ILAR Journal

ILAR Journal V37(4)
Fish, Amphibians, and Reptiles

Guidelines for the Care and Use of Fish in Research

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INTRODUCTION

This article focuses on the use of fish as a laboratory resource. The discussion should be useful to the principal investigator and the personnel responsible for fish husbandry, care, and management. It will also aid members of institutional animal care and use committees (IACUCs) when they evaluate the proposed use of fish species in various research projects.

During the last 10 years, the use of fish in research has been increasing (DeVita 1984; Post 1987; Powers 1989; Goodrich 1990; Evans 1993; Stoskopf 1993a). The development of fish as a food source has also grown. Aquaculture is one of the most rapidly expanding new food industries partly because fish are seen as a low-fat food source and as a replacement for meats that are fat-rich. Consequently, an increasing emphasis is placed on the amount of fish consumed, the quality of fish produced, and the efficiency of fish growth.

Research into the health and husbandry of food fishes is advancing with the development of the aquaculture industry and an increasing interest in maintaining fish as pets. At some point, the U.S. Department of Agriculture (USDA) or a similar group might inspect fish to ensure their quality as a food source. Research on fish is also expanding and intensifying as a result of a greater interest in establishing new aquaria for entertainment and educational purposes. Although aquarists are interested in a wider range of species than aquaculturalists, they are in a unique position to solve special husbandry problems with special species as well as identify valuable animal models or species with particular biological properties. The attention that fish have received from both hobbyists and the fish industry has increased awareness of fish health as a factor that impacts the environment and as a measurement of environmental health (McKone and Daniels 1991).

Fish research and more specifically research in aquaculture helps address questions regarding environmental pollution, conservation, and protection of the freshwater estuarine and marine environment. Fish are increasingly used in the laboratory as animal models in toxicology. Outside of the laboratory fish are also subjected to environmental stresses (man-made and otherwise) that can harm their health and well-being.

All of these factors should also act to stimulate field research, that is, research performed directly on fish in their natural habitat, even though the number of experimental variables is much greater. Recently, as a result of greater concerns about the humane use of higher vertebrates in research, fish have been evaluated as a replacement in toxicologic, pharmacologic, and genetic studies that might otherwise employ mice or other mammalian species. All of this will increase the use of fish in research and expand the knowledge base on the care and use of these species in the laboratory.

Comprehensive guidelines on the care and use of fish would be difficult to compile as there are so many different types of fish with a variety of husbandry requirements. Fish research in laboratory animal science explores and uses their incredible diversity (Pough 1992); the 20,000 species of fish worldwide constitute about half of all living species of vertebrates, making them the largest group of living vertebrates (Schaeffer

and others 1992). In addition to size, which varies from a few to 15 meters, fish vary significantly in their taxonomy, morphology, genetics, behavior, physiology, and ecology. Currently, the Animal Welfare Act does not cover certain animals, including cold-blooded vertebrates. However, all institutions funded by the Public Health Service (PHS) must follow the *Guide for the Care and Use of Laboratory Animals (Guide)* (NRC 1996), which covers all vertebrate species. The specific use of cold-blooded species including fish is not discussed in the *Guide,* but all institutions are expected to care for and use fish in research in a manner judged to be professionally and humanely appropriate for the particular species in question. Although fish differ from both warm-blooded and other cold-blooded species, like their endothermic counterparts they need to be maintained in a controlled environment with a limitation on stress.

SPECIES--MARINE AND FRESHWATER

Certain species of fish that are frequently used in research include rainbow trout *(Oncorhynchus mykiss)* (which range from 25-30 cm in length) and other salmonids, such as coho salmon (O. *kisutch),* sockeye salmon (O. *nerka),* Atlantic salmon *(Salmo salar)* and brook charr *(Salvelinusfontinalis)* (less than 25 cm long). Toxicity testing is frequently done with fishes such as the fathead minnow *(Pimphales promelas)* (up to 10 cm), sheepshead minnow *(Cyprinodon variegatus)* (8 cm), silversides *(Menidia beryllina* and M. *menidia)* (up to 14 cm), and the Japanese reedaka *(Oryzias latipes)* (up to 4 cm). The zebrafish *(Brachydanio rerio),* which is less than 5 cm long, is currently being used extensively in molecular and genetic studies. Other species frequently studied include catfish such as channel catfish *(Ictalurus punctatus)* (up to 1.2 m), brown bullheads *(Ictalurus nebulosus)* (up to 50 cm), sunfish *(Lepomis macrochirus)* (about 20 cm), tilapia *(Oreochromis mossambicus)* (up to 40 cm), Amazon molly *(Poecilia formosa)* (3-5 cm), and American eels *(Anguilla rostrata)* (up to 1.2 m). Ornamental species are also used such as goldfish *(Carassius auratus)* (up to 30 cm) and koi *(Cyprinus carpio)* (up to 60 cm).

The UFAW Handbook on the Care and Management of Laboratory Animals (Poole 1987) has useful chapters on freshwater fish and marine fish, which also address their potential laboratory uses.

AVAILABILITY OF FISH SPECIES

Choice of Species

When selecting fish for laboratory research, the first consideration should be choice of fish species. Depending on the type of research, the species will fall into three main types: marine, freshwater, or brackish. Making this decision will determine much of the life support structure needed, as well as begin to delimit husbandry measures.

Ease of maintenance is also a principal component of species choice. Delicate animals that require special care several times a day and those that suffer higher mortality rates may not be as desirable a choice as hardier fish that require less laboratory personnel time. Factors such as diet, temperature requirements, resistance to disease, and social compatibility all need to be considered before selecting a species. Space requirements, too, should be thought out. Some species, whether because of their size or an active or aggressive nature, need larger environments, which can take up laboratory space and require larger and more expensive life support equipment.

Sources of Fish

Once a suitable fish species has been selected, the investigator must then choose between acquiring captive-bred or wild-caught animals. Captive-bred fish are supplied mainly by hatcheries and laboratory supply houses, but some laboratories, aquariums, and hobbyists are also able to provide stock. Wild-caught fish can be bought from suppliers, or collected by oneself. Individual fish hatcheries are too numerous to list in this article, but they may be identified through each state's department of natural resources, or analogous office. For a description of how fish are collected, some of the challenges they face, and the associated medical procedures, investigators can refer to *Medical Procedures Used During the Capture and Transport of Fish* (Stetter 1992). See Table 1 for an abbreviated list of fish suppliers and other resources.

