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Field Sampling and Necropsy Examination of Fish

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ABSTRACT

This paper presents an overview of observational and fish sampling techniques for investigating fish lesions, morbidity and mortality. These sampling techniques and investigations are much like detective work and require attention to detail, common sense, technical proficiency and experience. To solve the mystery of a fish kill, the investigator must use available evidence and clues to piece together a series of events that often have long since passed. The cause of these field events may be chemical, biological or physical; more often, it is some combination of these. An initial categorization approach may be used to reduce the great number of possible causes of a fish kill to something more reasonable. Through proper observations, the most probable cause may be placed in one of four broad categories (although additional secondary relationships should also be recognized). These broad categories include oxygen related, toxics or water quality related, disease or population related and trauma related events, and may be based on defined criteria. Caution should be taken on making etiologic generalizations since many types of lesions or mortality events may appear similar. This paper provides support for making consistent observations; taking photographs, tissue and water samples; classifying external lesions and choosing appropriate necropsy methods. A bibliography is provided to reference information pertinent to fish kill investigations and fish disease, anatomy and taxonomy.

INTRODUCTION

In order to determine the cause of fish lesions, morbidity and mortality it is essential to make detailed field observations and examinations and specimen collections. The field investigator, whose job it is to determine the cause of such environmental events, frequently lacks needed historical information about the event in question. Although the science of fish disease and mortality investigation is not new, the diagnostic methodologies are constantly being refined and updated, particularly since they are applied under many different field circumstances. Fish lesions or mortalities may be

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caused by a variety of different stressors including disease, trauma, nutrient or organic enrichment, and toxics loading (Figure 1). Therefore, a consistent sampling approach is necessary in order to provide appropriate observations and samples for a trained diagnostician or fish pathologist.

One does not need to be a fish pathologist to make utilitarian observations or take fish tissue samples for diagnostic evaluation. Beyond the obvious loss of the organisms, large-scale fish kills may be indications of serious ecological disturbances. Kills are disturbing to the public at large and to resource managers because of the apparent sign that conditions within the affected water body have deteriorated to such a degree that fishery resources are no longer supported. Using a consistent approach, and with a little practice, field technicians, fisheries biologists and environmental managers can make relevant observations, take quality tissue samples, and make substantial contributions to field fish health studies. Making good observations, and making those observations in a way that cannot be *mis*understood by others, is an important aspect of data collection. Further, observations need to be systematic (follow a prepared, itemized "game plan") and consistent (treat all observations and samples in your study in accordance with accepted, standard methods) in your sampling strategy. The goal of this paper is to provide field investigators with an overview of observational and fish sampling techniques for investigating fish lesions, morbidity and mortality.

THE INVESTIGATION

Fish kill determinations are a primary responsibility of resource and environmental management agencies, and universities throughout the United States. Diagnosing the cause of fish mortalities can be one of the most technically challenging and complex duties of these groups. The investigation itself is much like detective work. It requires complete attention to detail, common sense, and a high degree of technical proficiency. Knowledge of fish behavior, scientific classification, and fish response to various pollutants is also a necessity.

To solve the mystery of a fish kill, the investigator must use the available evidence and clues to piece together a series of events that often have long since passed. Proficiency in such a specialized area is best obtained through experience. Toxic chemicals are by no means the only cause of fish kills. Any waste discharge or sequence of events that interferes with or alters the quality of an aquatic system can trigger acute or chronic mortalities. The cause can be chemical, biological or physical; more often, it is some combination of these.

THE NOTIFICATION

Diagnosing a fish kill begins at the time notification is received. Often times, the initial report comes by phone from a concerned citizen who has discovered dead or dying fish. All of the information provided should be recorded. This includes such details as the location of the mortalities, the number of fish involved, when they were first noticed, and any unusual events or environmental occurrences witnessed immediately before the fish were discovered. The name and phone number of the caller is important and should be recorded so that question that arise later can be asked and answered.

EVENT CATEGORIZATION

Once at the scene the investigator must reduce the great number of possible causes of the fish kill to something more reasonable. One manner in which this can be done is through an initial categorization approach. The approach and specific types of observation will likely vary between regions and waterway types. There may also be overlap in causative factors. For example, hypoxic conditions may be due to the presence of a toxic (or non-toxic) algae bloom, and/or an increased biological oxygen demand (BOD) due to a high biomass of decaying organic matter. However, through proper observations, the most probable cause may be placed in one of four broad categories. Additional secondary relationships should also be recognized. These broad categories include:

1. Oxygen related (anoxia, hypoxia, winter kill, algal blooms, increased BOD);
2. Toxics or water quality related (industrial or domestic pollution, pesticides, fertilizers, oils or fuels, suboptimal water quality including rapid thermal shift, changes in salinity, toxic algae, presence of irritating or noxious suspended materials);
3. Disease or population related (viral, bacterial, fungal, parasitic, spawning stress, over population);
4. Trauma related (sport or commercial fisherman discards, predation, explosive shock, turbine effects).

Unfortunately, conclusions cannot often be reached through any single observation. Conclusions are based on several supporting observations, each of which serves to verify another. The first step is simply to look at the species of fish involved. This information can provide valuable clues. Obviously the condition of those fish and the time frame in which they are observed also plays an important role in evaluating the cause(s) of a fish kill.

Oxygen related.

Periods of natural dissolved oxygen (D.O.) depletion can be caused by plant respiration at night, or it can occur as a result of oxygen demand associated with the decomposition of naturally occurring organic material. Additionally, hypoxic or anoxic waters can be brought to, or near, the surface as a result of bottom water inversion during spring or fall turnover, or during wind-induced seiche events. Non-toxic algal blooms can also severely affect fish populations due to the presence of high algae cell counts that may lease to clogging or irritation of fish gills. Further, algae blooms can be harmful to aquatic species since they can cause severe oxygen depletion due to respiration or increased biological oxygen demand.

