice of a particular system should include careful consideration of its associated tank types and how these types help users achieve their research and husbandry goals.

Construction Materials

As in rack construction, materials toxicity in tank construction is a concern in zebrafish research. Tanks are commonly made from glass, acrylic, fiberglass, polycarbonate, or polysulfone. In high-density installations, tanks are likely to be made from clear polycarbonate, primarily because it is durable, relatively inexpensive, and holds up to autoclaving. However, it

has been established that both polycarbonate and polysulfone can leach bisphenol-A, an estrogen mimic that can cause serious reproductive problems in vertebrates, including fish (Brotons et al. 1995; Duan et al. 2008; Howdeshell et al. 2003). This is of potential concern, especially for established zebrafish facilities using polycarbonate tanks, as it has been shown that old polycarbonate leaches significantly more bisphenol-A than new polycarbonate or polysulfone (Howdeshell et al. 2003). There is evidence that activated carbon can remove some bisphenol-A from the water (Bautista-Toledo et al. 2005), although the scale of carbon filters in most zebrafish applications is not likely to remediate the problem. Regular tank replacement may be the most reasonable strategy until a satisfactory bisphenol-A-free replacement material can be found.

Rubber-type seals commonly used in the plumbing industry are not all safe for zebrafish. For example, testing at the University of Oregon has shown ethylene propylene diene monomer rubber causes mortality in larval fish (B. Trevarrow, personal communication). There is no requirement for commercial rack vendors to test materials for fish safety, and therefore the burden is on the researcher or facility systems manager to ensure all tank materials are safe for fish.

Shape and Size

Although there are no published standards for the size and shape of tanks used for zebrafish, commercially available options generally fall into a distinct size and space footprint range (Table 3). This seems to be to some extent

Table 3 Space footprint and typical application of various commercially available zebrafish tanks

| Tank size, L | Maximum no. of shelves ^a | Typical application ^b |
|--------------|-------------------------------------|-------------------------------------|
| 0.8 | 18 | Isolation of 1 or 2 adults, nursery |
| 1.1 | 15 | Nursery, isolation of 1 or 2 adults |
| 1.4-1.5 | 20 | Isolation of 1 or 2 adults, nursery |
| 1.8 | 18 | Nursery, isolation, up to 18 adults |
| 2.8-3.0 | 12 | Nursery, up to 28-30 adults |
| 3.5 | 10 | Up to 35 adults, nursery |
| 6.0 | 6 | Up to 60 adults, nursery |
| 8.0 | 5 | Up to 80 adults |
| 9.0 | 5 | Up to 90 adults |
| 11.0 | 10 | Up to 110 adults |

^a Assumes a standard shelf width of 60" (152.4 cm)

^b Assumes a standard density of 10 fish/L for group housing of adults

dictated by the standard size of most commercially available rack units, which is typically 60 in wide × 90 in high × 14 in deep. This rack size limitation, the drive to maximize the number of animals in a given space footprint, and the general number of animals considered to be necessary to propagate a strain are the primary factors in determining tank sizes and shapes.

Holding densities are also a major determinant. Although there are no data-driven standards for the numbers of animals managers may hold in a given volume of water, an accepted range appears to be 5 to 10 fish per liter for adults (Matthews et al. 2002), with higher ranges acceptable for larval stages (Best et al. 2010; Harper and Lawrence 2010).

Consequently, smaller tanks with a volume of 0.8 to 3.0 L are used to house larval fish or smaller numbers of adults. Adults are generally housed in tanks ranging in volume from 3.0 to 10.0 L. These applications are somewhat arbitrarily determined, although biological and practical justifications can be made for these approaches. For example, it is both efficient and beneficial to house larval fish at higher densities in smaller tanks because they appear to tolerate being held at higher densities more than adults and because doing so in combination with large numbers of prey items facilitates the maximization of encounter rates between fish and prey (Best et al. 2010; Lawrence 2007). As fish grow into juvenile and adult stages, they must be held at lower densities to support growth rates and maintain conditions most conducive to welfare (Matthews et al. 2002). In some research applications, it becomes necessary to isolate adult fish individually or in pairs and it is much more practical to house them in lower volumes for space considerations.

