

**Investigation of the use of Osmotic Induction to Reduce
the Immersion Period for Marking Early Life Stages of Walleye
with Fluorescent Chemicals¹**

Dale E. Logsdon

*Minnesota Department of Natural Resources
Post Office Box 86
Waterville, Minnesota 56096, USA*

Abstract.—Preceding the immersion of walleyes in either oxytetracycline (OTC) or calcein with immersion in a concentrated salt solution (osmotic induction) allowed fluorescent marking of either fry or juvenile walleyes with a shorter immersion duration than commonly necessary. The fry immersed in 1.0-4.5% NaCl for up to 90 s followed by immersion for 1-3 h in 700 mg OTC/L appeared to tolerate the treatment well but the quality of the marks produced on their otoliths was inconsistent. Juvenile walleyes immersed in 2.5-5.0% NaCl for up to 4 min followed by immersion in 0.5% calcein for 4 min consistently formed marks but showed less tolerance to the treatment procedure and experienced variable mark retention. Weekly inspections of calcein treated walleyes revealed rapid mark degradation on structures exposed to sunlight, but at least some marks were visible on all of the walleyes treated by pre-immersion in 5% NaCl for 4 min at the end of the experiment 15 weeks post-treatment.

Key words: walleye, fry, oxytetracycline, calcein, osmotic inductions, fluorescent mark

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Introduction

The Minnesota Department of Natural Resources (MNDNR) stocks approximately 300 million walleye *Sander vitreus* fry into lakes and rivers annually. Evaluating the contribution of these stocked fry to the fisheries, however, can be complicated by annual variability in natural reproduction and the inability to discern between stocked and wild walleyes. Marking the stocked walleyes allows analysis of the stock composition, but conventional fish marking techniques such as fin clips or tags are not practical due to the small size of walleye fry at the time of marking.

Immersion in fluorochrome dyes may be a practical alternative for marking larval walleyes (Brooks et al. 1994). Oxytetracycline (OTC) and calcein are the most common fluorochrome dyes used to mark fish (Guy et al. 1996). These chemicals are readily absorbed by the fish and deposited in their calcified tissues (Thomas et al. 1995). When subjected to ultraviolet light, the marked tissues fluoresce at specific wavelengths. Tetracycline marked tissues appear yellow-gold (Weber and Ridgway 1962; Brooks et al. 1994; Logsdon et al. 2004) whereas calcein marked tissues appear bright-green (Brooks et al. 1994; Negus and Tureson 2004).

The earliest published fluorochrome marking experiments on fish were conducted with tetracycline by Weber and Ridgway (1962; 1967). They were able to produce visible marks on salmon by administering tetracycline as a food supplement, by injection, or by immersion. These marking techniques have been since replicated and modified to mark a variety of salmonids (Odense and Logan 1974; Bilton 1986; Hall 1991), as well as coregonids (Dabrowski and Tsukamoto 1986), sciaenids (Bumguardner 1991), moronids (Secor et al. 1991), clupeids (Lorson and Mudrak 1987; Hendricks et al. 1991) and percids (Scidmore and Olson 1969; Kayle 1992; Brooks et al. 1994; Unkenholz et al. 1997; Lucchesi 2002; Logsdon et al. 2004).

Despite early success in immersion-marking of walleye fingerlings (Scidmore and Olson 1969), a procedure for mass-marking walleye fry did not appear in the literature until described by Brooks et al. in 1994. Brooks et al. (1994) marked 4-5 day old walleye fry by im-

mersion in a pH buffered solution of 500 mg OTC/L. The OTC solution was first mixed in the bags used to transport fry, then the fry were added to the solution and allowed to immerse for 6 hours. This procedure resulted in fluorescence marks that were visible on 100% of the otoliths from walleyes that were inspected 11 months later using equipment similar to that described by Bumguardner (1991).

Poor success producing fluorescent marks on walleye fry less than 4 days old (Younk and Cook 1991; Brooks et al. 1994) led researchers to believe that otoliths of younger fish were not developed enough for deposition of OTC. Subsequent research, however, has demonstrated that the walleye otoliths could be successfully marked in newly hatched fry if the concentration of OTC was increased to 700mg/L (Logsdon et al. 2004). The ability to mark the otoliths of newly hatched fry in-transit greatly simplified the marking process by removing the need to separate and hold different aged fish in the hatchery and allowed the Minnesota DNR to mark over 30 million walleye fry annually for stocking into Red Lake with little interruption of standard hatchery procedures (Logsdon 2006).

Perhaps the best way to further improve the marking procedure would be to reduce the immersion period. A shorter immersion period would allow in-transit marking at higher fish densities and reduce the amount of chemicals required to mark a given number of fry. Use of osmotic induction (Mohler 2003) may allow for a significant reduction in the length of time necessary to produce a fluorescent mark on walleye fry by increasing the uptake rate of the OTC solution. The osmotic induction protocol differs from the marking procedure commonly used for walleye fry (Brooks et al. 1994; Fielder 2002; Lucchesi 2002; Logsdon et al. 2004) in that the fish are immersed in a salt solution prior to immersion in the labeling solution. Immersion in the salt solution produces an osmotic gradient and promotes the partial loss of water from the fish tissue to their environment. An abrupt movement from the salt solution to the labeling solution then reverses the osmotic gradient and results in a rapid uptake of the labeling solution as water is replaced in the fish tissue via osmosis across the skin and gills (Alcobendas et al. 1991; Mohler 2003). Osmotic induction has been suc-

cessfully used to reduce the contact time necessary to produce both tetracycline and calcein marks in the elvers of the European eel (*Anguilla anguilla*) (Alcobendas et al. 1991) as well as calcein marks in larval and juvenile salmonids (Mohler 2003; Negus and Tureson 2004).

The use of calcein as a labeling agent for walleye fry may hold some advantages over the use of OTC. Oxytetracycline marks undergo photolysis when exposed to extended periods of sunlight (Lorson and Mudrak 1987; Muth and Bestgen 1991; Macfarlane et al. 2002). This renders the external marks unreadable and requires the sacrifice of the fish for removal of internal calcified structures such as otoliths for mark detection. Calcein marks can also fade when exposed to light (Leips et al. 2001; Honeyfield et al. 2008; Honeyfield et al. 2011), however, calcein has been reported to produce marks on fin rays, scales, and other calcified structures that remain visible for several months via external examination with a portable detection device or epifluorescent microscopy (Mohler 2003; Negus and Tureson 2004; Honeyfield et al. 2011). If external visibility of the calcein marks persists over a reasonable period of time on walleyes then this technique could allow for a simplified, non-lethal method of assessing walleye stocking contribution.

