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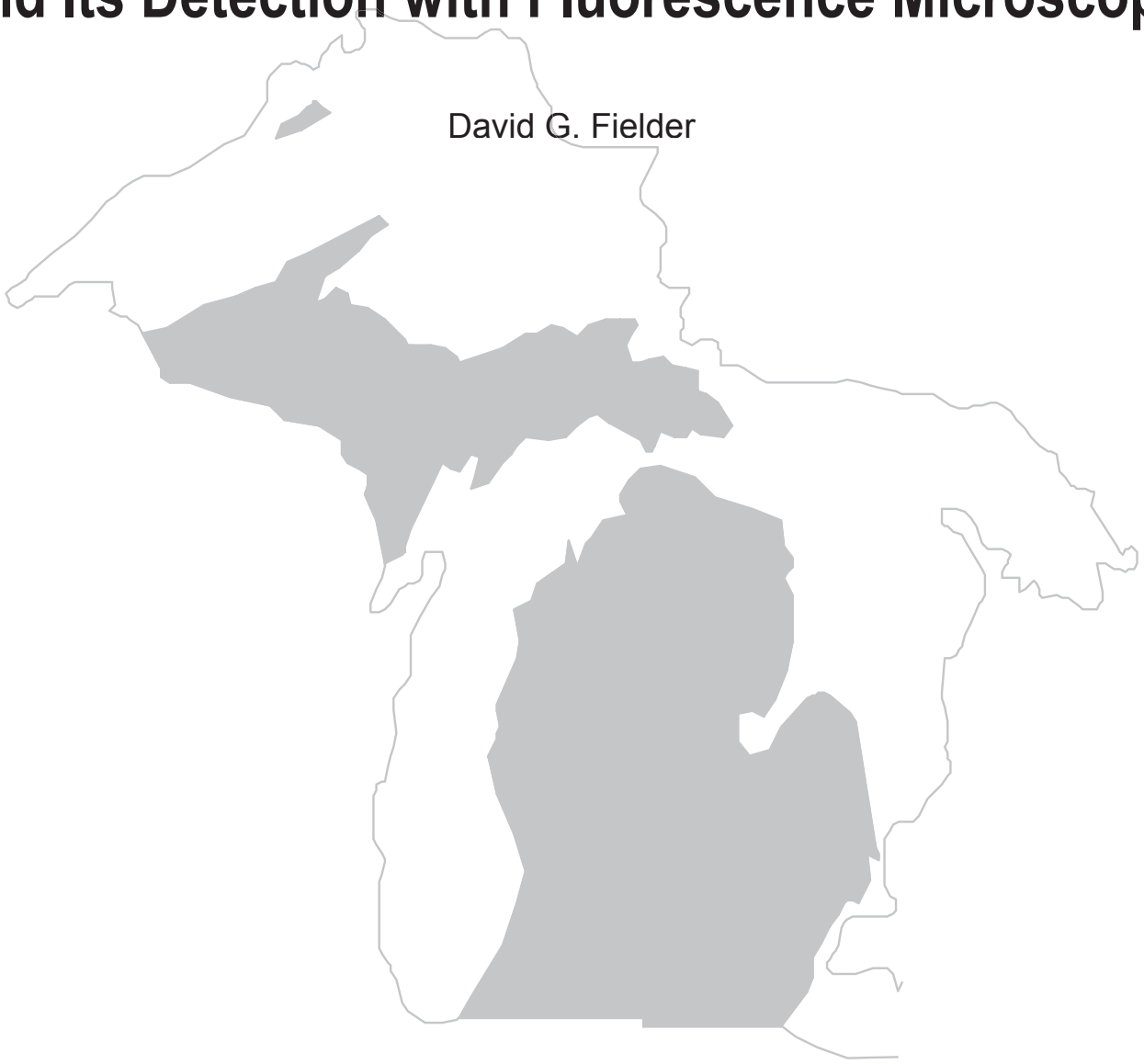

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MICHIGAN DEPARTMENT OF NATURAL RESOURCES
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Methodology for Immersion Marking Walleye Fry and Fingerlings in Oxytetracycline Hydrochloride and Its Detection with Fluorescence Microscopy

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Abstract.–This manual summarizes the process of conducting walleye stocking evaluations based on immersion marking with oxytetracycline hydrochloride. The summary includes methodology for application of the immersion treatment in the hatchery setting, detection of the mark using fluorescence microscopy and some considerations for planning evaluations based on the technique. The methodology described here was based on available literature and refined to meet the specific needs of the Michigan Department of Natural Resources, Fisheries Division.

Background

Stocking of walleye *Stizostedion vitreum* is a common management practice applied throughout the species' range (Fenton et al. 1996). Stocking has traditionally occurred using fry (newly hatched fish) or small fingerlings (approximately 1,134 - 363 per kg) (Heidinger 1999). Walleye management, however, has been slow to evolve because there has not been a convenient means with which to evaluate the contribution of stocked fish to existing populations. Past evaluations have primarily used alternate-year stocking schedules, designed to measure recruitment differences between stocked and non-stocked years. However, patterns in recruitment attributable to stocking can be difficult to identify due to variable environmental factors. Consequently, it typically requires a decade or more of alternate-year stocking to begin to determine the contribution of stocked fish (Younk and Cook 1991).

A suitable means to mark or tag hatchery-reared walleye has been lacking. Management of other species, particularly salmonids such as rainbow trout *Oncorhynchus mykiss*, lake trout *Salvelinus namaycush*, and chinook salmon *Oncorhynchus tshawytcsha* have long utilized a variety of tagging and marking methods. These include coded-wire tagging, fin clips, and oxytetracycline marking via laced feed (Guy et al. 1996). These techniques are impossible or impractical to apply to walleye fry or small fingerlings because the fish normally stocked are very small, numerous, and not typically reared on artificial feed. Some agencies do stock large walleye fingerlings (i.e. fall young-of-year [YOY]) as part of their stocking program. These fish, which may be as large as 10-15 cm, have been successfully tagged with coded-wire tags or freeze branded. Fry and small fingerlings, however, remain the primary hatchery product for many management agencies.

Oxytetracycline (OTC) is an antibiotic drug that has long been known to bind to bones and calcified tissues (Isben and Urist 1964). When bound in bones, this drug will fluoresce when exposed to ultraviolet (UV) light. Immersion marking is the most viable option for walleye, where the chemical is applied to fish in solution, and absorbed through the gills. This is a suitable technique because large numbers of fish can be cheaply treated as young as the fry stage (Guy et al. 1996). In walleye, the technique was first experimented with by Scidmore and Olson (1969) and was later refined by Brooks et al. (1994). Immersion marking has been applied to other species as well (Thomas et al. 1995).