Permits and Licenses

Wild-caught fish for laboratory use, whether captured by an investigator or a collector, may require scientific collecting permits. These include the federal and state scientific collectors permits and import permits.

Obtaining a state scientific collecting permit requires that one contact the appropriate governing agency (such as the department of natural resources), and request an application. The permits are not difficult to complete, last for 1 to 2 years, and are usually approved, although collecting protected or regulated species (such as striped bass, trout, or certain shellfish) may require special justification. The species sought will need to be listed by the applicant. Special collecting techniques such as electrofishing, use of certain types of nets, or use of chemicals may also call for a separate letter of authorization. The state office will require the collector to notify it where and when the collecting will take place, as well as to submit a year-end report on collection activity.

Federal permits are not needed unless one is working outside state boundaries, in federal waters, or with federally protected species. A telephone call to the Federal Wildlife Permit Office (see Table 1) will help to determine if a permit is required, as well as through which regional office the application should be routed. Having a federal permit for an endangered species will not automatically cover state requirements, however. The appropriate state agency will be able to determine if a special state license is needed in addition to the federal one. A federal listing of endangered and threatened wildlife and plants can be obtained from the U.S. Fish and Wildlife Service. Title 50 of the U.S. Fish and Wildlife Service restricts movement of certain food or sport fishes or their products from different parts of the world. Although the legislation applies only to food and sport fishes (including live and dead fish, fish products, and fish eggs), it is wise to be aware of diseases and their potential for transmission even when importing ornamental fishes for research.

When importing fish from outside the U.S., a special Declaration for Importation or Exportation of Fish or Wildlife (USFWS Form 3-177) must be completed and filed with the local U.S. Customs agent. This may be accompanied by an inspection by a Customs agent.

Fishes that appear on the Convention on International Trade in Endangered Species (CITES) lists are strictly controlled, and without the proper authorization, importation is prohibited. Appendix I of CITES lists animals that are threatened with extinction, and their acquisition is highly discouraged except under the most exceptional circumstances. CITES permits commercial trade in species listed in Appendix II, provided that the country of origin has issued an export permit. Appendix III species may be traded; listings are made in order to gain recognition and protection for species that are in danger of becoming threatened.

SUMMARY OF APPLICABLE ANIMAL WELFARE LAWS AND GUIDELINES

Any vertebrate research performed or sponsored by the U.S. government is covered by the *U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training (PHS Principles)* (PHS 1986 p 27). For guidance in following these principles, investigators are directed to the *Guide* (NRC 1996), which recommends that an "appropriate environment be provided" for nontraditional species and that "expert advice on the natural history and behavior of nontraditional species" be sought (NRC 1996, p 14 of prepublication copy). Except for a short bibliography, fish-specific information is not included in the *Guide.* Other useful guidelines include *The Care and Use of Amphibians, Reptiles and Fish in Research* by the Scientists Center for Animal Welfare (SCAW) (Schaeffer and others 1992) and *Guidelines for The Use offish in Field Research* by the American Society of Ichthyologists and Herpetologists, the American Fisheries Society, and the American Institute of Fisheries Research Biologists (ASIH 1987). Also useful is the Canadian Council on Animal Cafe's *Guide to the Care and Use of Experimental Animals* (CCAC *Guide)* (CCAC 1989). The Animal Welfare Act does not cover fish or any of the cold-blooded vertebrates.

The *PHS Principles* recommend selecting the appropriate species of the appropriate quality, as well as using the minimum numbers of animals required for valid results. Nonanimal alternatives, discomfort, distress, and pain must also be considered. Living conditions should contribute to the health and well-being of the animal, and animals should be cared for by trained and experienced personnel, inclusive of veterinary care. Investigators and personnel should be qualified, experienced, and receive training if necessary. Any exceptions to the *PHS Principles* must be reviewed by the IACUC. All of the above conditions are directly applicable to research with fish.

In addition to consulting with the attending veterinarian of the facility, it is frequently valuable for the IACUC to have access to regular ad hoc members that are full-time specialists in the maintenance of fish as research

animals. Individuals with relevant backgrounds could be scientists, aquarists, curators, directors of husbandry, or veterinarians working in aquariums or aquatic research centers.

DEVELOPING PROCEDURES FOR HOUSING, HUSBANDRY, AND BREEDING

Centralized Facilities

Since there are over 20,000 species of fish that live under many differing environmental conditions, husbandry will vary depending on the natural environment for a particular species. Life-support systems must be set up and "acclimated" prior to receiving animals (Spotte 1979, 1981; Alabaster and Lloyd 1980; Hawkins and Lloyd 1981; Munro and Roberts 1989; Goodrich 1990). During construction, thought should be given to providing adequate space and water quality. If a municipal water supply is used the water must be either dechlorinated by aeration or by filtration through activated carbon or chlorine-precipitating compounds such as sodium thiosulfate. All water should be analyzed prior to use, whether municipal or well water. Investigators must be aware of the appropriate levels of pH, ammonia, nitrate, and calcium in the water medium (Tucker 1993).

Laboratory fish rearing systems include static aquaria, flow-through, closed water recirculating, ponds or lakes, and a net or cage placed in a body of water (Schreck and Moyle 1990; Moe 1992). The first two methods are commonly used in fish research laboratories. Although flow-through systems have many advantages and are recommended, they are very expensive to set up and maintain. In the closed water system, which is more practical, water management is crucial.

Construction

Careful planning is crucial in choosing materials (concrete, plastic, or fiber) for construction of tanks, tank covers (to prevent fish loss and contamination), and plumbing. The tanks must be reasonably inert to the water. Care should be taken to ensure that concrete structures are properly treated so that there is minimal leaching and salt deposition (Hawkins and Lloyd 1981). Construction materials should not contain copper, nickel, cadmium, or brass. Polyvinyl chloride pipes are commonly used, but once installed the system must be adequately flushed to eliminate acetone, methylethylketones, and tetrahydrofurans that are released following gluing (Reimschuessel and Kane 1993). This may take weeks and requires water chemical analysis to confirm that the toxins have been removed.

Water Quality

Biological filtration, mechanical filtration, chemical filtration (adsorption), and disinfection are four major processes used to maintain closed water systems (Spotte 1979). Biological filtration involves heterotrophic and autotrophic bacteria that convert nitrogenous organic compounds (fish excreta, consisting primarily of ammonia) into nitrites and further to the less toxic nitrates (Stoskopf 1993b). Investigators should monitor the nitrification process, which is affected by many factors, including temperature, pH, dissolved oxygen, salinity, surface area of the filtrant, and bactericidal and parasiticidal agents.