The purpose of this study was to evaluate the use of osmotic induction for reducing the immersion period necessary to produce fluorescent marks on walleyes by determining: 1) the tolerance of walleyes to the osmotic induction procedure, 2) the quality of marks produced by OTC after osmotic induction in a NaCl solution, 3) the quality of marks produced by calcein using the osmotic induction procedure described in Option B under Investigational New Animal Drug (INAD) Protocol 10-987 (USFWS 2003), and 4) the retention and ease of non-lethal mark determination on calcein treated walleyes.

The original vision for this study was to evaluate osmotic induction using both OTC and calcein on newly hatched fry. However, the lack of calcification of tissues other than otoliths on newly hatched walleye fry (McElman and Balon 1979; Figure 1), suggested a very low likelihood of producing external marks during in-transit immersion in calcein. In addition to the necessity of having calcified structures present for mark

production, the size of the calcified structures present at the time of immersion has also been reported to affect later calcein mark detection. Negus and Tureson (2004) observed a corresponding decline in mark visibility as the proportion of marked to unmarked tissue decreased due to fish growth. Negus and Tureson (2004) reported that calcein marks produced on rainbow trout *Oncorhynchus mykiss* at 3.5 months post-hatch remained visible when inspected 35 months post hatch, whereas marks produced on trout 0.5 months post-hatch had faded noticeably when inspected at 12 months post-hatch and were not visible at all when inspected next at 22 months post-hatch.

Since non-lethal mark detection is the major advantage reported for the use of calcein over OTC, a life stage that maximized the probability of long-term mark detection was selected for initial calcein experimentation. Calcein has not been approved by the U.S. Food and Drug Administration (FDA) for use in marking food fish. It has, however, been granted a compassionate aquaculture investigational new animal drug (INAD) exemption that is administered through the U.S. Fish and Wildlife Service. This exemption, however, restricts the use of calcein to fish weighing less than 2 grams. The largest life stage of walleye that was available under the 2 gram restriction was that which MNDNR staff commonly refer to as “frylings”. Frylings are walleyes that are reared at high densities in drainable ponds at the MNDNR hatchery facilities at New London and Waterville then harvested from the ponds when the zooplankton levels in the ponds can no longer support rapid growth. Harvest generally occurs at 40 to 50 days post-hatch and the harvested frylings typically range between 30 and 40 mm TL.

Methods

Oxytetracycline

Treatment.—Newly hatched walleye fry (<24 h post-hatch) were treated by immersion in a NaCl solution, followed by immersion in an OTC solution. The treatments began by mixing a bulk solution of 700 mg OTC/L (Fielder 2002; Lucchesi 2002; Logsdon et al. 2004) with Terramycin 343 (Pfizer, New York, then buffer-



Figure 1. Photographs of a newly hatched (<24 h post-hatch) walleye fry. Photograph (a) is the ventral-lateral view of the entire fish, whereas photograph (b) is a close up of the head region showing the presence of calcified otoliths.

-ing the solution to pH 6.8 with sodium phosphate dibasic (Sigma, St. Louis, MO). A silicon-based surfactant (No-Foam, Argent Chemical, Redmond, WA) was also added to the solution at a rate of 0.04 mL surfactant/L to reduce foaming of the OTC solution. Various NaCl solutions were then mixed, depending on the treatment requirements, with Hi-Grade evaporated salt (Cargill, Minneapolis, MN). All solutions were mixed using the Waterville Hatchery supply water; which is ponded well-water that was pumped to a head/settling tank then allowed to gravity feed through the incubation batteries and fry tank.

The fish were first collected from the fry tank with a fine-mesh dip-net and allowed to drain until the water ceased flowing from the net in a constant stream. The fry in treatments that used 4,000 or less fry were then volumetrically enumerated by scooping 2.5, 9.9, or 19.7 ml fry with a kitchen-style measuring spoon. Then they were transferred to a larval concentrator (Secor et al. 1991) that consisted of a 100 mm diameter PVC tube with mesh-covered bottom that had been placed in a bucket of NaCl solution. The fry remained in the NaCl solution until the end of the prescribed immersion period; after which the larval concentrator was removed from the NaCl solution, the solution allowed to drain away from the fry, and the fry then poured into a container of OTC solution.

During treatments that used 65,000 fry, the entire contents of the dip net remained in the net and the bag of the net that contained the fry was lowered into a shallow container of the NaCl solution for the prescribed immersion period. After the NaCl immersion, the fry were allowed to drain again and then enumerated gravimetrically by pouring them into a container of OTC solution on an electronic balance. Both fry and OTC solution were poured into a container with additional OTC solution after weighing.

The containers used for the OTC immersion portion of the treatments were those commonly used by the MNDNR to transport walleye fry. These containers consisted of collapsible, 19-L clear plastic water jugs with the caps modified by the addition of automotive tire-valve stems to facilitate inflation with oxygen. The fry were combined with 11.4 L of OTC so-

lution in the containers, then the containers were partially collapsed to expel the ambient air, capped, and inflated with oxygen. The fry remained in the containers for the duration of the immersion period and care was taken to reduce fry exposure to sunlight and changes in temperature during the entire process. Upon completion of the immersion process, the fry were either separated from the solution with the larval concentrator and placed into 19 L aquaria, placed into fine-mesh screen boxes floating in a 1700 L raceway, or stocked into one of the 4 ha drainable ponds at the Waterville State Fish Hatchery. Upon the advice of Molly Negus (MNDNR, personal communication), the procedure was modified during 2008 and 2009 with the addition of a 5 s freshwater rinse between immersion in the NaCl solution and immersion in the OTC solution.

Treatment episodes.—The OTC treatments of newly hatched walleye fry were evaluated with an integrated sampling design that utilized samples of walleyes from seven different treatment episodes across three years. The general approach of the design was to first evaluate the effects of various combinations of NaCl concentration, NaCl immersion duration, and OTC immersion durations on small batches of fish held under controlled hatchery conditions; then to further evaluate, under production-level conditions, the treatment combinations that produced clear marks with low attendant mortality.

Because of the small size of the fry at the time of treatment and the stenohaline nature of fresh-water obligate species such as walleye (Smith 1982), I had concerns about their tolerance to the NaCl solution. Consequently, the initial treatments were conducted at a much lower NaCl concentration than that reported to produce successful marks on anadromous species such as european eels (Alcobendas et al. 1991), atlantic salmon (*Salmo salar*; Mohler 2003) Chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*O. mykiss*; Negus and Tureson 2004).

Two OTC treatment episodes were conducted during the first year of the study to evaluate mark efficacy and tolerance to immersion in 1% NaCl followed by 1 h immersion in 700 mg OTC/L. The OTC immersion period was set at 1 h because it represented a reasonable time pe-

riod for fry to be in-transit during trips from the hatchery to their final stocking destinations. The first OTC treatment episode was conducted on 26 April 2006 with NaCl immersion periods of 0, 10, 20, and 30 seconds. Approximately 500 walleyes at a time were treated at each of the NaCl immersion periods then subsequently immersed in the OTC solution for 1 hour. Three replicates were conducted at each NaCl immersion period. Upon completion of the immersion protocol, the treated fry from each of the replicates were transferred to aquaria. The treatment protocol from the first episode was repeated during the second OTC treatment episode, on 9 May 2006, except that the walleye fry were immersed in the NaCl solution for 0, 60, 120, and 180 seconds.