With regard to species involvement, physostomic fishes such as gar and bowfin have physical characteristics that enable them to use atmospheric oxygen. If oxygen concentrations within the water drop below the level needed to support most other species, physostomic fishes can survive by gulping air at the water surface. Other groups of fishes with superior-oriented mouths, such as topminnows and killifishes, respire at the water film just below the surface where there is usually enough oxygen to sustain them. It follows, then, that if there are no physostomic fishes or species with superior-oriented mouths among the carcasses, an oxygen-related problem begins to look like a possibility even before any water quality measurements are made (if the

habitat under investigation supports those species). In freshwater, oxygen-related fish mortalities often include largemouth bass, various sunfishes and shad, and some of the larger Cyprinids and Ictalurids typically found in these systems. In saltwater systems, mullet, red drum, sea trout, menhaden and almost any other species expected to be in the area are commonly found among the dead fish when oxygen levels drop too low. The aforementioned species observations should be made within the first few minutes after arriving at the scene of a fish kill. These observations should be followed with measurements of key water quality parameters.

Toxics or water quality related.

The size of the affected fish is also an important observation. Typically, a fish kill caused by depressed oxygen levels will not include smaller fishes unless critically low concentrations are reached. Toxics-related fish kills are typically characterized by the presence of all size classes, from juveniles to adults. It is helpful to rule out hypoxia as a differential in the case of potential toxic events. In pesticide-related kills, for example, one- and two-inch sunfish are often observed in freshwater habitats; however, they may not always appear during low-oxygen situations. The reason for this phenomenon is that younger, smaller-bodied fish require relatively less oxygen than larger fish. Consequently, the smaller fish can usually find enough oxygen in moderately depleted conditions to survive a temporary ecological upset. Larger fish, however, require much more oxygen from the water to sustain their larger body masses.

Certain physiological observations may be made from fish affected by certain contaminants. For example, moribund fish that have been poisoned by certain organophosphate pesticides will exhibit tetany (asynchronous muscle twitching under the skin); dead fish may be observed with their pectoral fins extended anteriorly. If toxic exposure is suspected, carcasses of fish should be collected, transported on ice and frozen for later chemical analysis. Care should be taken when transporting any biological material on ice such that the specimens are never in direct contact with ice or ice water; they should be contained within durable, labeled, air-tight bags prior to placing on ice.

Numerous species of unicellular and filamentous green and blue-green algae, diatoms and dinoflagellates, can release toxins that may lead to fish morbidity, behavioral anomalies and mortality. Shifts in water quality parameters may also be toxic to fish. In addition to decreased dissolved oxygen levels, abrupt changes in salinity, temperature or pH, or elevated forms of nitrogen, can be devastating to the homeostasis of fish. Acute loss of homeostasis may directly lead to mortalities, whereas chronic suboptimal water quality may have a more long-term effect on fish, leading to compromise of immune responsiveness and disease.

Disease and population related.

Fish kills involving diseases or parasites usually occur when a population is under some environmental stress. There may be outward signs on the carcasses that indicate the presence of external or internal parasites. Also, many disease-related mortalities often involve a single fish species or family. The spread of fish diseases is often opportunistic. That is, when conditions such as overpopulation, high water temperature, or low oxygen levels occur, fish populations can become stressed. Bacterial, viral, fungal and parasitic pathogens can then "take advantage" of this vulnerability. Fur-

ther, fish may become predisposed to disease by other environmental stress factors including over-population, inadequate food resources and spawning stress. Therefore, multiple etiologic factors may act synergistically to overwhelm the immune system of resident fishes. A brief outline for taking water samples, making appropriate external and internal fish observations, conducting a parasite survey and taking samples for microbiological analyses is provided in the sections below.

Trauma related.

Outward signs of physical trauma on fish carcasses, in conjunction with acceptable water quality and an apparently healthy fish population, characterize these types of mortalities. Trauma may also include angler-released fish as well as fish exposed to explosive or munitions testing. In some cases predator species, such as bluefish, striped bass and birds, do not always consume what they capture, thereby leaving uneaten carcasses in a feeding area.

By using the concept of initially placing the cause into one of the aforementioned broad categories, the goals of the fish kill investigation become somewhat simplified and the task of defining the cause(s) among a multitude of possibilities becomes more systematic and logical. Once the cause of a fish kill is categorized, the investigation takes a decidedly more complex direction. The investigator must use all observations, data from laboratory assays, and technical expertise to decide what is the most probable cause or causes of the kill. Secondary or follow-up investigations may be indicated.

DATA COLLECTION

What samples to take.

Depending on the body of water, the site history, insights into probable cause(s) of the event, and available time and personnel, the type of samples obtained for diagnostic pathology and chemical analyses may vary. Collect any and all evidence that may have related to the event. Collect any solution or solid material that may be suspected of contributing to the kill. Retain containers (bags, bottles or cans) that suspect solutions or solids may have been stored in. If toxics are suspected, collect fish for residue analysis. Obtain names of witnesses who may furnish additional information. Mortality counts should be estimated. American Fisheries Society publication #24 (AFS 1992), provides standard statistical techniques for enumerating affected fish.

Use of appropriate data sheets.

Different management and state agencies use different data collection formats for recording field observation variables. Regardless of the data collection methodology, good planning and appropriate use of data sheets play an important role in determining the final quantitative and qualitative outcome of your data. Remember, you can never go back and get data that was not collected! Several types of data sheets have been made available as templates through the website of the University of Maryland Aquatic Pathobiology Center (http://som1.umaryland.edu/aquaticpath)[1].

Sample identity and case history.