The mark is distinguishable by examining some bony part under UV light. Otoliths are the best material to examine for OTC immersion marks in walleye because they are the first calcified tissues to appear upon hatching, when many bones have not yet formed (McElman and Balon 1985). The otolith is a calcified tissue in fish that is not unlike human ear bones. Otoliths serve as orientation and movement sensors, and most fish have several pairs. Sagittal otoliths are the largest and most easily located in walleye. Otoliths are not attached to any other bone but rest in a fleshy cavity under and slightly behind the brain. Anglers catching fish marked with OTC will not be able to see any difference in the fish.

Walleye are most often marked as fry as opposed to fingerlings, even if the fish are to be stocked as fingerlings, because treatment is more easily and cheaply administered at the fry stage. It is typically not necessary to mark walleye as fingerlings (which can be differentiated from a fry mark) unless a stocking evaluation is comparing both fry and fingerlings in the same water body in the same year. Post stocking collections of walleye in the field then provide specimens for mark detection and evaluation of recruitment attributable to hatchery fish.

Walleye will retain an OTC mark for several years; however, it is most easily detected while the walleye is still YOY. For that reason, stocking evaluations based on immersion marking with OTC often first examine recruitment at the fall YOY age. Since recruitment often is not fully determined until later, the evaluation can continue to age 1 and beyond.

Otoliths marked by immersion of walleye fry can be viewed with a microscope at 100x to 400x under UV light to reveal a gold ring near the center. Fish marked as fingerlings will exhibit the ring farther out, onto the lobes of the otolith. The otolith from an unmarked walleye will lack this ring. By examining a sufficient sample size of fish, an estimate can be made of the percentage of the year class that is comprised of hatchery or wild fish.

The objectives of this document are to detail the procedures for conducting OTC immersion treatments of walleye in the hatchery setting, describe the protocol for accurate detection of marks in the laboratory, and offer some advice for conducting stocking evaluations based on OTC marking. The intent of this report is to serve as a manual to technicians and biologists.

OTC Treatment

Advance Planning

In Michigan, a watershed unit or research biologist should have previously submitted a "Marking and Tagging Study Proposal" form (Appendix 1) with their fish stocking request and have received approval from the Fish Marking Review Committee. Currently, immersion marking of walleye is an unapproved use of OTC by the U.S. Food and Drug Administration (FDA). Until FDA formally approves OTC for this use, all marking operations must be exempted by FDA under an Investigational New Animal Drug permit (or INAD). Michigan Department of Natural Resources (MDNR) currently participates in a multi-state INAD for OTC marking of walleye. Marking requests need to be coordinated with the state INAD administrator. Before proceeding with the marking, make certain the marking has been included in the FDA approval, and that any special FDA requirements have been followed.

Supplies and Quantities

OTC is typically available in pharmaceutical, research, and veterinary grades. Research grade OTC is usually adequate for fish marking and is typically a higher quality than veterinary grade drugs, which are widely available from feed stores. OTC is available from several sources (see Appendix 2 for vendor information).

To determine the amount of OTC needed, one must first know the volume of OTC solution required. This in turn will be determined by the number of fish to be treated. Densities of fish in treatment solution can approximate normal holding densities. A safe density for walleye fry is 1,000 fry per liter of water. For small fingerlings, the appropriate density will depend on the size of fish. Treatment is performed in standing water (using a holding tank, mini-raceway, or trough).

Calculate the exact volume of chamber water. Volume of a rectangular chamber like a raceway is calculated by LxWxH, where L is length, W is width, and H is height (measured to the top of the stand pipe). Measure the entire chamber, not just that restricted to fish movement (i.e. include the areas behind screens). For circular tanks, volume is equal to $\pi \times R^2 \times H$, where $\pi = 3.1416$, R is radius, and H is height.

As an example, suppose a treatment calls for 1,500,000 fry. At 1,000 per liter, they require a minimum of 1,500 liters (396 gallons). A circular tank is available with a radius of 91 cm with a 61-cm standpipe. Volume is calculated: $(91cm)^2 \times \pi \times 61cm = 1{,}586{,}947cm^3$ or 1,586.9 liters. At 1,000 fry per liter, this is an acceptable tank and volume.

The amount of OTC to secure in advance is a function of solution volume and concentration. Standard treatment concentration is 700 PPM (Parts per million equals milligrams per liter). Multiply 700 times the volume to determine the number of milligrams of OTC to acquire. In our example: 700 mg/l x 1586.9 liters = 1,110,830 mg or 1,111 grams.

Oxytetracycline hydrochloride, off-the-shelf, usually has less than 100% activity. One supplier catalog quotes the activity rate of their OTC to be $\geq$835 µg/mg of powder, or 83.5% activity. To determine the actual amount of stock chemical to order, divide your needed amount by the activity rate. In our example, 1,111 grams / 0.835 = 1,330 grams of stock chemical. Round up to nearest whole quantity for ordering or about 2 kg in this example. Active chemical ratio varies by lot so check the actual label before making final calculations.

The chemical vendor will generally supply OTC in 100-, 50- and 10-g increments. Bulk quantities (>1 kg) can also be ordered at substantial savings. It is generally a prudent precaution to have extra on hand in case of loss or spill. Ordering twice the necessary amount would not be inordinate since the opportunity to mark walleye comes only once a year in a fairly tight time span and it may not be possible to order replacement chemical in time. These chemicals should be used fresh each year or at least before their expiration date. Obtain a Material Safety Data Sheet (MSDS) and follow all precautions. Always store OTC away from sunlight.

OTC hydrochloride is very acidic (pH is 2-3) and would quickly kill fish. Therefore, the chemical must be buffered (neutralized) before applying it to fish. Buffering is done with sodium phosphate (also called sodium hydrogenphosphate, Appendix 2). While it is difficult to predict exactly how much buffer will be required, an approximate 1:1 ratio with the OTC is reasonable. The amount actually used will be determined by monitoring pH during the mixing process. Obtain an MSDS and follow all precautions.

Because preparation of the treatment solution requires careful monitoring of pH while neutralizing the acid, numerous pH readings will be required. pH paper is not adequate for this. An electronic pH meter in good working condition is required. Preparation of the solution often results in foaming. Commercial antifoaming agents such as No-Foam can be used for its control (Appendix 2).

Because the treatment is done in standing water (no fresh flow) for 6-8 hours, careful monitoring of the dissolved oxygen (DO) level is also necessary. Air or oxygen can be applied with air stones, and regular DO measurements help ensure the fish are not stressed. DO should typically remain in the range 8 - 14 PPM. Because OTC is in the solution, winkler

titrations are not possible. A reliable DO meter is required. If an air compression system is not available at the hatchery, a large tank of compressed air, regulator, and air stones are necessary.

Release of treatment solution to the environment is governed by the Michigan Department of Environmental Quality (MDEQ), Surface Water Quality Division. MDNR holds Rule 97 release authority from MDEQ, as well as a National Pollution Discharge Elimination Systems (NPDES) permit for release of OTC from most hatcheries and rearing ponds statewide. A specific list of approved release sites is maintained by the OTC INAD administrator in the Fisheries Division.