Oxytetracycline treatment episodes three and four were conducted in the second year of the study with a higher NaCl concentration and OTC immersion duration than OTC treatment episodes one and two. Approximately 500 fry were first treated on 7 May 2007 by immersion in 2.5% NaCl for 30 s followed by immersion in 700 mg/L OTC for 3 h. After the immersion process, the fry were distributed among 5 aquaria. Walleye fry subjected only to the OTC immersion portion of the process were also distributed among 5 aquaria to provide controls for evaluating the effects of NaCl immersion on mortality. The OTC treatment was then replicated twice with walleyes for stocking into rearing ponds at the Waterville State Fish Hatchery. Approximately 4,000 treated walleye fry were stocked into Pond 5, whereas Pond 4 received 2,000 fry. Pond 4 also received 2,000 untreated fry to serve as controls for growth and mortality evaluation. The OTC treatment used on 7 May 2007 was then replicated on 14 May 2007 with another 500 fry. Upon completion of the treatment, the fry were placed into a fine-mesh floating screen box.

The sixth OTC treatment episode replicated the treatment protocol from 2007 except for the addition of a freshwater rinse between the NaCl immersion and the OTC immersion. Approximately 65,000 fry were treated on 2 May 2008, by immersion in 2.5% NaCl for 30 s followed by a 5 s freshwater rinse and immersion in 700 mg/L OTC for 3 h; then stocked into Pond 3.

Two years of successful fry marking with low attendant mortality at 2.5% NaCl concentration prompted renewed optimism for marking walleyes with a 1 h OTC immersion period. Consequently, a series of trials was conducted during 2009 to first observe the fry's initial reactions to elevated NaCl exposure, then to compare the mark efficacy at the elevated NaCl exposure to the protocol that produced successful marks during 2006 and 2007. The seventh OTC treatment episode was conducted on 4 May 2009 with a 1 h OTC immersion period preceded by immersion in NaCl concentrations of 2.5, 3.5 or 4.5 percent for 30, 45, 60, or 90 seconds followed a freshwater rinse. Approximately 500 fry were treated at each combination of NaCl concentration and immersion period. Based on the condition of the fry observed after the 1 h OTC immersion period, another OTC treatment episode was conducted later that day with fry for stocking into rearing ponds. Approximately 65,000 fry were first treated by immersion for 90 s in 4.5% NaCl followed by a freshwater rinse and immersion for 1 hour in 700 mg/L OTC; these fish were stocked into Pond 4. Pond 2, conversely, received 65,000 fry treated with 2.5% NaCl for 30 s and a 3 h OTC immersion period to serve as controls.

Treatment effects on survival and growth.—My initial concerns about the effects of the osmotic induction procedure on the survival of walleye fry was evaluated by comparing the 24 h mortality levels of walleyes immersed in NaCl for various durations during treatment episodes one and two. Three replicate treatments were conducted at each NaCl duration level during the treatment episodes and the fry from each of the replicates were randomly assigned to one of twelve aquaria. The aquaria were placed in a raceway, and a constant temperature was maintained by bathing the aquaria in flowing water. Continuous aeration was also provided by releasing compressed air through stone diffusers in each of the aquaria. Visual estimates of the number of dead fry were conducted after 24 h by first counting 20 dead fry in each of the aquaria and then estimating how many multiples of 20 dead fry were present in each aquarium. Percent mortality was then determined by dividing the estimates of dead fry in each of the aquaria by the volumetrically-

estimated total number of fry stocked into the aquaria after treatment. Surviving fry were retained in the aquaria for an additional 9 d to allow for fluorescent mark formation on the otoliths. Visual estimates of 24 h mortality were also conducted for fry samples held in the hatchery after treatment episodes three, and four. Differences in mortality between walleyes treated at different NaCl immersion durations were determined through chi-square analysis.

Further evaluation of the effect of the entire treatment process on walleyes was evaluated by comparing growth and survival between the treated and untreated walleyes that were stocked into Pond 4 during 2007. The fry stocked into Pond 4 were measured volumetrically to maximize parity in numbers between treated and untreated fry. After 5 months, the pond was harvested and 100 fingerlings were collected. The total length of each walleye was measured to the nearest millimeter, and the otoliths were examined for the presence of a mark. Differences in mortality between the treated and untreated walleyes were determined through chi-square comparison of the ratio of marked fingerlings in the sample to the ratio of treated fry at stocking. Growth was analyzed by *t*-test comparison of the total length between the marked and unmarked groups.

Treatment efficacy.—Inspection of walleyes of various life stages for the presence of an OTC mark was conducted following the methods of Secor et al. (1991) and Brooks et al. (1994). The sagittal otoliths were first removed from the specimens and wiped dry. The otoliths were then prepared by securing them to a glass microscope slide with cyanoacrylate cement and polishing them with 600, 1,200, or 2,000 grit sandpaper until the inner growth rings became visible under 100 X magnification with transmitted light. Inspection for a mark was then conducted in a room with reduced lighting, under an epifluorescent microscope with fluorescent lighting and filter blocks designed to optimize tetracycline fluorescence (Bumgardner 1991; Brooks et al. 1994). The specific system employed was a Nikon Eclipse E-400 microscope with B-3A filter cube (505 nm dichroic mirror, 420-490 nm exciter filter, and 520 nm barrier filter), 10 X and 20 X objectives, and a 100 W mercury UV light source.

The intensity of the mark observed under 200 X was categorized using a rating system similar to that described by Weber and Ridgway (1967) where: absent = no mark evident, faint = the mark is present but not clearly visible, clear = the mark is readily visible but not vivid, and intense = the mark is both readily visible and vivid.

Oxytetracycline marks do not become visible on the otoliths of newly hatched fry until several days after treatment. Logsdon et al. (2004) observed that faint fluorescent marks were visible on 30% of their inspected sample 3 days post-treatment but that clear or intense marks did not become visible on the entire sample until 9 days post-treatment. Consequently, I evaluated initial mark formation on samples of fry from treatment episodes 1, 2, 3, and 4 only after they had been held in the hatchery for 10 days post-treatment. Further evaluation of mark efficacy/retention was conducted by inspecting samples of walleyes harvested from rearing ponds between 53 and 145 days post-treatment.