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1 Investigators who would like to contribute to our www-based datasheet database should contact Kane at the UM pathobiology Center, akane@umaryland.edu or 410-706-7230

In order to properly track samples and link samples to different field events, field accessions should have unique identification numbers. Use of a defined, consistent accession system to log individual animals and batch collections is paramount. Data sheets should include information on source location of fish, including GPS data, collection methods, water quality, time, date, weather conditions during collection, general environmental observations including anomalous behavior of affected fish (i.e., gulping at surface, flashing, morbidity, grouping in shallows, etc.) and the number of affected fish. Note nearby landmarks such as marinas, sources of potential contamination, and any unusual events prior to and during the course of the observations. Data sheets and samples should be labeled with pencil or waterproof ink. Also, consult historical fish kill files to see if any previous kill occurred in the area of the current investigation.

Water quality sampling.

Field water measurements should be performed at various stations in the body of water. Choosing appropriate station locations is vital to the success of the diagnosis. Stations within the affected area are an obvious necessity, but it is important to make sure that areas outside of the mortalities are also checked. This helps to ensure that the area of poor water quality is completely delineated. At each station testing should be performed at multiple depths since water quality often changes considerably from top to bottom. For example, oxygen levels are normally slightly higher near the surface as a result of active photosynthesis by the algal community as well as wind action. Temperature, dissolved oxygen, pH and salinity/conductivity are all important water quality parameters to measure at each sampling station. The measurements should be made in the field with instruments that have been properly serviced and calibrated prior to each use. Water samples should also be collected for algae identification if an algal bloom is observed or if presence of toxics is suspected. Use of appropriate sample containers, preservatives and refrigeration (i.e., ice chest) is important for sampling pesticides, metals, and volatile and semi-volatile organics; refer to specific instructions from the laboratory that will perform the analyses.

EXTERNAL EXAMINATION OF FISH

Observations of affected fish should provide data relative to the species, length and weight of sampled organisms, the condition of the fish (alive, moribund, dead), the presence of any areas of abnormal coloration, the color of the gills (healthy gills are usually a bright cherry red), the presence of excessive mucus on the skin, body shape alterations, abdominal distention, skeletal deformities or bulging eyes. The presence of lesions such as areas of reddening, frayed fins, ulcers and macroscopic or microscopic parasites should also be recorded. Further, overall body condition should be noted (use a scale of 1-5, where 1 = emaciated, 3 = average/normal, 5 = a "lunker"). Blood samples can be drawn from fish to assess hematocrit, plasma protein and white cell counts. The field investigator should make estimates of the percent of the sampled population that is affected. If fish are to be sampled for histopathology (as described below), at least 8 fish of each species should be collected for that purpose.

It is up to the investigator to be familiar with the normal anatomy, behavior and general physiology of the local fish fauna. Having a reference of normal anatomy for the indigenous species will aid in making observations relative to any potential

TABLE 1. Bibliography of references pertinent to (A) fish kill investigations; (B) fish disease, parasites, necropsy, pathology; (C) fish anatomy and physiology; and (D) fish identification.

Category:	Reference:
A	AFS (American Fisheries Society) 1992. Investigation and Valuation of Fish Kills. American Fisheries Society Special Publication 24, Bethesda, Maryland.
A	ASTM (American Society for Testing and Materials) 1996. Standard Guide for Fish and Wildlife Incident Monitoring and Reporting. ASTM E1849-96.
B	Austin, B. and D.A. Austin. Bacterial Fish Pathogens. John Wiley and Sons, NY 1987.
D	Bigelow, H.B and W.C. Schroeder. 1953. Fishes of the Gulf of Maine. Fishery Bulletin 74, Fishery Bulletin of the Fish and Wildlife Service, Volume 53, Washington, DC.
C	Bond, C.E. 1979. Biology of Fishes. Saunders College Publishing, Philadelphia, PA.
C	Evans, D.H. The Physiology of Fishes. 1993. CRC Press, Boca Raton, FL.
B	Ferguson, H.W. Systemic Pathology of Fish. Iowa State University Press. Ames, Iowa, 1989.
B,C	Gratzek, J.B. and J.R. Matthews. Aquariology. 1992. The Science of Fish Health Management. Tetra Press. Morris Plains, NJ.
B	Hoffman, G.L. and E.H. Williams. 1999. Parasites of North American Fishes. Comstock Publishing, NY.
D	Hubbs, C.L. and K.F. Lagler. 1974. Fishes of the Great Lakes Region. University of Michigan Press, Ann Arbor, MI.
B,C	Kane, A.S., *ed.*, FishGuts: A Multimedia Guide to the Art and Science of Fish Anatomy, Health and Necropsy. Mac/Win software on CD-ROM. APC Press, 1996. (http://som1.umaryland.edu/aquaticpath/fg)
C	Lagler, K.F., J.E. Bardach and R.R. Miller. Ichthyology. 1962. John Wiley and Sons, NY.
B	Lom, J. and I. Dyková. 1992. Protozoan Parasites of Fishes. Developments in Aquaculture and Fisheries Science, 26. Elsevier Press, Amsterdam.
A	Maryland Department of the Environment. 1999. Fish Kill Investigation Manual. Annapolis, MD
D	McClane, A.J. 1974. McClane's Field Guide to Freshwater Fishes of North America. Holt, Reinhart and Winston, NY.
D	McClane, A.J. 1974. McClane's Field Guide to Saltwater Fishes of North America. Holt, Reinhart and Winston, NY.
B	Noga, E. 1996. Fish Disease: Diagnosis and Treatment. Mosby Press, St. Louis, MO.
B	Reimschuessel, R., Bennett, R.O. and M.M. Lipsky. 1992. A Classification system for histological lesions. J. Aquat. Anim. Health.; **4**:135-143.
B	Reimschuessel, R. 1999. Necropsy Techniques in Aquarium Fish. In: Kirk's Current Veterinary Therapy, Philadelphia, Pa: W.B. Saunders Co., NY.
B	Ribelin, W.E. and G. Migaki. *eds.*, The Pathology of Fishes. The University of Wisconsin Press. 1975.
B	Roberts, R.J. *ed.*, Fish Pathology. 2nd. Ed. Bailliere Tindall. London, England, 1989.
D	Scott, W.B and E.J. Crossman. 1973. Freshwater Fishes of Canada. Bulletin 184, Fisheries Research Board of Canada, Ottawa.
B,C	Stoskopf, M. Fish Medicine. 1993. W.B. Saunders Company, Philadelphia, P.A.
D	Tomelleri, J.R and M.E. Eberle. 1990. Fishes of the Central United States. University Press of Kansas, Lawrence, KS.
B	Wolf, K. 1988. Fish Viruses and Fish Viral Diseases. Cornell University Press, Ithaca, NY.