Containment

A major operating goal of any zebrafish tank is to ensure that fish are completely contained within a given unit and do not intermingle with fish from other tanks. This issue is of no small concern because zebrafish tend to be very good at fitting through small spaces. Preventing escapes is critical for maintaining the genetic integrity of strains as well as for the control of specific communicable diseases. Tank design facilitates this via the implementation of tight-fitting lids as well as screens and baffles that, respectively, allow for water delivery and removal but prevent fish escape. The design strategies employed to achieve this goal change with the size and life stage of the fish being housed. Larval fish, in particular, present a specific challenge because of their small size. This challenge is usually met by employing specialized screens or "baby baffles" that exclude the passage of small fry out of tanks. Some tank designs also offer in-tank dividers as a means to isolate discrete groups or single animals from others in the same enclosure unit. Lids, too, can be manufactured to prevent fish escape, and vertical feed holes can discourage escape via jumping better than horizontal feed holes (T. Mason, personal observation).

Zebrafish systems vary to some extent in the manner that water is delivered to and removed from tanks on a rack. The general strategy that all commercial zebrafish housing systems employ is that tanks on a given rack are connected in some way to both supply lines and return gutters. In general, water is plumbed to racks in supply lines that run above tanks on each shelf. Water flows into tanks, and effluent overflows out through baffles or screens (that also serve to keep fish in tanks) into a gutter or raceway that drains to the treatment zone in the system.

The variation in current designs involves the manner in which the water is actually delivered into and drained from individual tanks. There are various strategies for delivery. In a few applications, water sprays directly into the tanks through holes drilled in the supply lines, meaning that control of flow is controlled at the level of the shelf (row) and not the tank. The supply lines may run within the tanks themselves, just above the water level, or spray down from above directly onto lids. Because this lack of control is not desirable, movement has been toward individual water tubes connected to the supply lines above the tanks. In this design the flow rate of water into tanks is controlled by valves or drip emitters. This is by far the most common approach, but it has known drawbacks. These valves or emitters are often a source of flooding because users incorrectly operate the mechanism or are never properly trained to do so. These devices may be difficult to adjust, which can be problematic for different life stages of fish, especially for larval stages that benefit from gradual increases in the rate of flow as they develop (Harper and Lawrence 2010). Delivery tubes running into tanks can become improperly placed, and, as a result, tube ends can come into contact with food on lids or fall into the water itself. Both of these occurrences pose potential health risks to the fish. For example, food on a lid can promote bacterial and fungal growth. Supply hose ends that come into contact with this growth may allow bacteria and/or mold to enter the stream and flow into the tank along with the supply water. When a supply hose is submerged, there is risk of cross-contamination between tanks, especially when tanks are moved from position to position on the rack shelf. These concerns can be mitigated through training, vigilance, and hose changes, which can, in turn, increase labor and costs for consumables. In some newer applications, delivery (and removal) of water into tanks is achieved by so-called “push and pull” locking valves. In this design, tanks are pushed into racks and locked into position where they then receive flow that can be adjusted by the user. In some designs, the locking mechanism is disengaged, and flow to the tank is automatically shut off when the tank is taken off-line, whereas in others, water continues to flow. The risk of improper placement is eliminated because, by design, the tank position is restricted to placement on the shelf under a supply valve. This greatly reduces user error that can contribute to flooding.

Effluent water removal from tanks is usually accomplished via tank overflow into a gutter or drain at the rear or front of a rack shelf. This is generally effective, although the location of the overflow will affect the efficiency with which solids are also removed from the tanks. Most tank designs move solids along the tank bottom, where they are either removed via a siphoning mechanism or flow beneath small gaps in baffles and exit through an overflow into a gutter. Designs that do not employ some version of either approach will not remove solids effectively. The connection between overflow and gutter or drain is also variable. In most instances, the tanks must be placed on racks so that overflow spills or trickles into gutters. In these cases, user error may be a problem; if tanks are not properly placed, flooding problems can occur. For this reason, tank designs that incorporate the coupling of overflow to drain via a direct, physical “locking” connection mentioned above are preferable.