Mechanical filters capture and eliminate undissolved particulate matter and organic particles that would otherwise contribute to the nitrogenous waste. These filters must be properly maintained to ensure that the water flows correctly and that detritus is eliminated. Chemical filtration, including granular activated carbon, foam fractionation, and ion exchangers are used to reduce organic carbon, ammonia, nitrate, and phosphate. Disinfection methods and ozonization and UV light treatment have been used to oxidize organic matter and to kill bacteria in both flow-through and recirculation systems (Schreck and Moyle 1990).

Temperature

The health, nutrient requirements, performance, reproduction and, in extreme cases, survival of fish are all dependent on the temperature of the water. Temperature requirements vary among species as well as between estuarine and freshwater fish (Tomasso 1993). Very gradual equilibration of water temperature is crucial when transferring, shipping, breeding, and acclimating fish, as well as when adjusting water

temperature. (Extreme caution must be taken with all electrical connections. Tanks should be well insulated and grounded and appropriate ground fault circuit breakers should be used.) An optimal temperature variation is about 1 °C/hour (Tomasso 1993), which is not considered stressful. Temperature variation also affects saturation of gases including oxygen (for example, there is less dissolved oxygen at higher temperatures). Gas content is inversely proportional to the water temperature. Sudden, large increases in temperature pose a hazard to the fish, particularly in a closed container aquarium.

Illumination

Both photoperiod and light intensity are important and requirements vary among species.

Periodicity. Although most species do well with a cycle of 12 hours of light and 12 hours of darkness, 8-10 hours of light is generally adequate for most fish, while 12-14 hours is appropriate for tropical fish (Moe 1992). This dusk to dawn system can be set up with a timer that turns a low light on and off for 30 minutes preceding and following the main tank lights.

Quality of light. Fluorescent lighting is commonly used in aquaria. Generally, an intensity of 10-12,000 lx of full spectrum lighting over the water surface can be used. A color temperature of 5000-7000 K and peak wave length ranging of 475 and 650 mm at proper intensity will provide good quality lighting. The readers are directed to an excellent extensive discussion on the quality of light by Moe (1992).

Diet

Fish are one of the most efficient animals in converting food nutrients into body tissues. This efficiency is due to a number of factors--they are poikilotherms, they excrete waste efficiently, and they require little energy for support and transportation (Halver 1989). In addition to the basal metabolic rate, temperature, stress, and health status are important determinants of energy requirements. Diets should be compounded with the above factors in mind while making sure that the energy requirements include all essential nutrients.

Essential amino acids (proteins make up 60-70% of fish tissue on a dry weight basis), vitamins, and minerals must be in proper ratios to ensure a well-balanced diet. Diet preparation must be appropriately performed to achieve specific research needs, to ensure sufficient stability in water, and to minimize pollution (Hardy 1989).

pH

Levels of pH between 6.5 and 9.0 are desirable, pH has multiple effects on the levels of dissolved gases and dissolvability of metals in the water as well as on oxygen uptake by the fish. It also affects organic acids and phosphates and the ratio of non-ionized to ionized ammonia. Fish vary in their tolerance to pH at different stages of their lives. Levels of 6.5 and higher are required for normal breeding and reproduction (Fromm 1980). There is also species variation in the pH requirement. Levels of ammonia, CO_2, and organic acids are all important for proper pH maintenance (Moe 1992).

Salinity, Alkalinity, and Hardness

The total amount of solid materials dissolved in the water is important. Fish need specific elements to carry out vital biochemical processes and they depend on their medium of existence for the same.

Salinity. The amount of dissolved salts in the water affects the density of water and temperature requirements of some species (Tucker 1993). When transferring fish, any changes in salinity should be gradual.

Alkalinity. The alkalinity of water is a measure of acid neutralizing capacity. Bicarbonates, carbonates, borates, phosphates, and other anions contribute to alkalinity, which is expressed by millequivalents per liter (mEq/L). Adequate alkalinity (0.2-10 mEq/L; sea water 2.5 mEq/L) ensures buffering of acid metals and proper functioning of bio filters (Moe 1992; Tucker 1993).

Hardness. Hardness is the measure of the mineral content (primarily calcium, magnesium, and other divalent cations). Water can be very hard to very soft depending on the levels of dissolved minerals (Moe 1992). Appropriate hardness may decrease stress, toxicity of dissolved metals, and ammonia (Tucker 1993).

Different species vary in their requirement of hardness, pH, and salinity.

Dissolved Oxygen (DO)

To maintain healthy fish, DO should be near saturation at any temperature and salinity. Oxygen is diffused into water by various means of aeration: agitation, liquid oxygen, air diffusers (using air compressors and blowers), U-tubes, air stones, and air lifts. A decrease in DO represents a sign of stress in fish. The amount of oxygen a fish requires depends on its life stage, species, size, as well as on the temperature of the water (Tomasso 1993; Tucker 1993). A flow rate of 0.7×10^{-3} sec^{d} assures saturation. The U.S. Environmental Protection Agency (EPA 1976) has set 5 mg/L as the minimum DO concentration for optimal fish health.

Nitrogen

Nitrogen is present in water as gas, nitrite, nitrate, and ammonia. Ammonia is the most toxic inorganic nitrogen produced by fish and by the heterotrophic bacteria of biological filters. EPA considers 0.02 mg/L of un-ionized ammonia as safe (EPA 1991). In recirculating water systems, nitrite toxicity can occur with improper biological filters (Schreck and Moyle 1990). Nitrite is formed in nitrification and denitrification processes and causes methemoglobinemia, and ultimately, hypoxia (Williams and Eddy 1986). Excess ammonia and nitrite levels are primarily responsible for "new tank syndrome" in fish (Moe 1992).

Artificial Sea Water

Many researchers need to purchase (and reconstitute) or prepare artificial seawater to meet their marine fish needs. Care should be taken to ensure that all essential elements are present and that the solution is properly mixed and stored (Spotte 1979). Water can be pretreated to reduce bacteria and remove disinfectants and algicides (Stoskopf 1993c).

Shipping

Following procurement either from the field or a commercial source, the water quality during transport must be taken into account. In general, when transporting fish, cooling the water tends to decrease the metabolic rate of the animal and thus decrease the amount of ammonia excreted into the water. In addition, lower temperatures reduce the requirement for oxygen.