← FIGURE 1. (*Left*) On-the-scene observations may include thousands of dead fish, as in seen with the menhaden example (bottom), or just few dead or dying fish as seen in the largemouth example (top). The cause of the event may involve disease, trauma, low dissolved oxygen, the presence of toxicants, or a combination of these stressors. Photos courtesy K. St Pé.

FIGURE 2. (*Above*) Gross photographs of goldfish taken from an aquaculture facility that was experiencing chronic mortalities. Lesions shown in these three specimens were typical of the twelve fish that were sampled for histopathology and microbiology. Whole fish pictures (left) indicate the location and extent of the different lesions while close-ups (right) provide detail. Case 124-99F depicts a 5 x 8 mm half moon-shaped ulcer located just posterior to the right pectoral fin. The margins of the ulcer are white and lack scales. Case 125-99F shows a 4 x 12 mm area of redness circumscribing the left pelvic fin; there is a loss of fin elements and a gray, rough-looking center. Case 126-99F shows a slightly raised, reddish-gray, irregular 5 x 7 mm firm, gelatinous mass on the maxilla, just posterior to the lips on the right side. A wet-mount microscopic preparation from a skin scrape of the lesion on each of these fish revealed "slightly waving haystacks" (observed at 40x objective magnification) as well as numerous highly motile bacilli. These motile "haystacks" are typical of *Flexibacter* bacteria, and are not necessarily the proximate or ultimate cause of the mortalities. Photos courtesy A. Kane.

deviations from normal. We have provided a bibliography (Table 1) that contains several texts and guides that review normal fish anatomy and physiology.

Making lesion descriptions.

It is not usually the responsibility of field personnel to make *diagnostic* observations (it is, however, often their responsibility to make *descriptive* observations). Good lesion observations that are descriptive and systematic, and that are not subject to misinterpretation, are invaluable for the diagnostician or fish pathologist. Record detailed observations of any lesion or area that may be abnormal. In any samples taken for histopathology, include pieces of the abnormal tissue attached to adjacent, apparently normal tissue. Include comments on lesion: **size** (give dimensions); **number**; **location** (where on body); **presentation** (focal, multifocal, diffuse); **shape** (round, oval, ribbon or tube-like, sharp edged, etc.); **consistency and texture** (smooth, granular, nodular, cheesy, boney, crumbly, tough, pitted, gelatinous, hard, firm); **color and opacity** (transparent, translucent, opaque; pale, mottled, streaked); **3-D description** (depressed, flat, raised); **severity** (minimal, mild, moderate, marked, severe); **extent** (% body coverage). An example of a lesion description might read: "multiple 0.5 to 1.0 mm white, slightly raised, round nodules; diffuse throughout the liver (moderate), spleen (moderate) and posterior kidney (severe). The spleen has rounded edges and the posterior kidney appears enlarged." Include a picture. The caption for Figure 2 provides additional examples of lesion descriptions.

Use the above comment categories and "food descriptions" to convey the lesion imagery, particularly when the anomaly which you are describing is unknown to you. Examples of material or food imagery which can be used to describe lesions include: cauliflower, cottage cheese, hamburger, cream cheese, pea soup, cream and coffee, marbles, sandpaper, etc. Imagery can also be used for size comparisons, e.g., the size of a nickel, dime, quarter, orange, etc. Draw a picture or use data sheets that contain a fish line drawing and draw in the abnormality indicating location. Complement the drawing with descriptive words. It is important to remember that many different types of fish lesions, such as ulcers, may look similar yet may be associated with a variety of different causative agents (Kane et al. 1999; Dykstra and Kane 2000).

Take photographs.

If you are going to participate in a field histopathology study that ultimately relies on field personnel to deliver samples and data for analysis, strongly consider incorporating photographic data. Note the photograph number of the lesion/specimen so that you can reference your specimens with specific photographic frames. Include in the picture a ruler or a common object such a coin to indicate scale. Photograph the whole, intact specimen (including information on accession number, scale/size and location of any lesions) in addition to close ups of the affected areas, i.e., lesions or abnormalities (see examples, Figure 2). Photograph specimens on non-white, non-reflective surfaces. Quality photographs provide the pathologist with the greatest possible descriptive information.

Anyone can learn to take quality pictures (particularly of gross lesions on dead fish!). The trick is to practice in advance. Make test rolls of film on a test subject such

as someone's thumbnail (as a "test lesion") before going out on a fish kill investigation. Bracket your exposures using a light meter. In other words, take a picture with the correct *f*-stop exposure and also take shots that are 1 *f*-stop underexposed and 1 *f*-stop overexposed. If using a disposable camera, practice with lighting and closest allowable distances. If using a flash unit try bouncing or diffusing the light to avoid bright hot spots. Tips on taking good photographs can often be gleaned from camera sales personnel. Enthusiastic photographers may be quite helpful when faced with the unusual task of talking about taking pictures of fish with lesions.

For best results with a 35 mm single lens reflex camera, purchase a macro lens which will permit taking close up (i.e., 1:1 ratio) pictures of lesions as well as whole fish pictures. A 60 mm macro lens offers good versatility for both close ups and general photography and can be purchased new or used for between $150 and $600, depending on your budget. A decent used camera body can be purchased from a reputable dealer for $150. Consider these items tools of the trade. They are well worth the investment.