Calcein

Treatment.—Walleye “frylings” (42-52 d post-hatch) were treated with calcein in accordance with Option B under the study protocol for INAD 10-987 (USFWS 2003); where the fish were first immersed in a NaCl solution then immersed in a calcein solution. The treatments began by mixing 4 L of 0.5% calcein solution in a 19 L plastic bucket using SE-MARK (Western Chemical, Ferndale, WA) concentrated calcein. Four liters of either 2.5% or 5.0% NaCl solution was mixed in another 19 L bucket using Hi-Grade evaporated salt (Cargill, Minneapolis, MN) and another 19 L bucket was filled with approximately 4 L of fresh hatchery water. Oxygen was then bubbled into the solutions through circular diffuser hoses.

The frylings were treated in batches of 35 to 100 fish. Each batch was first netted from a holding tank, allowed to drain until the water ceased flowing from the net in a constant stream, and then poured into a perforated 7.6 L plastic bucket that had been lowered into the NaCl solution. The fish remained in the NaCl solution until the end of the prescribed immersion period and then the perforated bucket was removed from the NaCl solution, the solution allowed to

drain away from the fish, then the bucket of fish transferred to the calcein solution. After the prescribed immersion period, the calcein was rinsed off the fish either with freshwater from a low pressure sprayer (Jerre Mohler, USFWS, personal communication) or by dipping again in a bucket of freshwater. Upon completion of the immersion process, the frylings were either placed in 19 L aquaria or transferred to a 473 L hauling tank and subsequently stocked into one of the 4 ha drainable ponds at the Waterville State Fish Hatchery. Upon the advice of Molly Negus (MNDNR, personal communication), the procedure was modified during 2008 and 2009 with the addition of a 5 s immersion in freshwater between the NaCl immersion and the calcein immersion.

Treatment episodes.—The sampling design for evaluating calcein treatments on frylings utilized samples of walleyes from five treatment episodes across three years. The approach mirrored that for the OTC evaluation in that the initial treatments were conducted with small batches of fish under controlled hatchery conditions, then expanded to be more representative of production-level conditions.

Two calcein treatment episodes were conducted during the first year of the study to evaluate mark efficacy and tolerance to immersion in 0.5% calcein for 4 min preceded by immersion in 2.5% NaCl. The first calcein treatment episode was conducted on 8 June 2006 with NaCl immersion periods of 0, 2, 3, and 4 minutes. Single scoops of between 37 and 87 frylings were treated at a time and three replicate treatments were conducted at each NaCl immersion period. Upon completion of the immersion protocol, the treated frylings from each of the replicates were rinsed with freshwater from a low pressure sprayer then transferred to aquaria. The 4 min NaCl immersion protocol from the first episode was replicated with approximately 3,600 frylings on 13 June 2006 and those fish stocked into Pond 3.

A single calcein treatment episode was conducted during 2007. On 7 June 2007, approximately 3,500 frylings were treated by immersion for 4 min in 2.5% calcein followed by a 4 min immersion in 0.5% calcein. This was a replicate of treatment episode two except that the final freshwater rinse was conducted by dip-

ping the fish in a bucket of freshwater instead of spraying them with a low pressure sprayer. Fish treated during calcein treatment episode three were stocked into Pond 9.

The remainder of the calcein treatments were conducted with both a higher NaCl concentration than the previous treatment episodes and a 5 s freshwater rinse between the NaCl immersion and the OTC immersion. Approximately 3,500 frylings were treated on 20 June 2008 (episode four) by immersion in 5.0% NaCl followed by a freshwater rinse and immersion in 0.5% calcein for 4 min. Upon completion of the calcein immersion, the frylings were rinsed with a 5 s dip in freshwater then transported to Pond 10 for stocking. Due to high mortality observed prior to stocking and the inability to later sample fish from Pond 10, another 1,444 frylings were treated on 11 July 2008 and stocked into Pond 10. This treatment episode (five) followed the same protocol as calcein treatment episode four except that the frylings were allowed to recover for 1 h in a hauling tank with oxygen saturated water prior to transportation and stocking into Pond 10. The protocol from calcein treatment episode five was replicated with 7,550 frylings on 26 June 2009 and the fish stocked into Pond 2.

Treatment effects on survival.—The effects of the osmotic induction procedure on acute mortality of walleye frylings was formally evaluated during the first year of the study. Three replicate treatments were conducted at each NaCl duration level during treatment episode one and the frylings from each of the replicates were randomly assigned to one of twelve aquaria outfitted with perforated covers and continuous flow-through hatchery water. The number of dead walleyes were enumerated after 24 hours and the fish were retained in the aquaria for another 6 days to allow for fluorescent mark formation on the otoliths. Differences in mortality between walleyes treated at different NaCl immersion durations were determined through chi-square analysis.

Treatment efficacy and mark retention.—All walleyes inspected for the presence of calcein marks were inspected following the study protocol for INAD 10-987 (USFWS 2003) using a SE-MARK handheld calcein detector (Western Chemical, Ferndale, WA). The wall-

eyes inspected immediately after treatment were inspected live, whereas those inspected later were sacrificed prior to inspection. Inspection was conducted in a dark, windowless room with only enough ambient light to locate equipment and fish in pails. Each fish was inspected at a distance of 1–2 inches and the intensity of the of the marks on the pectoral fin rays, pelvic fin rays, operculum, ventral surface of the jaw, and scales along the lateral surface for each fish were either rated using the same category system used for OTC marks (Weber and Ridgway 1967) or quantified by comparing them to calcein marks on a standardized colorimetric key (Honeyfield et al. 2008). A fresh colorimetric key, when used, was prepared within 6 h prior to examination of each sample of fish by serially diluting the stock calcein solution with distilled water and brushing a single stripe of each concentration onto a sheet of printer paper (sheets from same package used for all keys). The calcein concentration of the stock 10,000 mg calcein/L SE-MARK solution was first decreased to 1,000 mg calcein/L and then to 500 mg/L. After which, concentrations were reduced by 100 mg/l for each decremental concentration between 500 and 100 mg/L, by 5 mg/L for each concentration between 100 and 20 mg/L, and by 1 mg/L for each concentration between 20 mg/L and 1 mg/L. The key was then allowed to air dry in the dark and then laminated with clear plastic to keep it dry during use. Sagittal otoliths and scale samples from the ventral surface between the pelvic fins were also collected from a subsample of externally inspected walleyes and viewed under the same epi-fluorescent microscope used to inspect for OTC marks. The otoliths were processed using the same procedure as those inspected for OTC marks, whereas the scales were viewed under the fluorescent lighting without further preparation.

During the first year of the study, frylings were only inspected for calcein marks after being later re-sampled. A sample of fish treated during episode one was inspected after being held in an aquarium for 7 d and a sample of fish treated during episode two was inspected upon harvest of Pond 3 at 117 d post-treatment. Samples of fish from all subsequent treatments were inspected both immediately after treatment and when later harvested from the ponds.