The specimens should be taken off food for 2-3 days prior to transport, so that they will void their digestive tracts and not foul their shipping water. Depending on the species and length of time in transit, this fasting period will usually fall between 1 and 5 days, with two being common. Fish excreta lowers pH and affects the health of the fish.

The appropriate number of boxes and heavy polyethylene bags, depending on the size, compatibility of the fishes, and the length of their transport should be used. Small 3 cm long schooling fish, for example, could go together in one large bag while larger or more territorial fishes may need to be bagged separately. As a rough guide, one box can hold about fifty 2 cm long fish, or five 8 cm long fish, or one 25 cm long fish. Obviously, different species have different requirements, so good judgment is important. If in doubt, one should lean toward packing conservatively. Most fish shipping containers are of a standard size, 15" x 15" x 7" high. They can be ordered from a variety of sources (see Table 1).

With all but the very largest species, the best way to ship fish is in a plastic bag filled with water and oxygen and packed into a styrofoam insulated container. Special purpose square-bottomed bags should be used (see Table 1) as fish get trapped in the seams of conventional bags available in the laboratory. The general procedure is to fill the transport bag about half full with the fish' s original water of permanent residence, inflate the rest of the bag with oxygen or compressed air, and tie off with a rubber band so that no oxygen or water can escape. The bag should be fully inflated (unless fish are being shipped by air cargo), so that it is tight, almost like a balloon. At least one more bag should be put over the first bag and also secured by a rubber band. Special precautions should be taken when spiny fishes are transported as they may puncture the bag. Newspapers between the inner and outer bags may help. Once the bags are prepared, they can then be closely packed flat in the styrofoam fish box, which can be put inside a larger plastic bag, placed in the

cardboard outer box, and then sealed. A styrofoam box can be placed on a frozen ice block while shipping coldwater species. A good conservative packing job should maintain fish for 12 to 24 hours. Such containers can be sent by air freight and should be appropriately labeled. Multipurpose transport boxes commonly used by the Aquarium of the Americas are good for 48-72 hour transports (Schaeffer and others 1992). If possible, it is advisable to limit the transport time to under 24 hours by express shipping.

Fish can also be transported locally in bags or coolers (using appropriate sizes for the species) with air or oxygen bubbled through the water. State agencies such as natural resource departments frequently have transport vehicles adapted for delivering fish. Occasionally fish may be lightly tranquilized for transport with an anesthetic such as MS-222 (Brown 1993). The key to successful transport is to keep transport time to a minimum and reduce stress from handling, crowding, changes in temperature, low oxygen, and elevated ammonia.

Acclimation

Acclimation of new fish should ideally begin before they arrive. It is important to know as much about the quality of the water from which the fish are coming--temperature, salinity, pH, hardness--so that the laboratory environment can be adjusted to match these parameters. If need be, once the fish have settled in, these parameters can be changed.

Once fish are received in the laboratory they should be housed in quarantine tanks that have the correct temperature and salinity for that species. Fish in transport bags should be acclimated by floating the bags in the tanks until the water temperature in the bag is the same as the tank water temperature. The bags can be secured to the tank using clamps so that they can be opened to aerate the water during this time. Samples of the bag water should be evaluated for pH and ammonia to determine under what conditions the fish have been transported. Handling the animals as little as possible and keeping the lighting low will help reduce stress.

Newly arrived fish may also be acclimated by slowly transferring water into the transport bag or container from the new system. Aeration should be provided at this time. The water transfer should take place between 10 and 30 minutes, depending on how far apart the water parameters are between shipping water and system water. Acclimation can be considered complete when these measurable parameters are the same (or at least similar).

After the temperature in the bags have equilibrated with the tank water (approximately 30 minutes), the fish can be gently netted and released into the tanks, without the transport water. Prior to releasing the fish, it is advisable to conduct a rapid physical examination on several individuals. Fish can be gently held and examined for any external lesions and then some mucus can be scraped from the skin. A gill biopsy can also be taken. Samples should be examined for parasites. If possible, a full necropsy examination should be conducted on several fish to ascertain the general health of the population (Reimschuessel 1993).

As a group, the fish should be handled as little as possible, using only a smooth container such as a glass beaker or a fine mesh net. Situations where fish have to be chased around in order to be caught can create stress. With a little forethought, a quick trap can usually be made with a couple of hand nets or a beaker.

Quarantine

Ideally all fish should go through a 30-day quarantine period. This is especially important when the system into which the new fish will go already holds healthy populations. Having separate tanks with separate equipment and tools is important to avoid any transfer of disease. It is important that the quarantine systems should be separated from the holding systems with no cross-contamination from splashing, back-washing filtration systems, or aerosol infection. All siphoning or tank cleaning equipment and nets should be uniquely dedicated to each tank. During the quarantine period fish should be examined for signs of disease or parasitism. Signs of an unhealthy or stressed fish include wobbly swimming, severe undulations, problems with maintaining buoyancy, pale color, and folded fins. Treatments for parasites may be required during this time, especially for wild-caught animals. The CCAC *Guide* (CCAC 1984) contains a chapter on fish that can help in developing facility protocols. Tropical marine fishes are vulnerable to ectoparasites, and are most

often treated with copper sulfate at 0.2 mg/L for a period of 21 days. Freshwater fish generally need less proactive measures than tropical marine fish, so often a 30-day observation period will suffice (Whitaker and others 1994). If no disease manifests, the fish can be moved to the main system.

Breeding

Fish breeding protocols vary significantly among species. Small species extensively bred in the laboratory include Japanese medaka, fathead minnow, and zebrafish. The methods for producing transgenic fish for research continue to be developed (Yamamota 1975; Chen and others 1995).

Guidelines for raising fish in ponds can be developed from current aquaculture procedures (Boyd and Lichtkoppler 1979; Wheaton 1979; Piper and others 1982). When developing research ponds, several factors need to be considered including species requirements, land availability, incoming water (freshwater streams, well water, brackish or saltwater sources), and drainage. Discharge, especially for research protocols, must be in accordance with federal (EPA and USDA), state, and local regulations.

Research Laboratories

Guidelines developed for the research laboratory may be similar to those developed for a centralized facility. All incoming animals should be quarantined and acclimated before use in experiments. For toxicology and carcinogenicity research, the exposure route must be well evaluated. For aqueous exposures, tests may be conducted in static, static-renewal, or flow-through systems. The choice of system depends on the toxicant, the test species, the compound being tested, and available resources (EPA 1989,1991). In general, flow-through systems are preferable because they cause fewer fluctuations in water quality. Commercial flow-through toxicant delivery systems, however, are expensive and complex. Static systems have been widely used in research, especially with early life stages of small species such as the fathead minnow.