For the sake of simplicity, we have included a simple lesion classification system (below) and figures to demonstrate presentation of commonly observed lesions (Figures 3 and 4). A working knowledge of normal anatomy is an essential starting point, both with respect to normal anatomical variations as well as location reference points. Remember, by definition, a lesion may be defined as *any* alteration of a cell, tissue or organ that deviates from normal.

A SIMPLE CLASSIFICATION SYSTEM FOR EXTERNAL LESIONS

1. Observations. Describe the primary (or most obvious) lesion first. Make additional observations for other lesions. Many gross external lesions may be broadly classified according to the following descriptions (Figure 3):
   a) *Loss of Scales.* There is no reddening or other changes; lesion does not obviously penetrate beneath the surface of the skin and scales. May be due to net or capture trauma.
   b) *Reddening.* Blood or red color associated with the observation; not a penetrating lesion. May be on any portion of the fish including the fins.
   c) *Ulcer.* There is an obvious penetration through the skin and scales. Underlying dermis (deep skin beneath the scales), muscle or viscera may be visible.
   d) *Other.* Describe. See "Making lesion descriptions," above.
2. Location. (e.g., head, vent, left pectoral fin, lateral line, etc.)
3. Presentation. (see examples, Figure 4)
   a) *focal:* single affected area
   b) *multifocal:* more than one affected area
   c) *diffuse:* multiple affected areas widely distributed over a large area(s) of the body
4. Severity. Rank the severity of each observed lesion on either a scale of 0-3 **or** 0-5, where 0=no lesion. These two ranking systems are presented below. Obviously the use of a ranking system to describe severity is rela-

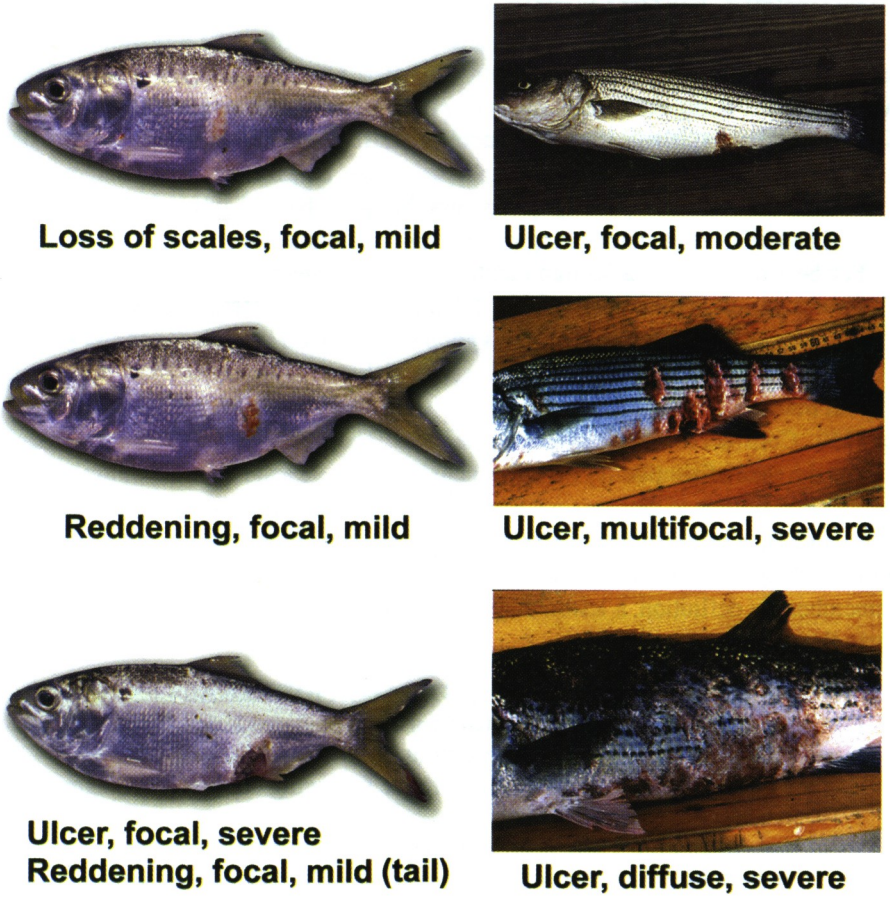


FIGURE 3. Photographs of menhaden (left) and striped bass (right) lesions including loss of scales, areas of reddening and ulcers. Although only three commonly observed types of lesions are shown here, there are many different types of lesions. It is very important to make descriptive observations rather than diagnoses (e.g., area of reddening versus area of hemorrhage), taking care not to identify what you cannot see or determine with a high degree of certainty. Note that the middle striped bass picture shows a multifocal presentation of the ulcers since the primary, largest ulcers are discrete. There are, however, some coalesced ulcers; at times the difference between multifocal and diffuse is not easily defined. Photos courtesy A. Kane and Maryland Department of Natural Resources.

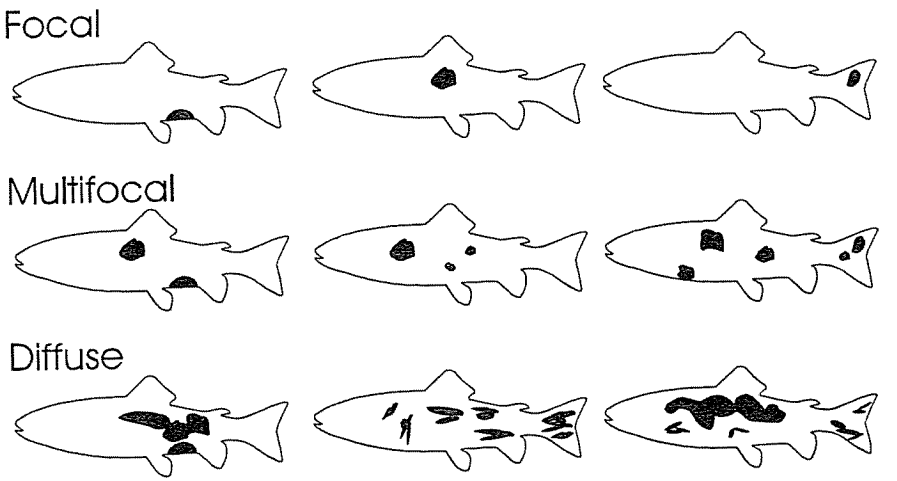


FIGURE 4. Cartoon describing the distribution of lesions (focal versus multifocal/diffuse) and degree of coalescence (multifocal versus diffuse). Figure courtesy Maryland Department of Natural Resources.

tive. However, with practice, you can make consistent lesion severity observations that will be in agreement with other observers.