In absence of the necessary release permit, work can proceed by filtering the treatment solution. Treatment solution should be filtered through a column of activated charcoal before release into the hatchery effluent. Commercial units are available (Appendix 2). Another small-scale alternative is to replace the bottom of a five-gallon bucket with a screen and fill it with activated charcoal, then allow treatment solution to gravity drain through the bucket to a floor drain.

Many biologists feel that walleye mortality in holding can be minimized by providing some circulation. In absence of a fresh flow, circulation can be achieved by using a pump. A standard sump pump can provide suitable circulation for large holding tanks. A short section of garden hose will also be necessary to direct the pump discharge. Set-up of the pump is described in a later section.

Additional supplies needed include five-gallon buckets, tablespoons, stirring sticks (a drill-driven stirrer like those available for mixing paint is preferred), a flour sifter, plastic measuring cups with handles, plastic gloves, dust masks, and safety glasses.

Preparing Treatment Solution

Begin by planning the day, allowing sufficient time for preparation, treatment, clearing of solution, packaging, and transport. Treatment requires 6-8 hours, and it is necessary to shield fish and treatment solution from sunlight, which will degrade the chemical. Treatment of fry is best done on fish that are as old as possible, ideally five days old. Walleye fry do not begin exogenous feeding until 5-8 days of age at normal hatchery temperatures (R. Summerfelt, Iowa State University, personal communication). However, younger fry (i.e. 3- to 4-day-old) may be used if necessary, but 1- to 2-day-old fry are inadequate. This may require additional planning in timing of egg collection and separation of daily cohorts.

Fish should be in the treatment tank before stopping the fresh flow. Remove any dead fry before beginning. Begin air flow and position air stones to ensure even distribution. In a long trough or mini-raceway, an air stone may be necessary at each end and in the middle. Air stones can be positioned to keep fry away from metal screens, which can injure fish. If using a sump pump to provide circulation, seat the pump behind the standpipe screen, with the return flow directed back into the tank. Besides providing circulation, the flow will help mix the chemical. It will be necessary to replace the open standpipe with a capped pipe to allow sufficient depth for the pump intake to sit fully immersed. Record beginning DO level and pH as the pre-buffer pH level on the OTC marking field form (see Appendix 3). Fill in the beginning information on the OTC marking field form. A procedural checklist is offered in Appendix 4.

Remove approximately 2 1/2 gallons of water from the treatment tank with a five-gallon bucket. It is important to draw water from the treatment tank, as using any other source will alter the final tank volume used for calculation of chemical amounts. Large treatment tanks (requiring large volumes) may necessitate dividing the OTC between two or three buckets. While wearing plastic gloves, dust masks, and safety glasses, slowly add the OTC powder (previously weighed) to water in the bucket. Begin stirring using a power paint stirrer to help the powder dissolve. No-Foam may be necessary to reduce foaming (a capful is usually sufficient). Once all the OTC is dissolved in the bucket(s), note pH.

Begin neutralization by adding sodium phosphate powder, using a flour sifter to prevent clumping, while continuing to stir. Leave the

pH probe in the bucket to obtain a continual readout of the buffering process. Slowly add buffer, and observe the rising pH. Pause occasionally to allow the stirring to take effect before adding more buffer. The original solution is a clear yellow color, and the addition of buffer will turn it cloudy yellow. Ideally, you will return pH to the pre-buffer level previously measured and recorded. Record the approximate amount of buffer used on the field form.

Some water sources may have a pH of 8.0 or even higher. It will be difficult to raise the pH beyond 7.0 with a buffer. Overuse of buffer can be lethal to fry. The addition of OTC and buffer at pH of 7.0 will not greatly alter the overall pH due to the small volume of mixture.

Sodium hydroxide (a base) could be used to neutralize pH in place of the buffer and would allow higher pH to be achieved, but is not recommended. Sodium hydroxide can easily elevate the pH well beyond the intended end point. If sodium hydroxide is used, it should be added very slowly.

Treating Fish

Begin by adding the buffered OTC to the fish container. Use plastic cups with handles to scoop the slurry from the bucket and slowly pour it into the treatment tank. Add the chemical to the fish and not the fish to a prepared chemical solution. Pour the chemical onto bubbling water at the site of the air stones or at the hose if using a pump. Let the bubbling or circulating action help mix the chemical with water. If necessary, add some solution between air stones to ensure an even mixture. When finished, there will often be residual buffer in the bottom of the bucket. It is best not to add this to the treatment tank. Instead, rinse the residue with a small amount of tank water (by now, solution) and decant the rinse into the treatment tank. Repeat this several times and then discard the residue. Note start time on the field form.

Continue monitoring DO, pH, and fish for signs of stress for the next 6 to 8 hours, making adjustments to air flow as necessary. A bubbling action that is too vigorous can injure fry, so it is better to have several air streams than a single powerful one. Continue the control of foaming by adding capfuls of No-Foam at each bubbler or the pump flow if necessary.

Monitor pH during treatment. Some change is acceptable and will probably happen slowly enough that fish can adjust. Large changes in pH are rare. Also monitor temperature. In the indoor hatchery setting, temperature should not be a problem.

A good means for examining fish is with a flashlight directed down into the water column. One can check for mortality by sweeping the bottom with a feather or fry net.

If fish show signs of stress or mortality, one will have to make a judgment call on what to do. If the number of fish being treated is not great and their potential loss could be absorbed, you might continue with treatment. The decision to continue may depend on how much time remains. If stress is occurring, and the potential loss of fish is unacceptable, you may have to begin clearing procedures prematurely. Fortunately, exposure for as little as six hours may be sufficient to establish a good mark, although 8-hour treatments are preferred. Generally, treatment mortality is rare.

Overnight treatments are possible and can sometimes be logistically more convenient for purposes of delivering fish. Overnight treatments, however, will still benefit from monitoring.

If mortality under these procedures becomes a problem, several options and alternatives exist. Treatments can be conducted at a lower concentration of 500 PPM with acceptable results (Brooks et al. 1994). Treatment exposure can be limited to the minimum duration of 6 hours. Lastly, marking in bags during transport (described later) may prove less stressful. Bag Marking requires less total volumes of chemicals.

Clearing Procedure

At the conclusion of treatment, record final pH and any other information on the OTC marking field form. Then resume fresh flow to the treatment tank. Remove the pump and/or air stones and replace any plugs with the open stand pipe. Resist the temptation to remove the fish

prematurely. They will perform best if given time to gradually move from solution back to fresh water. There may also be DO, pH, and temperature acclimation taking place.

Full clearing may require several hours depending on volume and tank type. Circular tanks do not exchange water as efficiently as raceways. Once clearing is complete, fish are ready for transport and stocking. If fish are to be held further, ensure that unmarked fry are not added to the marked lot. Retain the OTC marking field form for your records. Ensure shipping boxes are labeled with "OTC marked fish", and that all stocking and transport records reflect the marking.