The inability to detect external marks on walleyes harvested from the ponds during 2006 and 2007 raised doubt about the persistence of calcein marks. Consequently, the sampling regime and mark rating system were modified during 2008 to assess the rate and extent of mark degradation. Samples of walleyes from treatment episode five were collected weekly from Pond 10 and the intensity of the marks on the pectoral fin rays, pelvic fin rays, operculum, ventral surface of the jaw, and scales along the lateral surface of each fish was quantified by comparing the intensity of the observed marks to a colorimetric key. A sample of 100 walleyes was collected when the pond was harvested at 15 weeks post-treatment. Ten walleyes were inspected immediately with the SE-MARK detector, whereas the remaining walleyes were frozen for later inspection of the otoliths under an epi-fluorescent microscope. In addition to 5 body structures that were quantitatively examined during the previous inspections; a qualitative examination of all the external surfaces of the fish as well as the inside of the mouth and gills, was also conducted with the SE-MARK detector to identify the body structures with the most persistent marks.

Samples of at least 100 scales were also collected from the ventral surfaces of each of the 10 walleyes that were immediately inspected after harvest of Pond 10 and the scales stored in various media to evaluate the potential for delayed inspection of the mark using epi-fluorescent microscopy. Five different media were used to store the scales: 1) mineral oil, 2) glycerin, 3) 70% isopropyl alcohol, 4) distilled water, and 5) dry in paper coin envelopes. Samples of scales from each of the media were stored either in a freezer or in a closet protected from exposure to the light. Ten scales from each of the fish were stored in each of the resulting 10 storage combinations for 2 weeks and then inspected with the same epi-fluorescent microscope used to inspect for OTC marks. The intensity of the marks on the scales was qualitatively rated using the same category system used for OTC marks (Weber and Ridgway 1967). Differences in mark intensity between frozen and non-frozen scales were evaluated with the Mann-Whitney rank sum test, whereas differences in mark quality among scales stored in

different media within the frozen and non-frozen groups was evaluated with Kruskal-Wallis rank sum test followed by post hoc pairwise comparisons using the Tukey test. Otoliths were also inspected from a sample of 20 walleyes collected from Pond 10 using the above microscope and rating system.

Results

Oxytetracycline

Treatment effects on survival and growth.—The walleye fry appeared stressed (lethargic, pale and congregating near the surface) when immersed in the NaCl solution but became motile, well pigmented, and well distributed in the transport jugs following immersion in the OTC solution. Less than 1% mortality was observed immediately following treatment. Mortality after 24 h, however, ranged from 0% to 50% among individual aquaria. The highest mortality occurred following the first treatment episode; during which I had the least experience with the marking process and kept the fry out of the water the longest during transfer between the tanks, the NaCl bath, and the transport jugs.

Treatment episode one compared 1 h immersion in OTC preceded by immersion in 1% NaCl for 0, 10, 20, or 30 s. The resulting mean 24 h mortality ranged from 8.3% for those immersed for 10 s to 23.3% for those immersed for 30 s (Table 1). Chi-square testing indicated significant differences in mean mortality among the immersion periods ($P < 0.001$). Mortality did not appear to be dependent upon NaCl immersion duration, however, since fry not subjected to the

NaCl pre-treatment actually suffered higher mean mortality than all but the 30 s immersion group. In contrast, mortality during the 24 h following treatment episode two ranged from only 0% to 5% among aquaria; with those not pre-treated in NaCl suffering the lowest mortality (Table 1). Chi-square test results indicated significant differences in mortality among immersion periods ($P = 0.016$)

Mortality evaluations during 2007, however, failed to identify significantly higher ($\alpha = 0.05$) mortality of walleyes pre-treated in the NaCl solution even though the NaCl concentration was raised to 2.5%. Pre-treated fry from treatment episode three averaged 3.8% mortality after 24 h but those subjected to only OTC immersion averaged 4.8% mortality (Table 2). Similar results were observed for fry treated during treatment episode four. Fry held in floating baskets after treatment episode four suffered 4.6% mortality during the first 24 h whereas those subjected to only the OTC immersion suffered 5.0% mortality (Table 2). Treated walleyes were also recovered from Pond 3 in the same proportion (50:50) that they were stocked 145 d prior ($\chi^2 = 0.0200$, $P = 0.888$); indicating similar survival between those subjected to the osmotic induction/OTC immersion process and those not treated at all. Further mortality testing was truncated due to space limitations in the hatchery but no mortalities were observed within 1 h after any of the various combinations of NaCl concentration and immersion periods during treatment episode 6. Less than 1% mortality was observed during treatment episode 7.

Table 1. Comparison (Pearson chi-square) of walleye mortality 24 hours after being treated as newly hatched fry by immersion for various durations in 1% NaCl solution followed immediately by 1 hour immersion in 700 mg/L OTC solution. Approximately 1500 fry were treated at each NaCl immersion duration.

| OTC treatment episode | Mortality (%) by NaCl immersion duration | | | | χ^2 | P |
|-----------------------|--|-------------------|--------------------|--------------------|----------|--------|
| | <u>0 seconds</u> | <u>10 seconds</u> | <u>20 seconds</u> | <u>30 seconds</u> | | |
| 1 | 16.7 | 8.3 | 11.7 | 23.3 | 50.29 | <0.001 |
| 2 | <u>0 seconds</u> | <u>60 seconds</u> | <u>120 seconds</u> | <u>360 seconds</u> | | |
| | 1.3 | 5.0 | 4.0 | 4.0 | 10.26 | 0.016 |

Table 2. Comparison (Pearson chi-square) of mean total mortality of walleyes 24 hours after being treated as newly hatched fry by immersion for 0, or 30 seconds in 2.5% NaCl solution followed immediately by 1 hour immersion in 700 mg/L OTC solution. Approximately 1500 fry were treated at each NaCl immersion duration.

| OTC | | | | |
|-------------------|--|------------|----------|-------|
| treatment episode | Mortality (%) by NaCl immersion duration | | χ^2 | P |
| | 0 seconds | 30 seconds | | |
| 3 | 4.8 | 3.8 | 0.389 | 0.533 |
| 4 | 5.0 | 4.6 | 0.022 | 0.882 |

No apparent effect of the osmotic induction/OTC immersion process on the growth of walleyes was observed during this study. Mean total length of the treated walleyes harvested from Pond 3 in 2007 was 138.1, whereas the mean length of the untreated controls was 140.1 ($t=0.318$, $P=0.751$).

Treatment efficacy and mark retention.—One hour immersion in 700 mg OTC/L preceded by immersion in 1% NaCl failed to produce suitable marks on the walleye fry treated in 2006. Although many of the inspected fish possessed discernible marks on their otoliths, all the samples included many marks that were faint and difficult to detect. All the samples except for those immersed in NaCl for 20 s also included fish without marks (Table 3).