0 normal
1 mild
2 moderate
3 severe

1 | 2 | 3
mild moderate severe
Rank scale 0-3

0 normal
1 minimal ("barely perceptible")
2 mild
3 moderate
4 marked
5 severe ("cannot imagine it being worse")

1 | 2 | 3 | 4 | 5
minimal mild moderate marked severe
Rank scale 0-5

Parasite observations.

Alterations in the normally-occurring parasitic fauna may be due to disease incidence, changes in organic loading, or the effects of contaminants. Microscopically examine a skin scrape, a gill biopsy and a fecal mount or gut scrape from at least 6 freshly sacrificed specimens. Note that parasites often "jump ship" from moribund and dead hosts. External mucus and skin epithelium can be sampled with a microscope coverslip by gently scraping the side of the fish from anterior to posterior. A small biopsy taken from the red filamentous portion of the gills should also be observed under the microscope. Start with low magnification (4x objective) and work your way up to higher magnification (10x and 40x objectives). If electricity is not available for a traveling microscope, a small field microscope may be handy (e.g., FM-31 field microscope by Swift Microscopes, Inc.). If you cannot taxonomically identify the parasite(s) (as accurately as possible to phylum, class, order, family, or genus), draw

a picture of your observations and make notes relating to parasite number (rank the infestation using a severity scale), location, presence of cilia or flagella (if any) and type of movement (if present).

INTERNAL EXAMINATION OF FISH

Euthanasia.

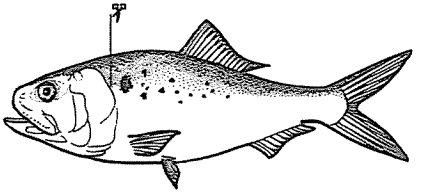
Fish should be humanely sacrificed prior to performing a necropsy or taking microbiological samples. Two methods are commonly used to euthanize fish. These include an overdose of a water soluble anesthetic, methanetricane sulfonate (MS222) and cervical transection. MS222 is a wetable powder which can be dissolved to a concentration of 100-500 mg/L, depending on the species and the hardness and salinity of the dilution water. Advantages of MS222 include ease of use, particularly in batch application. Disadvantages include the need to use protective gear (gloves, goggles) and disposal. Advantages of cervical transection is that the method is rapid and effective, and there is no chemical disposal afterwards. Disadvantages of the technique become obvious when working with larger catfish and other species with well-developed crania that are difficult to cut through. Further, cervical transection cuts may be distracting and messy when photodocumenting lesions on euthanized animals.

Microbiological sampling.

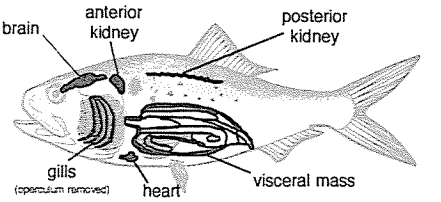
Well-defined sampling techniques are also used for the detection and identification of different bacterial pathogens of fish in populations showing signs of disease. Ideally, at least five moribund fish or those showing clinical signs typical of the disease outbreak should be sampled. Fish that are found dead at the time of the sampling are not acceptable for bacteriological examination unless they are known to have died within 30 minutes of sample collection (contaminating bacteria can grow quickly in dead fish, particularly in warm water/warm weather).

The organs most commonly sampled are liver, kidney, and spleen. A sample of the brain should also be sampled if *Streptococcus* or *Corynebacterium* are suspected (when in doubt, sample it). If fluid is present in the body cavity it should also be collected for culture.

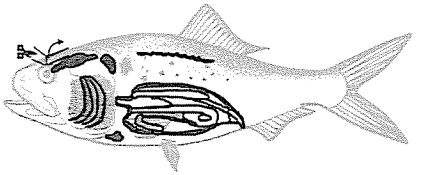
Samples for attempted culture of bacteria must be taken aseptically using sterile techniques. Disinfection of the body surface is recommended before external incisions are made to expose the organs. Disinfect the surface of the fish by wiping it with gauze soaked in 95% alcohol. Adequate disinfection has been achieved when the surface of the fish turns slightly opaque. Sterile tools must be used for making all incisions and removing samples for culture. Sterilize instruments by dipping in 95% ethyl alcohol followed by flaming before each use (i.e., *each time* it touches the fish). This may be challenging if conditions are breezy; a wind shield may be necessary. If samples can not be inoculated immediately onto appropriate culture media they can be collected with a special transport swab and sent to a diagnostic laboratory by overnight priority mail. Use a sterile scalpel blade to stab each organ to be swab-cultured. Touch the sterile culture swab to the organ tissue exposed by the scalpel stab. Replace the swab back into its holder and activate the media to wet and maintain the sample on the swab by crushing the ampoule. Label and identify culture swabs with a unique identification numbers and organ site before you start with the necropsy. This will help keep you organized and insure proper sample processing. Keep samples out of direct sunlight



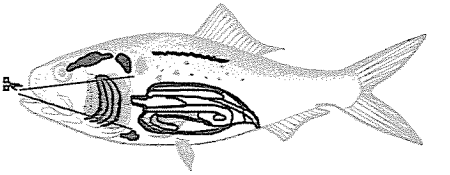
1. Euthansia by severing the spinal cord just behind operculum (Atlantic menhaden shown).