Bag Marking

An alternative marking methodology for fry is to mark fish during transport in plastic bags, rather than in a tank at the hatchery. Typically, OTC solution is mixed fresh the day of application using the same technique as described for tank or raceway marking. In this instance, however, a separate tank or container of water is brought to treatment concentration. This solution then serves as the water for packaging the fry. The principal difference is that the fry will be added to the solution rather than the concentrate to the fish. The volume of solution prepared need only be enough to accommodate bagging, which is often less than tank or raceway volumes. The OTC solution for bagging should be saturated with oxygen in advance of shipping to ensure adequate DO levels during transport.

This technique can save some effort in the hatchery, but still requires fry to be a minimum of 3-4 days old. Thus, bag marking does not eliminate holding of fry.

Transport time must be estimated and additional holding time allowed if necessary to ensure the minimum 6-hour treatment time. For example, if it requires four hours to deliver fry to the rearing pond, then fry might be retained in the bag at the hatchery for at least two hours before transport. Alternatively, fry could be held at the pond for the additional time; however, conditions are usually better at the hatchery, as it is often cooler and less exposed to sunlight.

Fingerling Marking

When marking fingerling walleye, the procedure is essentially the same as with fry. Often, however, larger quantities of water are involved. Like fry marking, treatment may take place in holding tanks or raceways. One fingerling marking option is to mark in a transport truck. This can be advantageous because transport trucks often are already equipped with an air or oxygen delivery system. The treatment, however, still needs to be shielded from sunlight. Walleye fingerling marking is performed at the same concentration and duration as fry marking.

Detection

Accurate detection of OTC marks requires careful removal and preparation of the sagittal otoliths and examination with fluorescence microscopy. The detection process in the laboratory is as critical a step as initial treatment of fish. Whether examining known marked fish for quality control purposes or scoring unknowns from field collections, the process is the same.

Supplies

Detection of OTC marks with a fluorescence microscope requires a high intensity beam of UV light focused at the mark. Because immersion marking is less efficient than marking with laced feed, and because it spans only a very narrow marking duration, low intensity, hand-held black lights are not sufficient for detection. A fluorescence microscope is needed, with a mercury vapor light of at least 100W intensity. Quartz halogen fluorescence microscopes are inadequate.

Detection is done under 100x, 200x, or 400x power, so objectives of these magnifications are necessary. A beginning, or locating, lens of 40x is also very helpful. Manufacturers of

fluorescence microscopes often offer objectives that are specifically engineered for fluorescence detection work. These objectives are desirable although not entirely necessary. The fluorescence microscope must be equipped with a filter assembly that will limit wave lengths to 450-490 nm. The Nikon B-2H filter cube is suitable although the B-2A is also effective (Appendix 2). The scope must be set up in a room that can offer complete darkness during detection.

Other supplies needed include a dissection tray, scalpels, probes, forceps, petri dish, squirt bottle, cyanoacrylic glue (super glue), glass microscope slides, slide box, and wet/dry (emery style) sandpaper of 400 and 600 grit. A dissection scope may be necessary to remove otoliths from very young walleye.

Removing and Mounting Otoliths

Specimens should be frozen before removal of otoliths rather than using a chemical preservative. Specimens should be stored in the dark and examined within six months of capture.

There are three main techniques for removal of otoliths: cut off the head and remove otoliths from the back; split the fish head laterally and examine each half; or remove the dorsal portion of the skull and brain to access otoliths. The latter method is believed to be superior, and is presented alone in the following paragraphs (Figure 1). While the other methods work, they require greater cutting of the specimen and are less predictable in locating otoliths.

Begin by thawing specimens and taking any required measurements such as length and weight. Next, while working in the dissection pan, locate the two oval pigmented areas on top of most walleye heads (Figure 1A). They will be dorsal and just posterior to the eyes. This is approximately where the otoliths are located, only deeper inside, under the brain. Using the pigmented areas as guides, make a transverse (cross) cut just posterior to them about 20% into the body of the fish (Figure 1B).

Using this first cut as a means to position the scalpel, cut forward shaving off the top of the fish's head, exposing the interior cavity (Figure 1C). This may also cut into the eye sockets. For yearlings and older fish, a small fillet knife may be required. Next, using forceps, remove the brain. Remove small amounts at a time so that you do not accidentally dislodge the otoliths underneath. With the brain removed, one will see two parallel depressions. These hold the sagittal otoliths covered with a thin layer of mesentery. Using a probe, penetrate the mesentery and gently push to the edge of the depression. This will turn the otolith onto its edge where it may easily be gripped with forceps. Repeat with the second sagittal otolith.

Place the otoliths in a petri dish with a small amount of water. Using a probe, remove any remaining mesentery. Next, dry the otoliths by dabbing onto a paper towel. Finally, set the otoliths in a dry petri dish where they will be protected until ready for mounting.

During this entire process, ensure that the otoliths are shielded from sunlight. A single specimen should be prepared from otolith removal through mounting before beginning another. Take care with otoliths, as they are easily lost and surprisingly fragile.

For otolith removal from fry or small fingerlings, a dissection scope may be required. For fry, lighting should be adjusted so that the light shines through the fish. Fry are transparent enough that sagittal otoliths will appear as tiny oval objects in their heads (Figure 1D). Carefully disarticulate the head with a probe and try to tease out the otoliths. One may be able to find only a single otolith with this method.

For mounting otoliths, start with a clean glass slide. An etched writing slide will allow you to record the fish number, date, location, or other data used to identify individual specimens. Place a drop or two of cyanoacrylic glue on the center of the slide. Immediately place each otolith in the glue. It is not critical which side is down (concave or convex), but be consistent with both otoliths (Figure 2). Position the otoliths so that they rest parallel to each other with their longest axis in line with the length of the slide. Allow to air dry. The slide may be placed under a hair dryer set at low power to speed drying. Once dry, otoliths are ready for sanding and reading.

Mark detection

Sanding the otolith is a delicate part of detection process. The amount of sanding required will depend on age of the fish. Otoliths from fry and small fingerlings may not require sanding. Fall YOY and older fish will require some sanding. The biggest challenge in this process is sanding far enough to expose the mark, but not erase it. Often, the difference is just one or two extra passes with sandpaper, so the process requires frequent checking of progress with a microscope.

For most YOY, sand with 600-weight wet/dry emery paper. Large YOY or older fish may require starting with 400-weight paper. Use small, easily held pieces of emery paper and apply water. Hold the slide down on a flat surface with one hand and press the sandpaper with the other hand, moving in a longitudinal direction (along the length of the slide). The otoliths should be glued close enough together that both otoliths are sanded at once. If otoliths break free, not enough glue was used.

It takes practice to learn how much sanding to do before checking progress. Generally, for fall YOY, otoliths can be given about 30 passes before examining. Rinse and dry the slide and place it on the scope. Starting with white light, examine the otoliths on low power. Daily rings should be clearly visible where sanding has occurred. The goal is to expose the center rings where the OTC mark will be located (or to view rings farther out if marked as a fingerling, Figure 3). Once the otolith is oriented under white light, darken the room and switch to UV light. After your eyes adjust to the lower light intensity, the otolith appears green.