For toxicology and carcinogenicity research, protocols must be developed to deal with the discharge water depending on the toxicant used. This is less of a problem if the toxicant is administered by another route (such as orally or by gavage or injection). For other types of research, the protocols will vary greatly depending on the species and experimental design. For example, in vitro studies using fish tissues mostly require maintenance and sacrifice of the experimental fish. On the other hand, studies measuring physiological parameters may require surgical manipulations or catheterizations. Such procedures must be developed on a case-by-case basis.

DANGEROUS AQUATIC ANIMALS AND SAFETY CONSIDERATIONS

The key to human safety when working with any animal is a combination of awareness and common sense. Even the smallest of creatures is equipped with defense mechanisms of some sort. In addition, workers may be exposed to diseases, illnesses, and infestations that may be transmitted from and to the animals. By using proper precautions, the risk of zoonotic infection can be greatly minimized.

Dangerous Aquatic Animals

Aquatic animals have evolved numerous mechanisms for self-preservation. While some inflict injury through trauma, others are capable of delivering venom or a severe electric shock. Avoiding injury of both worker and animal requires proper handling and feeding techniques, special equipment, and a basic understanding of the unique habits of each species. Animal housing should be designed for safety and ease of use. For example, an overhead hoist and specially designed stretcher reduces the chance that large sharks will fracture their extensive liver or injure personnel when restrained. Emergency procedures must be in place and practiced on a routine basis so that all personnel are comfortable with managing an injury. Finally, at least 2 people should be present when working with animals capable of inflicting a life-threatening wound.

Traumatogenic Animals

Traumatogenic animals cause injury via a bite, sting, electric shock, puncture, or other physical mechanism. Although the list is extensive, examples include sharks, rays, barracudas, moray eels, sawfish, large groupers, piranhas, and surgeon fish. In many cases, secondary bacterial infection (Table 3) presents a greater risk to the recipient than the initial trauma. Poor hygiene and husbandry practices augment the likelihood that a serious infection will occur. All wounds, no matter how small, should be cleansed thoroughly with a disinfectant. If any question exists as to the extent of an injury or risk of secondary infection a qualified physician should be consulted.

Venomous Fish

Many venomous fish exist in both the cartilaginous (elasmobranchs) and bony (teleosts) fish groups (Table 2). Most of these venomous animals use spines capable of inoculating their victim with toxin. Although many of the teleosts inject their venom, the elasmobranchs (stingrays) carry a spine at the base of the tail that has an integumentary sheath that contains the toxin. Every attempt must be made to remove this sheath as well as the spine from the wound by various means (Russell and others 1958). Venomous fish stings should be treated immediately by soaking the wound in very hot water for up to 90 minutes, which denatures the venom's proteins (Halstead and others 1990). Venom associated with the integumentary sheath of the spine of marine rays is capable of producing significant cardiovascular effects including irreversible cardiac standstill (Russell 1957). Secondary bacterial infections often present a more serious problem for people than the toxin delivered (Table 3).

Electrogenic Animals

Electrogenic fish, of which there about 250 species, possess a specialized organ that can generate very high voltages when needed for protection or to stun prey. Proper equipment such as rubber-soled shoes and rubber gloves provides some protection against shock when handling these animals. Additionally, these animals can be agitated, causing them to produce a series of shocks leading to electrical discharge, which makes them safer to handle. Examples of electrogenic fishes include electric eels (Electrophoridae), knifefish (Notop-teridae), and catfish (Malapteruridae) in freshwater and electric rays (Torpedinidae) in saltwater.

Zoonoses

Aquatic animals live immersed within an environment of potential pathogens. The presence of microorganisms alone is a danger to caretakers. In closed systems, however, the concentration of microorganisms may be amplified increasing the risk of human infection. Typically, bacteria associated with lesions are gram-negative organisms such as *Aeromonas hydrophila* in freshwater and *Vibrio spp.* in saltwater. Other organisms can be acquired from aquatic animals (Table 3).

Once exposed, infection or disease will occur depending on the virulence of the organism and the susceptibility of the host (Geraci 1991). Following several commonsense guidelines can minimize the likelihood of a serious illness. First, and most important, is the practice of proper hygiene. Hands should always be washed with an antimicrobial soap after handling animals or working in their environment. Second, any open wounds should be covered to prevent inoculation. Third, immunosuppressed individuals should avoid exposure to potential pathogens. Fourth, ill employees should not come in contact with animals or their environment. Finally, if an injury does occur while handling an animal or working in its environment, proper first aid must be applied.

Working with all animals presents some level of danger from injury or zoonotic infection. The likelihood that an injury or infection will occur is dependent upon the individual's ability, proper hygiene, and common sense. Therefore, it is essential to adopt proper husbandry practices, use equipment designed for the specific task, train inexperienced personnel, and develop emergency protocols tailored to the animals being used.

EXAMPLES OF USES OF SPECIES IN BIOMEDICAL RESEARCH

Fish have been used in biomedical research for many years (Klontz 1971; Wolke 1984; Powers 1989). With their diverse sizes and their myriad of anatomical variations, fish offer the scientist opportunities to explore novel organs and structures. These studies can have profound implications for understanding mammalian biology and physiology. For example, one of the first investigations demonstrating the role of renal tubular secretion in the excretion of xenobiotics was accomplished using the aglomerular toadfish (Marshall and Graffiin 1982). Until then it was almost heresy to suggest that substances appearing in the urine had come from anything but glomerular filtration. More recently, nephron neogenesis following toxicant-induced injury, not found in mammals, has been demonstrated in goldfish kidneys (Reimschuessel and others 1990).

Other specialized features in fish of interest to biomedical researchers include antifreeze-like molecules in the blood of arctic species (Eastman and DeVries 1986), electrical activity in muscles of the electric eel (Meszler and others 1974; Fendler and others 1993), survival of dehydration in the African lung fish (Sawyer and others 1982), and copper accumulation in white perch (Frazier 1984). Fish are also extensively studied as models for research on aging (Anonymous 1991; Patnaik and others 1994), vision (Djamgoz and Wagner 1992), locomotion in cells (Lee and others 1993), and leukemia (Mulcahy 1992). Species are also evaluated for pharmacologically active compounds such as Indian catfish venom (Auddy and others 1994), and angiogenic inhibitors and antineoplastic agents in shark tissues (Snodgrass and others 1976; Pettit and Ode 1977; Oikawa and others 1990). Fish are also studied as indicators of environmental pollution (McKone and Daniels 1991) using parameters such as neoplasia (Black and others 1982; Baumann and others 1987), and immunological function (Anderson 1990; Blazer and others 1994; Muhvich and others 1995).