Increasing the OTC immersion period to 3 h and the NaCl concentration to 2.5% (30 s NaCl immersion duration) in 2007 succeeded in increasing the quality of marks produced through osmotic induction. All of the walleyes inspected 10 days following OTC treatment episodes three and four possessed intense fluorescent marks, whereas 98% of the sample of walleyes from treatment episode three also possessed either clear or intense marks when inspected 135 d later. Replication of the 2007 treatment regimen in 2008, albeit with the addition of a freshwater rinse between the NaCl and OTC immersions, also produced easily detectable marks on all the walleyes inspected 53 d post-treatment.

Increasing the NaCl concentration to 4.5% in 2009, however, failed to produce suitable marks on walleyes when followed up with only a 1 h immersion in the OTC solution. Of the 50 walleyes inspected 45 d post-treatment, 86% of the marks were either absent or difficult to detect. Surprisingly, replication of the treatment regime successfully used in 2007 and 2008 also

failed to produce suitable marks on the walleyes in 2009. As many as seventy-six percent of the marks were either absent or difficult to detect.

Calcein

Treatment effects on survival.—The frylings first swam erratically, then lost equilibrium and floated to the surface when first immersed in the NaCl solution. During the first calcein treatment episode, all of the treated walleyes retained vigor after being transferred to the aquaria. Only two walleyes died within the first 24 hours, and one of those was a fish from the control group that was not immersed in the NaCl solution (Table 4). Chi-square testing failed to indicate a significant difference in mortality among the NaCl immersion durations ($P=0.596$).

All subsequent treatments were conducted under more production-level conditions; with many more fish treated, more sequential dips in the same solutions, and more crowded conditions preceding and immediately following treatments. The appearance of fish after transfer to freshwater differed among treatments but, in all cases, the subsequent abundance of walleyes harvested from ponds stocked with calcein-treated frylings was lower than expected by the hatchery manager (Bruce Pittman, MNDNR, personal communication). All the walleyes treated during episode three retained vigor after transfer to freshwater but many of the walleyes treated during episodes two, four, five, and six remained pale and lethargic. Only seven walleyes treated during episode two were recovered during harvest of Pond 3, no feeding activity was observed or walleyes sampled from Pond 10 following treatment episode four, and no walleyes treated during episode six were recovered during harvest of Pond 2.

Table 3. Quality of fluorescent marks observed on the otoliths of walleyes treated as newly hatched fry at various combinations of NaCl concentration, NaCl immersion duration and OTC immersion durations. The numbers represent the percentages of walleyes that were assigned to each mark quality category based on the visibility of the mark under 200X magnification.

| Treatment | | | | Inspection | | | | | | | |
|-----------------------|------------------------|-----------------------------|----------------------------|------------|---------|--------------|----|--------|-------|-------|---------|
| OTC treatment episode | NaCl concentration (%) | NaCl immersion duration (s) | OTC immersion duration (h) | n | Age (d) | Mark quality | | | | | |
| | | | | | | | | Absent | Faint | Clear | Intense |
| 2006 | | | | | | | | | | | |
| 1 | 1.0 | 0 | 1 | 20 | 10 | 10 | 55 | 20 | 5 | | |
| 1 | 1.0 | 10 | 1 | 20 | 10 | 30 | 60 | 10 | 0 | | |
| 1 | 1.0 | 20 | 1 | 20 | 10 | 0 | 45 | 40 | 15 | | |
| 1 | 1.0 | 30 | 1 | 20 | 10 | 20 | 60 | 15 | 5 | | |
| 2 | 1.0 | 0 | 1 | 20 | 10 | 15 | 65 | 10 | 10 | | |
| 2 | 1.0 | 60 | 1 | 20 | 10 | 20 | 75 | 5 | 0 | | |
| 2 | 1.0 | 120 | 1 | 20 | 10 | 50 | 45 | 5 | 0 | | |
| 2 | 1.0 | 180 | 1 | 20 | 10 | 30 | 60 | 10 | 0 | | |
| 2007 | | | | | | | | | | | |
| 3 | 2.5 | 30 | 3 | 12 | 10 | 0 | 0 | 0 | 100 | | |
| 3 | 2.5 | 30 | 3 | 100 | 145 | 0 | 2 | 52 | 46 | | |
| 4 | 2.5 | 30 | 3 | 10 | 10 | 0 | 0 | 0 | 100 | | |
| 2008 | | | | | | | | | | | |
| 5 | 2.5 | 30 | 3 | 100 | 53 | 0 | 0 | 48 | 52 | | |
| 2009 | | | | | | | | | | | |
| 7 | 2.5 | 30 | 3 | 50 | 43 | 6 | 70 | 20 | 4 | | |
| 7 | 4.5 | 90 | 1 | 50 | 45 | 14 | 72 | 12 | 2 | | |

Table 4. Comparison (Pearson chi-square) of walleye mortality 24 hours after being treated as 42-day post-hatch frylings by immersion for various durations in 2.5% NaCl solution followed immediately by 4 minute immersion in 0.5% calcein solution. One hundred sixty five walleyes were treated at each NaCl immersion duration.

| OTC treatment episode | Mortality (%) by NaCl immersion duration | | | | χ^2 | P |
|-----------------------------|--|-----------|-----------|-----------|----------|-------|
| | 0 minutes | 2 minutes | 3 minutes | 4 minutes | | |
| 1 | 0.68 | 0.0 | 0.0 | 0.38 | 1.889 | 0.596 |

Treatment efficacy and mark retention.—All of the calcein treatments successfully produced fluorescent marks on the treated frylings. The entire bodies of treated frylings possessed a distinctive green glow that was visible to the unaided eye immediately following treatment and all of the frylings inspected with the SE-MARK detector within a week of treatment possessed either clear or intense fluorescent green marks on the outer surfaces of their pectoral fin rays, pelvic fin rays, operculum, jaw, and scales along their lateral surfaces.

The frylings treated by pre-immersion in 2.5% NaCl for 4 min, however, failed to exhibit visible marks on their external surfaces when inspected again at 117 or 119 days post treatment. This apparent lack of mark retention prompted the increase in NaCl concentration to 5% in 2008 in hopes of producing longer lasting marks. Weekly comparison of mark intensity to the colorimetric key was also conducted during 2008 to document the rate and extent of mark degradation.

Frylings treated during 2008 exhibited distinct differences in initial mark intensity among the structures examined. Mean mark intensity on the pelvic fin rays, jaw, and operculum ranged from 620 to 730 ppm calcein (relative to the colorimetric key), whereas the mean mark intensity of the pectoral fin rays, head, and lateral scales ranged between 140 and 220 ppm calcein (Figure 2). Mark intensities decreased by at least 50% during the first week and continued decreasing exponentially through Week 15. The dichotomous grouping of the mark intensities among structures continued to some extent throughout the study. By week 12, many of the inspected walleye failed to possess discernible marks on pectoral fin rays or head, but at least

faint marks were visible on the pelvic fin rays, operculum, jaw, and lateral scales throughout the 15 weeks of the project. Whole body inspections conducted at Week 15 also documented that marks persisted on the upper roof of the mouth, the gill arches, and the scales between the pelvic insertions (Figure 3).