If still on low power (40x), the mark will probably not be visible even if present. Use an objective of the next higher power (100x or 200x). At this power, the mark should be visible on a marked fish if otoliths have been sufficiently sanded. Ensure that the slide is still positioned correctly after the objective is switched. This may require reorienting under white light. For fish marked as fry, focus at the center of the otolith in order to view the first several rings at once. For fish marked as fingerlings, slowly move the slide, exploring one side of the otolith for the mark. It may help to count (under white light) the daily rings to where the mark is expected. For example, if the fingerling was marked at 40 days, count out 40 rings from the center.

Several reading and sanding cycles may be necessary before being able to make a final determination. When no mark is seen, the difficult part is deciding if it is because the fish is unmarked or because it has not been sanded far enough. It takes practice and skill to know the difference. One way to decide is by examining under white light. Rings in the area of expected mark should be clearly visible. If they are, and no mark is seen, then the fish is unmarked. Usually, the mark will slowly become apparent and the last one or two sandings only make the mark more obvious. If you are sure you see a mark, you can stop there. Utilize both otoliths in the detection process.

An unmarked otolith never reveals a gold line or ring despite slow, methodical sanding. In an attempt to reveal the mark, one may eventually sand too far. An otolith sanded too far will appear bright, clear, and lack daily rings (Figure 3D). If the over-sanding was the result of multiple reading and sanding cycles, then the fish is unmarked. If, however, the otolith was over-sanded as a result of impatience, without frequent reading, the otolith is scored as "undeterminable" and should be omitted from analysis.

Having the second otolith is a big help. Although both otoliths are sanded simultaneously, they rarely will diminish at the same rate. Often, if one is over sanded, the other is still readable.

Attempts to speed up the sanding/reading process by using a power grinder such as a Dremel tool almost always moves too fast and goes too far in the first sanding. It is best to use to the hand method or obtain a very low speed grinder.

Errors in mark detection can include detecting false marks as well as failing to recognize a true mark. False marks most often result from cracks and checking in the otolith. A check is a deeper or more pronounced daily ring and is thought to sometimes result from handling stress. Cracks and checks can gather the background light (autofluorescence) and concentrate it in a mark-like illumination. At

least one study (Kayle 1992) that attempted to use OTC marking is reported to have mistaken false marks or autofluorescence for true marks (Brooks et al. 1994).

A true OTC mark appears gold or yellow in color while autofluorescence appears green. Close inspection of the mark at a higher power (400x) can sometimes help discern true color. Pond stocking or transport can often induce a stress check at the same location one might expect to see a true mark. While this is a nonsignificant error (because only the hatchery fish would be exposed to such handling), wild fish have been observed to have checks on occasion as well. As a rule, an otolith failing to include both the gold color and the expected ring or band should be scored as unmarked (even if a bright green mark appears). Beginning readers should have a selection of known marked and known unmarked otoliths to practice with before attempting determination of actual unknown otoliths.

One should organize detection work using the Sample Analysis Form (Appendix 5). Store the slides in a closable slide box away from sunlight. Marks typically remain visible for about six months but begin to deteriorate immediately. Marks are most visible the first day the otolith is removed from the fish. A photomicrograph (photo-through-the-microscope) of the otolith specimen can be used to archive the image if a long-term record is desired.

Quality control

As OTC marking has evolved in recent years, it is more consistently achieving a 100% mark on treated fish. However, anomalies, such as less than a 100% mark or poor mark quality, are possible. To ensure quality control, a small sample of fingerlings (25-50) should be retained and frozen. If questions or concerns arise, this sample can be examined for mark frequency and quality. Subsequent results can be interpreted accordingly. If fish are marked and stocked as fry, and you wish to ensure quality control, rearing a small subsample to fingerling size will allow evaluation of mark frequency and quality. For fish marked and stocked as fingerlings, consider rearing a small subsample for a few weeks and then preserving by freezing.

Stocking Evaluations Based on OTC Marking

OTC marking can be used to determine what percentage of the year class is wild and what percentage is from stocking. Such information has been previously impossible to determine for walleye. If you sampled 123 fall YOY and 98 were judged to be marked, then about 88% of the year class can be attributed to stocking.

The ratio of wild to hatchery fish may be influenced by several biases, such as where hatchery fish were released and where recaptured fish were sampled. In a very large lake with many bays and inlets, hatchery fish may all reside in a single nursery area near the stocking site. Migration or mixing may not occur for walleye until after their first winter or even until they become sexually mature. If sampling is performed mainly in the stocking area, there may be a bias toward larger contribution of hatchery fish. If fish did not spread out and sampling was done evenly across the lake, there may be a bias toward larger contribution of wild fish.

Bias can be minimized with a geographic distribution of releases of hatchery fish and dispersal of sampling effort. Many large lakes have more than one access location. Perhaps fish could be released at each ramp. Better still, could fish be transported by boat to a variety of stocking locations within the lake? Sampling can then be done on a random or stratified random basis. This will allow a more confident determination of source of recruitment.

Sampling can be done in many ways. For YOY, one might sample in late summer or fall by electrofishing, bottom trawling, or small-mesh gill nets. Choice of gear can also provide a means to evaluate year class strength. Electrofishing can provide a Serns' index of recruitment (Serns 1983). Any gear, fished uniformly, can offer catch rates to compare among years. This information may prove valuable in concert with mark composition of the year class. One can then determine not only

what made up the year class (hatchery or natural reproduction), but also whether it was strong or weak. For determining percent composition of the year class, catches from different gear types can be combined.

Such evaluations are best done over several years. A number of environmental variables influence year class strength, and they are rarely constant among years. Conditions may favor natural reproduction one year, but suppress it in others. Hatchery fish may perform well in absence of natural reproduction but fail in its presence. A strong year class can suppress subsequent year classes. To tease out these differences in recruitment, the alternate-year design may still prove a useful stocking strategy and can be used together with OTC marking.

When a blend of wild and hatchery fish comprise a year class, consider examining for differences in condition, average length, or weight. These differences may indicate competitive advantages. Assessment of fall YOY is a good means to begin the evaluation process, then follow up next year with the same year class as yearlings. Did percent composition of hatchery fish remain the same over winter? Concern over patchiness of distribution diminishes for yearling and older fish.

Once composition of a year class is known at the yearling stage, it will usually not change. This percentage can be assumed to apply when the year class recruits to the fishery. If there is a creel survey in place, one has estimates of harvest. Creel data can be used to estimate what percent of the harvest is attributable to the year class under study (by aging). The percent of marked (hatchery) fish can then be multiplied by the number of that year class harvested giving the number of hatchery fish harvested.