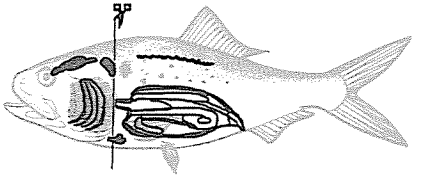
2. Cartoon depicting generalized location of internal anatomical features in menhaden.



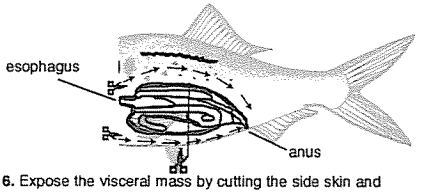
3. Expose brain by making cut between eyes across top of head, and then making two smaller cuts starting at the top of the eye sockets going posteriorly. Peel back the brain case.



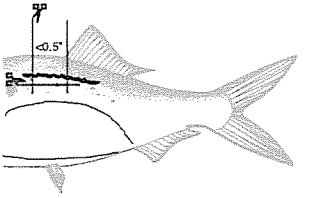
4. Expose gills and oral cavity on left side by making two cuts (dorsally & ventrally) through the operculum.



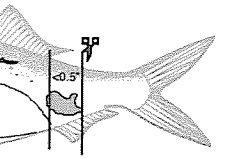
5. Cut off head just behind the operculum. This should expose the anterior kidney and heart. If heart not exposed, make small cut to expose but not cut it. Place the head in formalin.



6. Expose the visceral mass by cutting the side skin and musculature. Remove the visceral mass by cutting at the esophagus and anus. Take care not to rupture gall bladder. Make a cut in the visceral mass (solid line) to enhance fixative penetration. Place visceral mass in formalin.



7. Sample a wedge of the dorsum, just anterior to the dorsal fin (as shown). This will include skin, muscle and posterior kidney (depicted as the "dark wavy tissue." The posterior kidney in other species may be a saddle-shaped organ or it may be inter-digitated against the backbone as in menhaden (backbone not shown).



8. If there is a lesion or anomaly sample that area as well as a small amount of normal adjacent tissue.

FIGURE 5. Rapid field necropsy techniques for relatively small (5-15 mm) fish shown with Atlantic menhaden. Sample animals immediately after collection since tissues deteriorate quickly. To sample live animals, euthanize by severing the spinal cord (panel 1) or an overdose of MS222. The internal organ placement as seen in menhaden (panel 2) may vary significantly in other species. Use appropriately sized forceps and sharp scissors for all manipulations and cutting to allow proper fixative penetration to all tissues including the brain (panel 3), the gills and oral cavity (panel 4), the heart and anterior kidney (panel 5), the visceral organs (liver, intestine, cecae, spleen and mesentery - panel 6), posterior kidney (panel 7) and any observable lesions (panel 8). Although this technique saves time in the field, the resulting preserved tissues often require additional cutting prior to histological processing. Procedure and image courtesy A. Kane

TABLE 2. Suppliers for necropsy equipment and other supplies. Request a catalog from these and other companies so that when you need to make budgeting decisions and/or order supplies, the information is at your fingertips. Mention of suppliers in this paper does not constitute endorsement.

Argent Chemical Laboratories - MS222 and other chemicals/drugs
Telephone: 425-885-3777/800-426 6258
Fax: 425-885-2112
Internet: www.argent-labs.com
Address: 8702 152 Ave. NE Redmond, WA 98052

Aquanetics Systems Inc. - Aquaculture hardware. Great catalog
Telephone: 619-291-8444
Address: 5252 Lovelock Street, San Diego, CA 92110
Fax: 619-291-8335

Biomedical Research Instruments, Inc. - medical and dissection instruments
Telephone: 301-881-7911/800-327-9498
Address: 12264 Wilkins Avenue, Rockville, MD 20852
Fax: 301-881-8762
Internet: www.biosupplynet.com

Fisher Scientific - General scientific supplies
Telephone: 1-800-766-7009
Address: 600 Business Center Dr., Pittsburgh PA
Internet: www.fishersci.com
Fax: 1-800-926-1166

IDE Interstate Inc. - medical equipment and instruments, pharmaceuticals and proprietaries
Telephone: 1-800-666-8100
Address: 1500 New Horizons Blvd., Amityville, N.Y. 11701
Fax: 1-800-IDE-FAX1
Internet: ideinterstate.com

Sigma Chemical - biochemicals and reagents; excellent technical assistance
Telephone: To place an order- 800-325-3010, General Info. 800-521-8956
Internet: www.sigma.sial.com/sigma/techlib.htm.
Address: P.O. Box 14508, St. Louis, MO 63178
Fax: 800-325-5052

Thomas Scientific - General scientific supplies
Telephone: 609-467-2000/800 345-2100
Fax: 609-467-3087
Internet: www.thomassci.com
Address: 99 High Hill Rd. at I-295, P.O. Box 99, Swedesboro, NJ 08085-0099

VWR Scientific Products - General scientific supplies
Telephone: 1-800-932-5000
Address: 405 Heron Drive, P.O. Box 626 Bridgeport, NJ 08014
Fax: 410 418- 8398
Internet: www.vwrsp.com

and between 18 and 25°C until they can be processed in the laboratory. If necessary, ship samples to a microbiology laboratory by overnight priority courier, notifying the laboratory in advance of shipment.

Specimens used for microbiological sampling can subsequently be used for histopathological tissue sampling (but not *vice versa*). Alternatively, and particularly with small fish, it may be easier to sample separate specimens for microbiology and histopathology.

Samples may also be taken for viral culture. Collect samples of brain, liver, spleen and kidney in pre-labeled aluminum foil. Refrigerate until getting back to the laboratory and keep samples frozen until they can be processed by a diagnostic laboratory.