Small species of fish have been used in many studies because their size allows large numbers to be kept in a limited space and their short life cycles provide the opportunity to examine multiple generations (DeVita 1984; May and others 1987a,b). Fish have also been used to investigate carci-nogenicity and toxicity of various compounds (Iwan and Cella 1981; Ishikawa and others 1984; EPA 1989, 1991). Japanese medaka and zebrafish transgenic specimens are being used to evaluate the roles of multiple genes in development (Streisinger and others 1981; Brenner and others 1993; Chen and others 1995)

The Armed Forces Institute of Pathology (AFIP) in A *Handbook: Animal Models of Human Disease* (AFIP 1989) lists the following species as models: multiple schwannomas of bicolor damselfish, type I diabetes in carp, DNA damage in the Amazon molly, Wilson's disease in the white perch, hepatocellular carcinoma in rainbow trout (Ayres 1971), and malignant melanoma in platy/swordtail hybrids.

Methods for Fish Biology (Schreck and Moyle 1990) presents an excellent overview of the concept and design of research methods employing fish including field experiments, fish genetics, systematics, and taxonomic methods using morphology and electrophoresis, chromosome analysis, histology, anesthesia, surgery, and hematology. It also includes specific areas of study on respirometry, growth, bioenergetics, the nervous system, stress and acclimation, aquatic toxicology, reproduction, behavior, antecology (the study of single-species ecology), community ecology, as well as a section on maintaining fish for research and teaching.

ANESTHESIA, ANALGESIA, AND EUTHANASIA

Anesthetics, when used in a judicious and appropriate manner, may provide great benefit in the relief of pain, which helps both in maintaining and handling fish. With increasing anesthetic concentrations, sedation, immobility, loss of equilibrium, and loss of consciousness can be achieved in a controlled fashion allowing for a variety of procedures to be safely carried out, both for the animal as well as the handler. Anesthetics are used in teleosts and elasmobranchs to perform surgical and diagnostic procedures, and to facilitate capture, handling, and transport.

A good anesthetic should provide predictable results including effective analgesia, good immobilization, and rapid induction and recovery, while allowing for a wide margin of safety (Brown 1993). Although pain and suffering in fish are poorly understood, it is clear that the proper use of an agent can minimize the stress experienced by a fish and therefore prevent the cascade of physiological and biochemical changes that result from a fright and flight situation (Davis 1992; Iwana 1992). Avoiding such an internal upheaval within a fish is key in preventing the disruption of osmoregulation, loss of immune function, and decreased reproduction.

While topical and local anesthetics have been used on occasion in fish, general anesthetics are more commonly applied. The majority of chemicals used as general anesthetics mix well with water and allow for minimal restraint once the fish have been placed into a designated induction pool. The water quality of this chamber should match closely that in which the fish have been kept. Of particular interest are salinity, hardiness, pH, dissolved oxygen, and temperature. The easiest way to achieve this is to remove water directly from the fish's environment and then add anesthetic. Under other circumstances, it may be desirable to add anesthetic directly to the fish environment. This is done by achieving the appropriate anesthetic concentrations in the given amount of water. This is particularly advantageous when large numbers of animals are to be sedated for movement from one system to another.

The stages of anesthesia (Table 4) are similar to those observed in other animals. The dosage used depends on the species, as well as the metabolic ability and overall health of the individual fish. It may be helpful to use conservative doses on I or 2 representative animals, adding anesthetic as needed to move quickly through the excitement phase where injury is most likely to occur.

Anesthesia is maintained by continued exposure to varying concentrations of the agent. Intermittent administration of the anesthetic solution over the gills may be done by simply using 60 cm^3 syringes. Alternatively a drip system can be created using 2 bags connected by IV tubing and a 3-way stopcock for mixing. More advanced anesthetic machines can be easily constructed that allow for a continuous flow of well-oxygenated and titrated anesthetic to the animals.

Monitoring the depth of anesthesia becomes increasingly difficult as the fish loses its equilibrium, stops swimming, fails to respond to deep pressure, and subsequently ceases any opercular activity. Slow and steady opercular movements without response to physical stimuli are desirable. On larger animals, an electrocardiogram (ECG) may provide valuable information about heart rate as well as atrial and ventricular activity. With hypoxia for instance, the T-wave amplitude will increase as an irregular rhythm is observed (Harms and Bakal 1994).

For recovery, the animals can be placed in a well-oxygenated, anesthetic-free environment. Jaw tone will return before opercular activity (Harms and Bakal 1994). Propelling the fish head first through the water will force water through the mouth and over the gills effectively removing the drug while oxygenating the fish.

The list of potential fish anesthetics is long. When deciding upon a particular chemical one must take into account its intended use, availability, cost, legal issues, and personal preference. To date only Finquel (tricaine methanesulfonate) has been approved for use in food fish by the Food and Drug Administration. Even so, its label prohibits the immediate release or consumption of fish within 21 days of treatment. Carbon dioxide and bicarbonate have also been used as anesthetics in food fish and although they are not labeled for this use, they are food additives that are generally recognized as safe (Summerfelt and Smith 1990).

Tricaine Methanesulfonate (Finquel, MS-222)

This fine white crystal is highly soluble in water and is related to Novocain, procaine, and benzocaine. It is absorbed rapidly via gill diffusion or by coupling to specific enzyme systems (Summerfelt and Smith 1990). In teleosts, MS-222 is biotransformed in the liver and probably the kidney (Harms and Bakal 1994). The metabolic transformation of tricaine in elasmobranchs, such as the spiny dogfish, however, appears minimal (Dunn 1990). A stock solution of 10 g/L of carbon filtered fresh or distilled water may be prepared. This solution may be stored at room temperature in opaque plastic or brown glass containers. With exposure to sunlight the solution will turn brown.

The anesthetic dosage of MS-222 in fish ranges from 50-200 mg/L. Like many of the anesthetics, there exists a wide range of species sensitivity. Aeration should be provided in the anesthetic solution as hypoxia is a potential side affect. Because of its acidic nature, a solution of 100 mg/L has a pH of 5.0 in very soft water. Buffering the stock solution is not feasible due to formation of a white precipitate. Adjustments must therefore be made after making the appropriate dilution. The pH of seawater does not appear to be significantly affected by the addition of stock solution.