The cost of supplying a hatchery fish to the creel has long been an elusive value in walleye management. Armed with estimates of hatchery fish proportions of the harvest, one could determine cost by dividing total production costs by return to creel. By applying mortality assumptions over the life of the year class, total hatchery contribution to the fishery from the original stocking investment can be estimated. The ability to mark hatchery walleye will enable other types of evaluations such as investigations of release timing, release locations, brood source comparison, size at stocking, transport, or rearing techniques.

Contingencies

In the event that quality control analysis determines that less than 100% of the treated fish show a visible mark, evaluation may still be possible. If a sufficient sample of quality control fish can be analyzed to determine what percent can be confidently recognized as marked, that ratio can be used to adjust subsequent samples from the field.

For example, if only 75% of the treated fish yield a visible mark (as determined from analysis of known marked fish from rearing ponds), then an additional 25% of the subsequent field collections can be assigned to hatchery origin. If 100 specimens were collected post stocking from the wild and 50 showed visible marks, then another 25% would be assumed to be hatchery fish that failed to take the mark. In this example, marking success is 75%. If 50 field marks were detected, then the number of marked fish (50/.75) should be 67 out of the 100 (67%). This approach assumes that the hatchery fish without a visible mark survived and performed identically to marked fish.

Similarly, a problem may occur when marked fish are stocked in a lake or river also stocked with unmarked fish the same year. Providing one knows the numbers of each group stocked, the above approach can also be used to perform the stocking evaluation. However, the same assumption applies and may be more tenuous if the fish were not stocked at the same size or came from different sources.

A more common problem encountered is poor mark quality. The mark may be visible on 100% of treated fish, but faint. The evaluation might still proceed but will likely require a much more careful detection effort. Sanding may require many more inspections to detect the faint mark. Ultimately, the investigator will have to determine if the results are reliable. One might choose to proceed, noting that the estimate of hatchery contribution from such a marking is minimal.

Conclusion

Immersion marking with OTC is a substantial advancement in walleye stocking evaluation, but requires skill and expertise to accurately apply and interpret. Critical evaluations of stocking should utilize multiple years and consider additional evaluation techniques. Publication of evaluation results should discuss marking methods and their effectiveness to help ensure the further evolution of this procedure.

Acknowledgements

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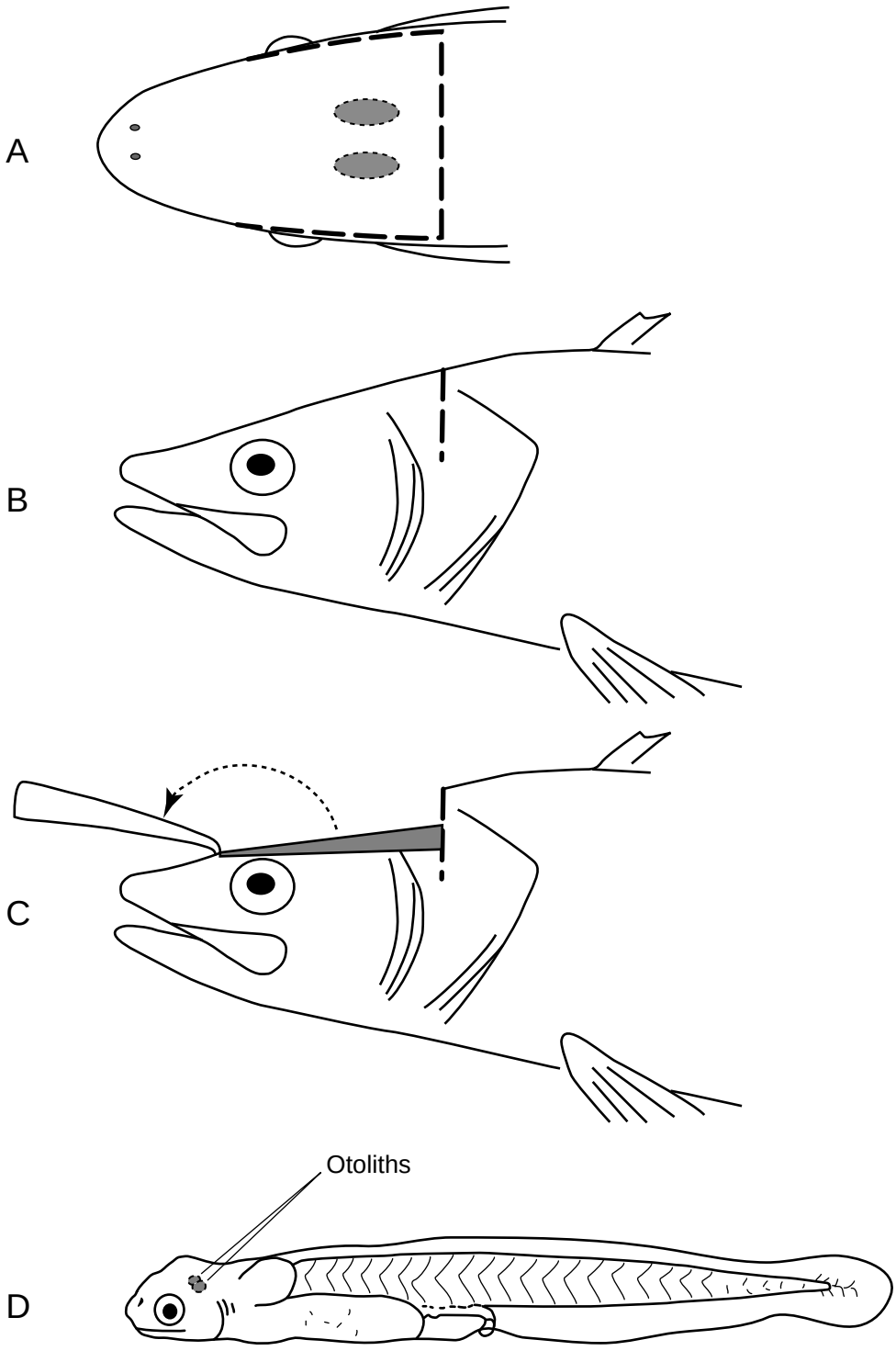
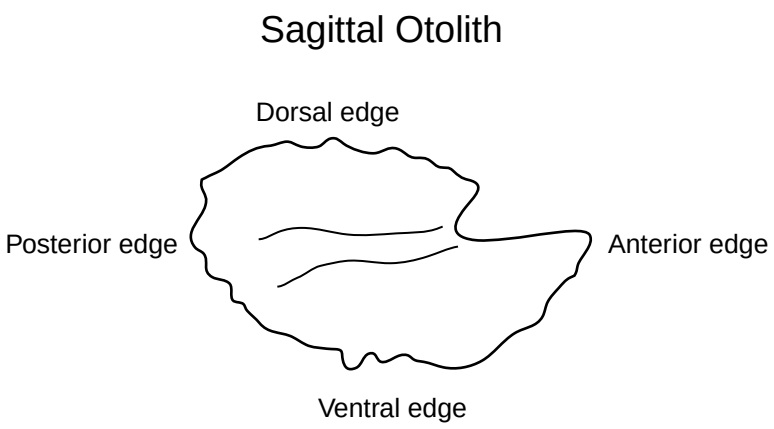


Figure 1.–Extraction procedure for walleye saggital otoliths. A: Positioning of ototliths and location of cuttings. B: Initial cut. C: Second cut to expose brain, otoliths lie beneath brain in fleshy cavities. D: Position of the otoliths in a larval walleye.