All of the walleyes whose scales were sampled at Week 15 had visible marks on at least some of the scales from each of the storage media, but not all scales examined for each media had discernible marks (Figure 4). A higher percentage of the scales stored in frozen media possessed visible marks than those that were kept at room temperature. In addition, Mann-Whitney testing indicated that the marks of the frozen scales were generally brighter than their non-frozen counterparts ($T=199956.5$, $P<0.001$). Kruskal-Wallis tests indicated significant differences in mark quality among storage media for both the frozen scales ($H=51.9$, $P<0.001$) and the non-frozen scales ($H=122.5$, $P<0.001$). Scales stored in alcohol possessed the highest percentage of marks, whether frozen or not, but post hoc Tukey tests failed to detect a significant difference ($P<0.05$) between the quality of marks on scales stored in alcohol and those simply stored dry in a coin envelopes (Figure 4).

All of the otoliths from walleyes treated during 2008 also showed discernible marks when viewed under the epi-fluorescent microscope. The calcein marks on the otoliths differed in shape from that of the OTC marks on otoliths in that the entire center section of the otolith fluoresced; whereas the OTC marks appeared as a fluorescent band associated with the daily growth ring that was being formed at the time of treatment (Figure 5). Many of the

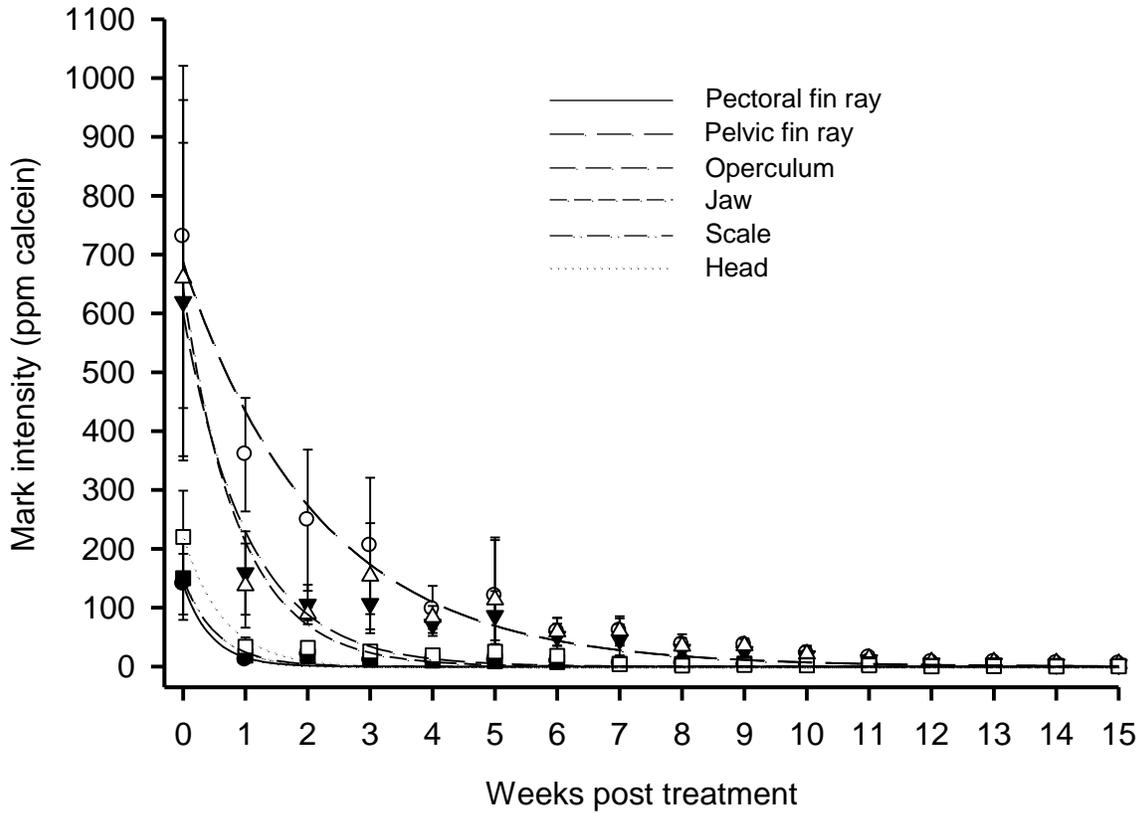


Figure 2. Mean (\pm SD) mark intensity (y) across time (x) of various anatomical structures of walleyes treated by immersion in 2.5% NaCl solution followed by a 5 second fresh-water rinse and immersion in 2.5% calcein solution for 4 minutes (osmotic induction) then held in Pond 10. Trend lines represent the exponential regression equations best fitting the mean observed mark intensities of each structure: pectoral fin ray ($y=139.75e^{(-2.21x)}$, $r^2=0.95$), pelvic fin ray ($y=687.53e^{(-0.46x)}$, $r^2=0.97$), operculum ($y=601.55e^{(-0.96x)}$, $r^2=0.92$), jaw ($y=643.47e^{(-1.11x)}$, $r^2=0.85$), scale ($y = 149.53e^{(-1.82x)}$, $r^2=0.96$), and head ($y=218.35e^{(-1.53x)}$, $r^2=0.94$).