Quinaldine Sulfate (2-Methylquinoline Sulfate) and Quinaldine (2-Methylquinoline)

Quinaldine sulfate is a highly soluble light yellow powder that has a wider margin of safety than tricaine. It is not metabolized by fish and is excreted unchanged (Harms and Bakal 1994). Unlike its cousin Quinaldine, which is a slightly water-soluble, oily liquid, the salt Quinaldine sulfate has no odor. Both are irritating to mucous membranes. Like tricaine, Quinaldine depresses sensory centers of the central nervous system. Because it is lipid soluble, Quinaldine accumulates preferentially in the brain (Summerfelt and Smith 1990). Quinaldine and stock solutions of Quinaldine sulfate must be stored in tightly sealed containers that protect them from light.

Fish anesthetized with either Quinaldine sulfate or Quinaldine maintain a response to pressure that dissipates after approximately 20 seconds of contact. In addition, their analgesic qualities are questionable making them a poor choice for most surgical procedures. Quinaldine doses range from 5-12 mg/L in salmonids, and 2.5-30 mg/L or higher for warm water fish. Tilapia are insensitive to the effects of Quinaldine requiring concentrations of 50-1000 mg/L to reach an appropriate plane of anesthesia (Summerfelt and Smith 1990).

Although Quinaldine sulfate acidifies the water like tricaine, induction and recovery times are faster than experienced with Quinaldine. Effective concentrations are 25 mg/ L for salmonids and 15-60 mg/L for warm water species. While carp are sensitive to this drug large-mouth bass are less so (Stoskopf 1993d).

Benzocaine (Ethyl Aminobenzoate) and Benzocaine Hydrochloride

Benzocaine is a highly insoluble powder that must be dissolved in either ethanol or acetone. A stock solution of 100 g/L is generally prepared. A water soluble form of the drug, benzocaine hydrochloride, is available. Like tricaine, buffering benzocaine hydrochloride working solutions may be necessary. Both solutions must be stored in light protected, airtight containers. Because benzocaine is hydrolyzed to para-aminobenzoic acid, this anesthetic should be avoided in animals receiving sulfonamides (Brown 1993). Concentrations typically range from 25-200 mg/L.

Metomidate (Marinil)

Metomidate is an imidazole-based nonbarbituate hypnotic agent that has been used in fish. The drug was initially thought to eliminate the stress response as elevated cortisol peaks were absent in anesthetized patients. This appears not to be the case as it is thought that metomidate suppresses cortisol synthesis through the suppression of 11-B-hydroxy-lation of cholesterol (Stoskopf 1993d).

Doses for transport range from 0.06-0.20 mg/L. Anesthesia is achieved in most fish using 2.5-5.0 mg/L. Induction is rapid although recovery may be prolonged in animals exposed to the initial dose for long periods of time.

2-Phenoxyethanol

2-Phenoxyethanol is an oily liquid with slight solubility that is added directly to the water. Anesthetic concentrations range from 0.08--0.5 ml/L. Unfortunately, this chemical has a narrow margin of safety and sublethal exposure may cause liver and kidney damage (Summerfelt and Smith 1990). For these reasons, 2-phenoxyethanol is not a desirable anesthetic for use in fish.

Ketamine Hydrochloride (HCL) and Ketamine HCL/Xylazine

Ketamine provides excellent analgesia and anesthesia in teleosts when injected intramuscularly at a dose of 60-80 mg/ kg (Williams and others 1988). Because of the large volumes associated with this dose, ketamine may be lyophilized for reconstitution in smaller volumes. Lower doses of 12-20 mg/kg are effective in carcharhinid sharks. Although species specific, induction generally takes 10-20 minutes and provides 10-20 minutes of surgical anesthesia. A modified spear gun may be made to carry darts swiftly through the water allowing the acquisition of select individuals from a large body of water. Bruising or scale loss may occur at the injection site. If available, tricaine, benzocaine, or Quinaldine sulfate are easier, quicker, and safer to use than ketamine.

Ketamine HCL (12 mg/kg) when mixed with xylazine (6 rog/kg) provides an excellent anesthetic cocktail for sharks. Muscle spasms that occur with ketamine alone are no longer observed with this combination (Stoskopf 1993e). Doxapram HCL and yohimbine given intravenously are effective reversal agents.

Carbon Dioxide

Carbon dioxide has been used as a fish anesthetic for many years. It is readily available in the form of Alka-Seltzer™, which when added to water at a rate of 3 tablets per 400 ml produces an equilibrium of carbon dioxide, bicarbonate, and carbonic acid. Despite its ease of use and lack of toxic residues this method of anesthesia is stressful as it alters blood gases and acid-base balance within the animal (Iwama and others 1988). Alternative anesthetics should be used.

Electroanesthesia

There is limited research in this area. Electroanesthesia provides a very rapid tool to immobilize fish for minor procedures. It is most useful where large numbers of animals need to be collected quickly or the use of chemical agents may interfere with the research being done. Although the technique is considered safe, occasional trauma or cardiac fibrillation may be seen.

Euthanasia

Several methods of euthanasia have been used in fish including hypothermia, electrocution, overdosing with tricaine or carbon dioxide, and a sharp blow to the head. Of these, tricaine administered at 500 mg/L is most desirable as it does not alter blood cortisol, catecholamine, or glucose levels commonly associated with stress (Harrell 1992). Where chemicals may alter experimental data, cranial concussion followed by some other physical method may be employed (Andrews 1993).

For fish, the *Report of the A VMA Panel on Euthanasia* (AVMA 1993) lists as acceptable tricaine methanesulfonate, benzocaine, and barbiturates, and as conditionally acceptable, stunning by blow and decapitation, and decapitation.

IACUC Protocol Review

When reviewing protocols involving fish, it is of utmost importance that the IACUC have the benefit of direct knowledge and experience in the use of fish in the laboratory or in the field and with the particular species (or related species) concerned. The committee should feel free to use outside reviewers for proposed projects with which they have limited experience. Another critical part of the IACUC review is to be certain that all laws and regulations are followed. The acquisition offish may require licenses and permits, and failure to obtain these could adversely impact the study and the institution.