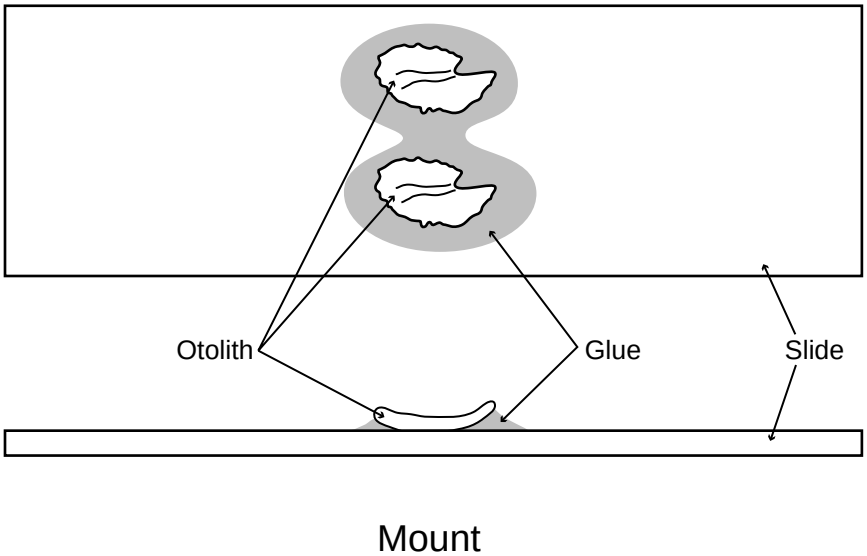


Figure 2.–Walleye otolith positioning and mounting for preparation (sanding) and detection of oxytetracycline.

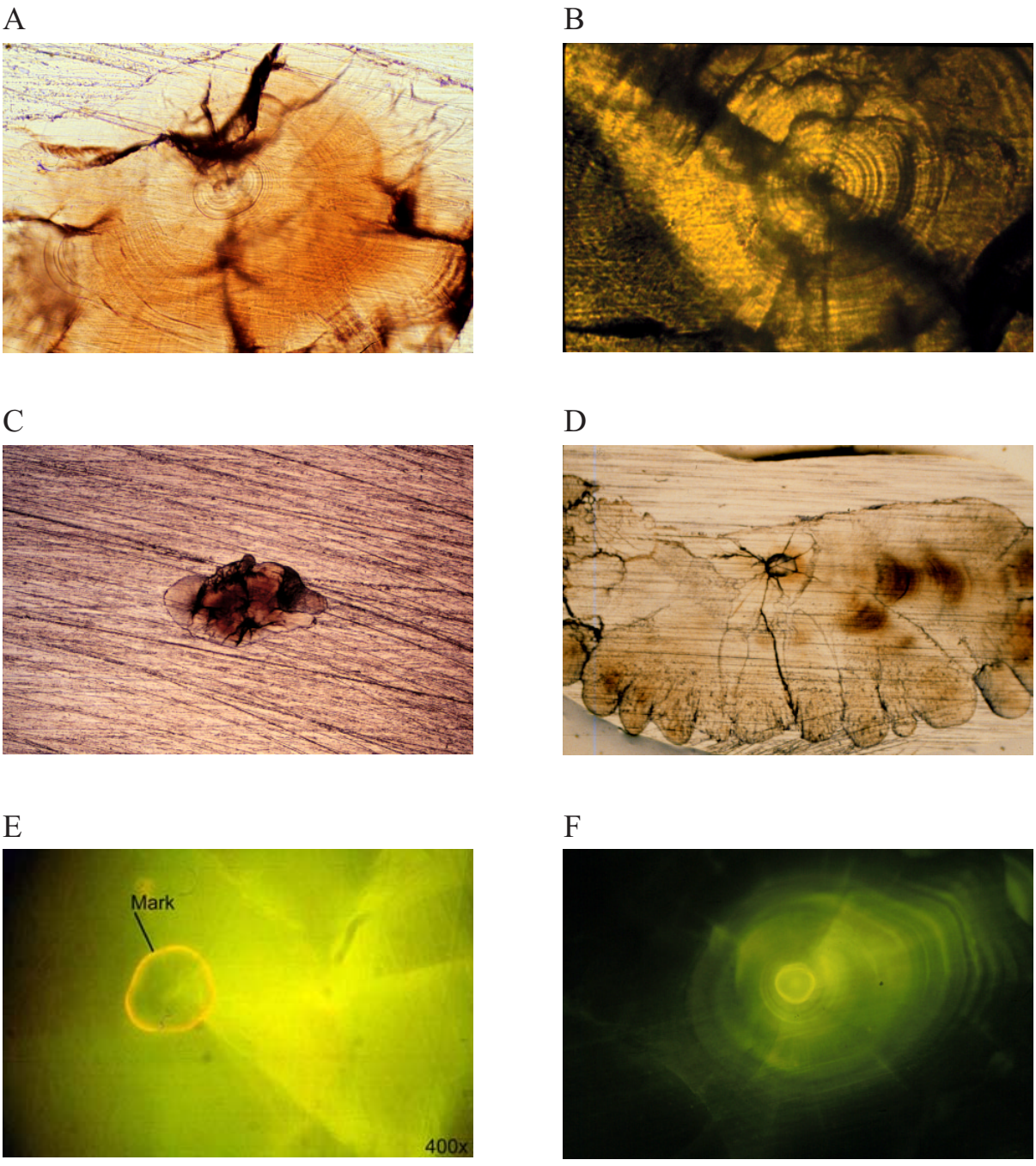
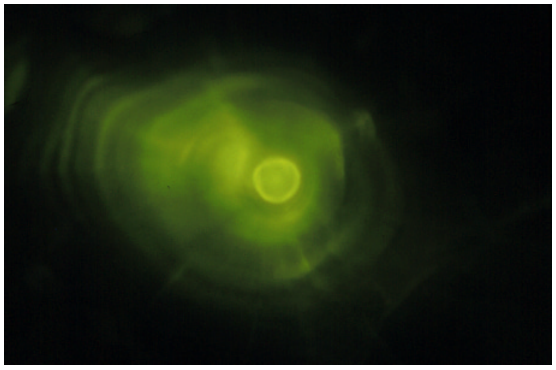


Figure 3.–(This graphic is best viewed in color, and will be available on the Institute for Fisheries Research web site.) Walleye otolith preparation and examination. All views are 100x unless otherwise specified. A: Otolith ready for examination (seen in white light). B: Close up of otolith center (focus) ready for examination (400x). C: An otolith from a small fingerling (40x). D: An otolith over sanded. E: OTC fry mark under UV light (400x). F: OTC fry mark under UV light. G; OTC fry mark under UV light. H: OTC fry mark (400x). I: Unmarked otolith under UV light. J: OTC fingerling marked otolith.