Perform a systematic necropsy.

Sample all organ systems including gills, heart, skin, muscle, bone/cartilage, brain, eye, lateral line, esophagus, stomach, intestine, anterior kidney, posterior kidney, swim bladder, liver, mesentery/pancreas, spleen and gonad. Observe the amount of internal body fat (use a scale of 0-3, where 0 = none, 1 = minimal, 2 = moderate 3 = a lot). It is not possible to adequately cover necropsy procedures within the context of this article. However, several references are provided for the reader in the bibliography section. One reference,(Kane, 1996) "*FishGuts: A Multimedia Guide to the Art and Science of Fish Health and Necropsy*," provides a digital overview of the subject including QuickTime movies of systematic necropsy techniques. Proficiency comes with practice and experience.

The type of necropsy procedure may need to vary with the available amount of time and personnel, the required area and total number of fish to be sampled, and the size of the fish. Under optimal conditions, a complete necropsy should be performed, where tissues from all organ systems are properly dissected, sampled and preserved (Kane, 1996; and Reimschuessel, 1999 in Table 1). The time required to perform a complete necropsy, including parasite and microbiological samples may range from 10-45 minutes depending on the size of the fish and the proficiency of the technician. It is helpful to have two persons involved in the processing; one to perform the necropsy and one to take notes, label samples, make organ "stabs" for microbiology, and make observations for microscopic parasites.

There are instances where time cannot permit complete necropsy processing in the field. As a substitute, we offer an abbreviated necropsy protocol that can be used with small fish (5-15 cm total length). This technique is not appropriate for larger fish because individual organs need to be cut in order to insure adequate tissue fixation. This abbreviated technique is shown in Figure 5 and illustrates euthanasia by cervical transection, opening of the cranium to expose the brain, removing an operculum to expose the gills, cutting off the head to expose the anterior kidney and heart, reflecting the side of the fish to remove the visceral mass, sampling the posterior kidney and any grossly observable lesions. Although the cartoon in Figure 5 exemplifies these techniques in Atlantic menhaden, the methods are applicable to other species as well. However, the anatomical placement of organs may vary from species to species.

When sampling fish ≤ 4 cm total length, and time is of the essence, an even further abbreviated procedure may be used. With a small, sharp, pointed scissors make a midline incision from the vent up to the branchiostegal membranes (just beneath where the opercula meet at the ventral midline). Make a shallow cut on the forehead, between

the eyes, to permit formalin to access the brain. With small forceps grasp the posterior-most portion of the gut and gently reflect the gut package, including all the visceral organs, anteriorly. This will expose the swim bladder and the posterior kidney. Make a single cut through the gut package, avoiding the gall bladder, to provide better fixation to the intestine and portions of the liver.

It is helpful to have decent necropsy tools, and choose the correct tools for the job. For example, use bone cutters for cutting through large bones, heavy scissors and forceps for cutting through and manipulating skin, muscle and small bones, and fine scissors and forceps for soft tissues. As a courtesy, Table 2 provides a list of suppliers of necropsy equipment and related supplies.

Sampling and preservation techniques.

Regardless of the technique used to necropsy the fish, it is important to insure that all preserved tissues are small enough to permit fixative penetration. Tissues that are not properly preserved are "as good as no tissues," and possibly worse due to the added time and expense in processing them. To insure adequate fixative penetration, specimens should not exceed 4 mm in any one dimension. Only small tissue samples are necessary for histopathology. Several fixative types may be used for preserving samples. We provide a recipe for 10% neutral buffered formalin. This is a good, all-purpose fixative and the ingredients are readily available. Always place tissues in at least 10 volumes of fixative (i.e., a 1:10 ratio of tissue:fixative) to insure proper concentration of active ingredient as the tissues fix.

Recipe For 10% Neutral Buffered Formalin:

Formaldehyde (37%)	100 mL
Distilled water	900 mL
NaH_2PO_4 (sodium phosphate, monobasic)	4.0 g
Na_2HPO_4 (sodium phosphate, dibasic)	6.5 g

Protective Clothing

Gloves, apron, goggles and other protection should be worn as needed, particularly if working with formalin or there is evidence of an active toxic event. Note that formalin is irritating, toxic, and may accumulate in contact lenses; it should be used under a hood or in a well-ventilated area.

Sample shipment.

Preserved samples should be shipped in sturdy, wide-mouth plastic jars that are appropriately sized for the specimen. Ship jars inside sealed plastic bags inside a very sturdy box. Fill the box Include copies of all pertinent data sheets and accession numbers for samples. If using Federal Express or other special service, be sure to include recipients proper address and telephone number and verify with the recipient that the package will be arriving at a specified time and location. If you are collecting live animals to bring to a collaborating diagnostic laboratory, they should be transported as soon as possible in insulated, oxygenated containers. If the ambient temperature is very hot, use of a small ice block, that does not come in direct contact with the transport water, is helpful. One easy way to accomplish this is to place a frozen,

water-filled 1 or 2 L plastic soda bottle inside a transport cooler that contains an oxygenated transport bag.

Cleanup.

Common sense applies. Double bag all carcass remnants, bloody materials and gloves. Have a dedicated container for "sharps," i.e., syringes, scalpel blades, broken glass, microscope slides and cover slips. Properly labeled, large lidded coffee cans work well as a sharps container. Good housekeeping is important from a public relations standpoint: do not leave *anything* behind and clean up any blood/mucus from working surfaces.

CONCLUSION

The concepts and methods outlined in this paper were assembled in order to support appropriate and consistent sampling methodology in fish kill investigations. Most of the methods that are outlined are to assist with making descriptive observations that must convey accurate, meaningful information. These techniques with their associated terminology are acquired through experience and proficiency gained only by practice.

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Reimschuessel, R. 1999. Necropsy Techniques in Aquarium Fish. In: Kirk's Current Veterinary Therapy, Philadelphia, PA: W.B. Saunders Co., NY.