Capture techniques, such as the use of seines and traps, gill netting, ichthyocides, electrofishing, and hooks or spears must be carefully reviewed as to their necessity and the possibility of capture distress (ASIH 1987). The fish habitat should be as undisturbed as possible to support the concept of habitat conservation. Restraint and handling in the particular proposal should look to limit the amount of time of handling, especially with large or venomous fish. A team approach when working with these fish should be encouraged or required depending on the particular circumstances. In any case, the investigator (or investigators) should be experienced in handling these species or have a plan to receive training prior to the initiation of the protocol. In general, a protocol should not be approved by the IACUC unless the investigator has appropriate training in the particular handling, care, and use of the species proposed. Prolonged restraint should be avoided (see above, under Anesthesia, Analgesia, and Euthanasia).

Marking fish for field study is important to analyze their movements and population biology (Schreck and Moyle 1990; ASIH 1987). One must consider the effect of the marking on fish behavior and health. There are a variety of identification methods, including fin-clipping, electrocauterization or freeze branding (under a local or a general anesthetic), tattooing, acrylic paint injections, tagging, radiotelemetry, and radioisotope injections. Tagging is used most frequently and has been widely investigated.

Release of feral fish back into the wild should not be approved unless it is in accord with all federal, state, and

local requirements. The fish must be in excellent health, able to function in the new environment, released only within its native range of distribution, and unlikely to spread any pathogenic agents. In the event that the fish must be euthanized, it is essential to use acceptable criteria to ensure death prior to disposal (ASIH 1987).

ACKNOWLEDGEMENT

The authors wish to thank Mrs. Debbie Day and Ms. Michelle Beach for their help with information searches and in the organization of the article.

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TABLE I Fish suppliers and other resources

Saltwater fish vendors & collectors	***Fish shipping bags***
Pacific/Indian Ocean Tropicals	SeaI-Tite Plastic Packaging Co. Miami, FL Tel: 305/264-9015
Avista International, Inc. Falls Church, VA Tel: 703/573-4136	***Saltwater sources***
Cortez Handcaught Marines, Inc Inglewood, CA Tel: 310/215-0303	Forty Fathoms. Marine Enterprises, Inc. 8755 Mylander Lane Towson MD 21204 Tel: 301/321-1189
Quality Marine Los Angeles, CA Tel: 310/645-1107	H.W. Marine Mix Hawaiian Marine Import, Inc. PO Box 218687 Houston, TX 77218 Tel: 713/492-1189
Atlantic Tropicals	
Captains' Marine Key West, FL Tel: 305/294-7683	Instant Ocean. Aquarium Systems 8141 Tyler Blvd. Mentor, OH 44060 Tel: 216/255-1997
Dynasty Marine Marathon, FL Tel: 305/743-7666	Jungle Ocean 50. Jungle Laboratories Corp. Box 630 Cibolo TX 78208 Tel: 800/327-2200 or 512/658-3503
Gulf Specimen Marine Laboratories, Inc. Panacea, FL 904/984-5297	Kahl Sea Salt Kahl Scientific Instrument Corporation PO Box 1166 El Cajon, CA 92202-1166 Tel: 619/444-2158 or 5944
Cold Marine	
Marine Biological Laboratory Woods Hole, MA Tel: 508/548-3705, ext. 375	Marine Environment. Import Associates, Inc. P.O. Box 16350 San Francisco, CA 94116 Tel: 415/591-2200
Pacific Bio-Marine Labs, Inc. Venice, CA Tel: 310/677-1056	Rila Marine Mix. Rila Products P.O. Box 114 Teaneck NJ 07666 Tel: 201/836-0855
Sea Life Supply Sand City, CA Tel: 408/394-0828	
Laboratory supply (fish)	***National aquaculture suppliers***
Carolina Biological Supply Co. Burlington, NC Tel: 919/584-0381	are listed in: Aquaculture Magazine Buyer's Guide PO Box 2329 Asheville, NC 28802 Tel: 704/254-7334
Ward's Biology Rochester, NY Tel: 800/962-2660	
Fish shipping boxes	***Federal Wildlife Permit Office***

J.V. Industries Hialeah, FL Tel: 305/885-4666 Lifelike Plastics Miami, FL Tel: 305/835-0646	U.S. Fish & Wildlife Service 4401 N. Fairfax Dr. Arlington, VA 22203 Tel: 800/358-2104

TABLE 2 Venomous/spiny fish

Elasmobranchs stingrays or whip rays devil rays or mantas eagle rays or bat rays cownose rays round stingrays *Freshwater rays* S. American river rays Spiny Dogfish Ratfish or Elephantfish	*Dasyatidae* *Mobulidae* *Myliobatidae* *Rhinopteridae* *Urolophidae* *Potamotrygonidae spp.* *Squalidae* *Chimaeridae*	**Teleosts** Catfish Ariidae, Clariidae, Plotosidae Weeverfish Trachinidae Scorpionfish Scorpaenidae includes 60 genera and over 300 species Scorpionfish Lionfish Stonefish Sculpin Rockfish Toadfish Surgeonfish[1] Dragonets Rabbitfish Stargazers Leatherbacks[2]	Ariidae, Clariidae, Plotosidae Trachinidae Scorpaenidae Batrachoididae Acanthuridae Callionymidae Siganidae Uranoscopidae Carangidae

[1]Some surgeonfish have venomous spines at the base of the tail, others do not, but all can inflict a wound.
[2]*Scomberoides sanctipetri* is the only carangid (which includes jacks, scads, and pompanos) known to have venomous spines (Halstead and others 1990).

TABLE 3 Aquatic microorganisms associated with zoonotic infections

Aeromonas hydrophila
Atypical Mycobacteria
M. fortuitum
M. marinum
M. chelonia
Campylobacter spp.
Edwardsiella tarda
Enteropathogenic *E. coli*
Enterotoxic *E. coli*
Erysipelothrix spp.
Legionella pneumophila
Pseudomonas spp. Salmonella spp.
Vibrio cholera
Vibrio parahemolyticus
Vibrio vulnificus
Vibrio fluvialis
Yersinia enterocolitica

TABLE 4 Stages of anesthesia

Stage I: Induction Erratic swimming, excitement phase, some loss of equilibrium, disorientation, increased respiration, some loss of tactile response, reduced activity
Stage II: Sedation Loss of equilibrium, slow swimming without direction, decreased respiration
Stage III: Anesthesia 1. Complete loss of equilibrium, swimming and respiratory activity slowed, still responsive to stimuli 2. Surgical plane, unable to swim, respiration becomes shallow, no response to stimuli 3. Cessation of opercular movements
Stage IV: Pre-Mortem Spasmodic over-distention of opercules, cardiac failure