G



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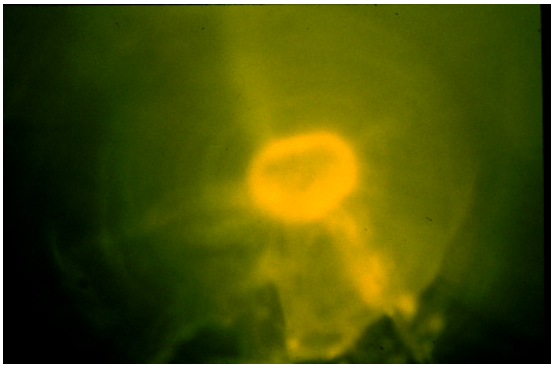

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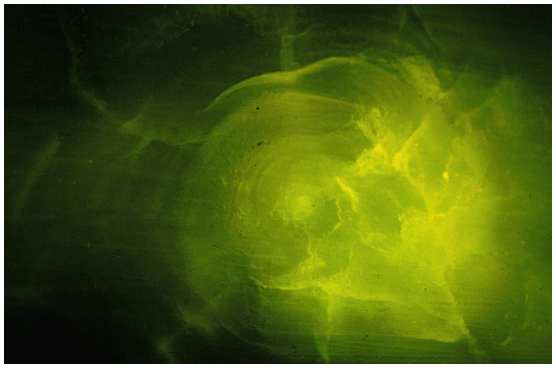

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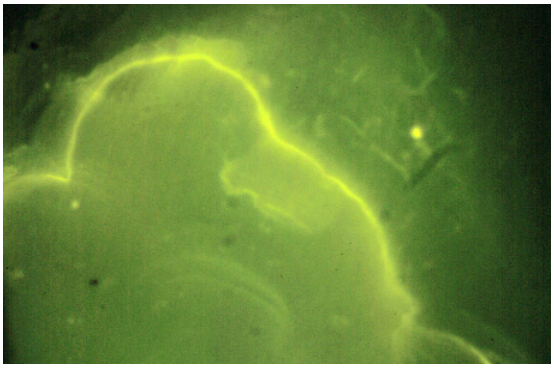

Figure 3.–Continued.

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Appendix 1.–Study Proposal form for fish marking and tagging.

Prescription # _____
Stocking Request # _____
_____

Michigan Department of Natural Resources
Fisheries Division

Fish Marking/Tagging Proposal Form

Project Title _____ **Date** _____

Project Leader _____ **Unit** (MU, Research, etc.) _____

Project/Study Objectives _____

_____

_____

Mark/Tag Evaluation Procedure (include method, i.e. creel census; costs; duration; etc.)

Method _____

_____

_____

Est. cost of evaluation _____

Source of funding _____

Marking and Stocking Schedule

Waterbody _____ County _____ FMU _____
Species/Strain _____ Age _____

Stocking Year						
# Fish Marked						
# Fish Unmarked						
Mark/Tag Desired						

Waterbody _____ County _____ FMU _____
Species/Strain _____ Age _____

Stocking Year						
# Fish Marked						
# Fish Unmarked						
Mark/Tag Desired						

Does this project have Basin Team Approval? (yes/no) _____ **Date approved** _____

Fish Marking Review Committee Approval _____ **Date** _____
(fish stocking specialist)

Appendix 2.–Vendor information.

- **OTC (oxytetracycline hydrochloride)**

  USB Corp.
  (800) 321-9322
  Catalog number US23659
  www.usbweb.com

- **Sodium phosphate, dibasic (sodium hydrogenphosphate)**

  Aldrich Chemical Inc.
  (800) 558-9160
  Catalog number 21,988-6
  www.sigma-aldrich.com

- **No-Foam**

  Argent Chemical Inc.
  (800) 426-6258
  www.argent-labs.com

- **Disposorb activated charcoal Filter**

  Calgon Corp.
  (412) 787-6700
  www.calgon.com

- **Fluorescence Microscope**

  Nikon Corporation
  Mager Scientific Inc.
  (313) 426-3885
  www.nikonusa.com

Appendix 3.–Field form for oxytetracycline marking.

Field Form for Oxytetracycline Marking

Date _____ Hatchery _____

Researcher _____

Species _____ Size _____ #/lb. _____

Number marked _____

Water volume _____ OTC assay _____

OTC amount _____ OTC concentration _____

Buffer or base _____

Type of buffer or base _____ Amount of buffer or base _____

No foam? _____ Water hardness (if known) _____

Prebuffer pH _____

Start pH _____

Finish pH _____

Treatment start time _____

Treatment finish time _____

Total treatment time _____

Total marking mortality (no. or %) _____

Marked fish destination _____

_____

Comments _____

_____

_____

Appendix 4.–Supply and procedure checklist for oxytetracycline immersion in tanks.

Supply and Procedure Checklist for Oxytetracycline Immersion in Tanks

1. Ensure sufficient supplies and equipment in advance.
   a. OTC supply; enough OTC to achieve 700 mg of active chemical per liter of final solution. Total volume will typically be 1 liter per 1,000 fry.
   b. Sodium Phosphate, will need about 1:1 ratio with OTC.
   c. No-Foam, one pint should suffice.
   d. Working and calibrated DO, pH meters and electronic scale.
   e. Activated charcoal filter, if necessary.
   f. Air supply, air stones and sump pump.
   g. Other supplies, buckets, spoons, flour sifter, stirring rod or sticks, handled cups, plastic gloves and safety glasses.
2. Place the required number of fry or fingerlings in the treatment tank the night before if possible. Start flows, shield work area from sunlight. Ready all equipment. Coordinate transport, delivery and stocking.
3. On morning of treatment, stop fresh flow to fish and start air stones and sump pump.
4. Measure pH in treatment tank and record on OTC field form.
5. Measure out OTC and mix with about 2.5 gallons of water in a five-gallon bucket.
6. While measuring pH in bucket, slowly add sodium phosphate using the flour sifter, stirring all the while.
7. Stop adding buffer when pH reaches and holds at 7.00.
8. Using plastic cups, slowly pour neutralized OTC solution in tank by pouring over bubblers and pump flow.
9. Note time on field form once all the OTC is added.
10. Continue to monitor fish for stress, pH, and DO for 6 - 8 hours.
11. After a minimum of 6 hours, resume fresh flow, remove air stones and sump pump, restore stand pipe, and note time on field form.
12. Direct run off into charcoal filter if necessary.
13. Once treatment solution is completely cleared, package and transport fish.

Appendix 5.–Analysis form for oxytetracycline marking.

Analysis Form for Oxytetracycline Marking

Collection No.	Date	Location	Length (mm)	Collector	Reader	Mark	Comments