Additional Concerns

The humid, moist environment in zebrafish housing rooms encourages fungal growth, and the standard method for feeding fish tanks is to individually distribute the food. Lid cleanliness is a common problem in zebrafish labs, and lids designed with features that encourage food to fall into the water are preferred.

Algal and/or cyanobacterial growth are also concerns for fish husbandry. The nutrient-rich water that encourages zebrafish growth and health can also encourage the growth of algae and cyanobacteria. Some tanks have been designed to discourage the growth of these organisms through color choices for tank lids and walls, although this method is not entirely effective. Many tanks are designed with shapes that encourage waste to lift and exit the tank along with the effluent water (see “Solids Removal”). Waste may encourage the growth of algae and cyanobacteria as well as other organisms that may be pathogenic to the fish (R. Wagner, personal communication). Some designs are more efficient than others at removing waste, and, in any case, some solid waste will collect and necessitate tank cleaning or replacement.

Additional Rack Features

Racks and tanks can be complemented with other equipment, such as options for automated feeding, mass breeding, and sentinel tanks, among others. Commercial manufacturers often have the ability to custom build equipment for research projects, and this can offer facility managers and researchers greater flexibility to meet various management or experimental goals.

Feeding Equipment

There is no standard zebrafish feed (Harper and Lawrence 2010), and current commercial solutions for feeding equipment

are generally able to accommodate various dry feeds, although they are often designed for a particular tank system. Choices for feeders include both manual (handheld) and automatic. Handheld feeders range from a simple spoon or spatula to a small battery-driven vibrating dispenser. In general, automatic fish feeders include those types found in the aquarium hobby trade designed to deliver dry feeds (flakes and/or pellets), although a promising development in this area is the recent advent of a robotic feeding unit with the ability to deliver measured quantities of both live and dry feeds. Designed for a specific tank system, this is a major advance for larger facilities and could signal a trend for future automatic feeders in other tank systems.

Breeding Equipment

Embryo production drives many different zebrafish research applications. The need for embryos has fueled the development of specialized equipment designed to encourage zebrafish adults to breed. Simple, traditional 1- to 2-L static crossing cages (e.g., Mullins et al. 1994) have long been used to breed pairs or small groups of adults. More recent advances have been made to facilitate mass spawning involving hundreds of males and females. Several options for this are now available from commercial vendors and are designed as integrated units within housing systems. The same sanitation, durability, and material toxicity concerns that apply to racks and tanks apply to breeding equipment.

Sentinel Tanks

Health monitoring or sentinel programs are an integral part of a professionally run zebrafish research program (Matthews et al. 2002). The goal of a sentinel program is to detect and monitor pathogens in a fish colony by routinely (i.e., semiannually or quarterly) collecting samples of fish and running disease diagnostics on them (usually histopathology). In most program designs, sentinel tanks containing the fish to be analyzed are set up within housing systems, both prefiltration and postfiltration. This approach not only allows for the monitoring of disease but also allows managers to assess system function and ultraviolet performance. Depending on the vendor, specialized housing tanks plumbed to receive effluent (postfiltration) water either are available as options for currently offered systems or can be customized for individual applications.

Conclusions

Zebrafish research programs have matured in size and complexity far beyond the flow-through water system and glass aquariums Dr. George Streisinger used in the 1970s and 1980s. With the increased demand for life-support, housing, and husbandry equipment, the zebrafish community has seen innovative solutions to its needs and will undoubtedly see

improvements in the future. As scientists find novel ways to use the zebrafish in vertebrate research, projects will drive industry, and new advances in equipment design will facilitate the further growth and utility of the zebrafish model system.

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