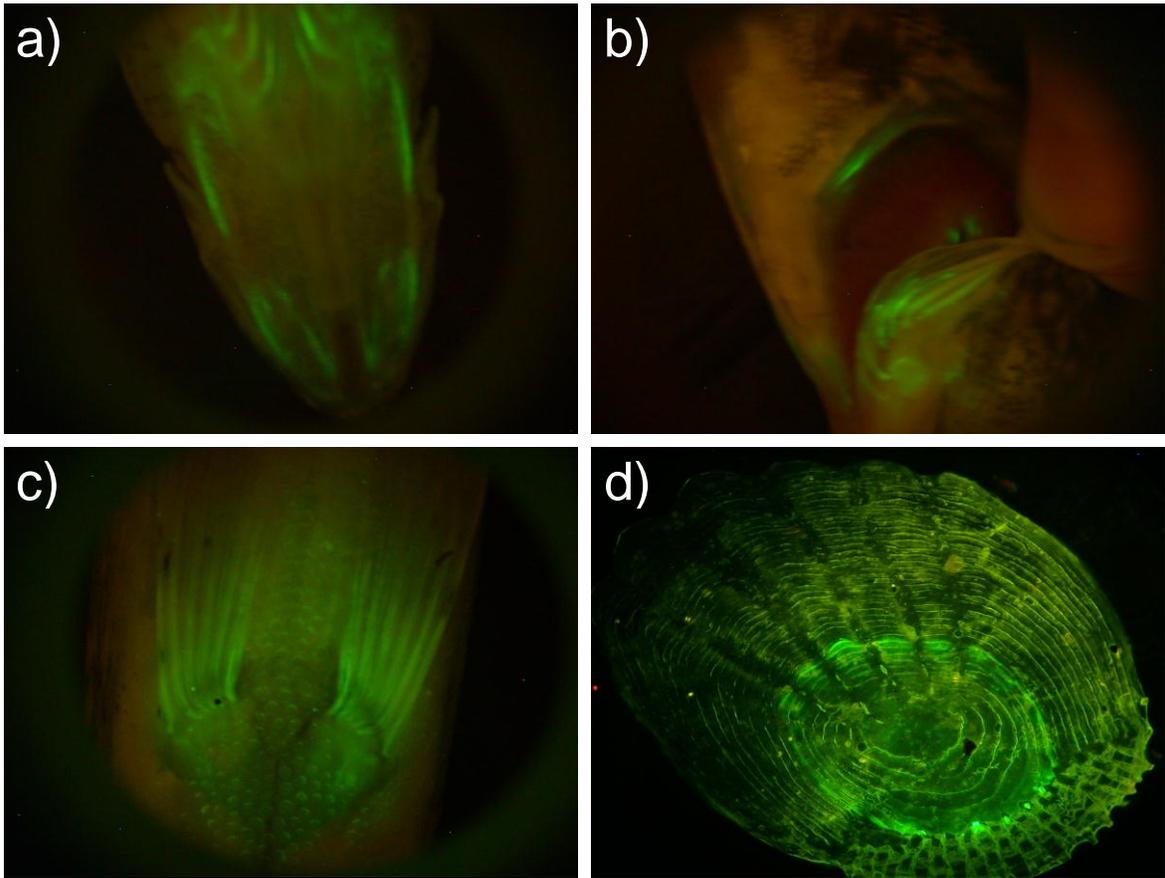


Figure 3. Calcein marks on walleyes 4 months after treatment by immersion for 4 minutes in a 5% saline bath followed by immersion for 4 minutes in a 0.5% calcein bath. Photograph (a) shows the ventral surface of the jaw, (b) a lateral view of the gill area with operculum lifted up, (c) the pelvic area of the ventral surface, and (d) 100X magnification of a scale from the pelvic area of the ventral surface.

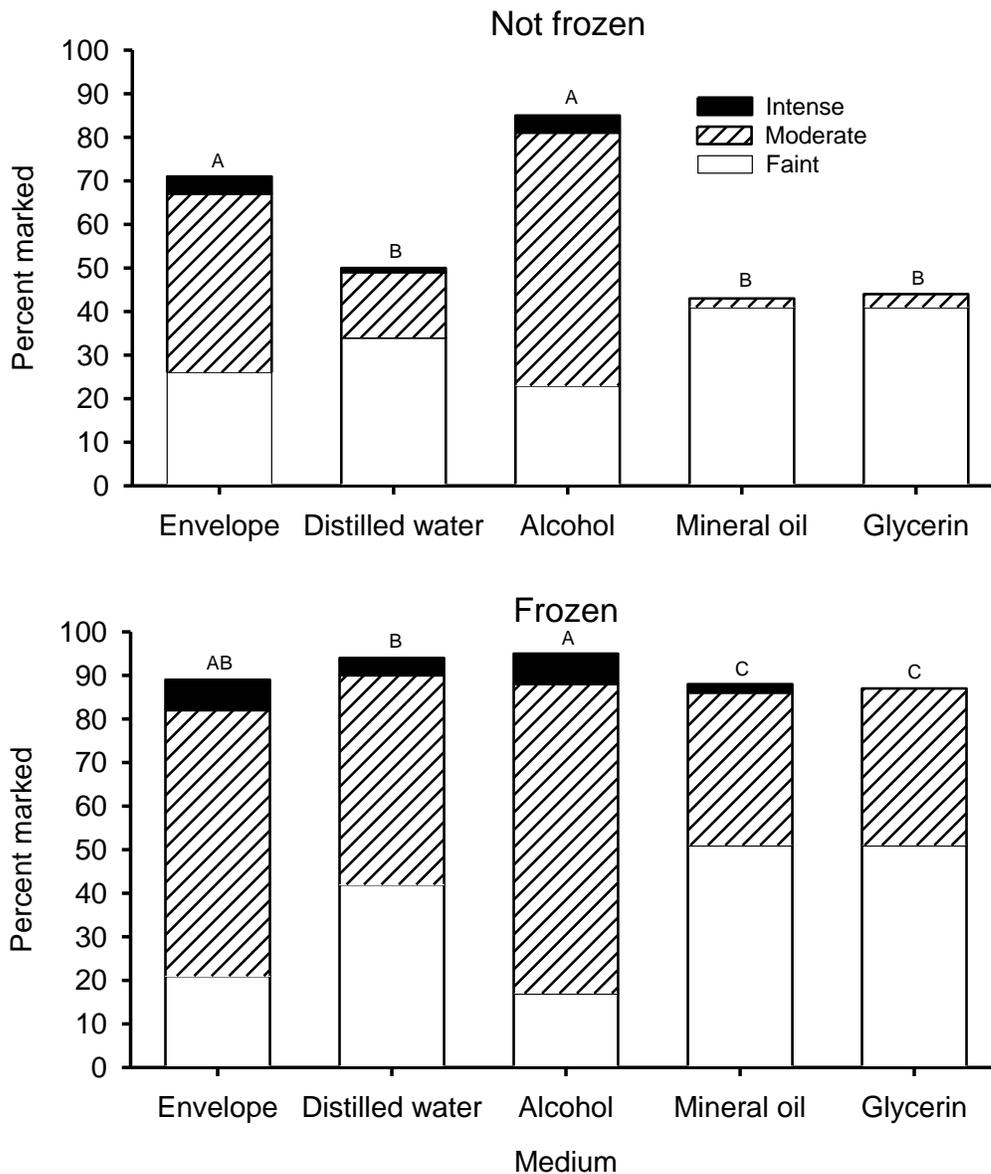


Figure 4. Quality of osmotic induced fluorescent marks observed on scales after removal from the walleyes at 15 weeks post-mark and stored either frozen or unfrozen in 5 different media for 2 weeks. Percent marked represents the percentages of scales that were assigned to each mark quality category based on the visibility of the mark under an epi-fluorescent microscope at 100X. A total of 100 scales, 10 from each of 10 fish, were inspected from each medium. Values with the same letter are not significantly ($P < 0.05$) different within each storage medium (Tukey test).

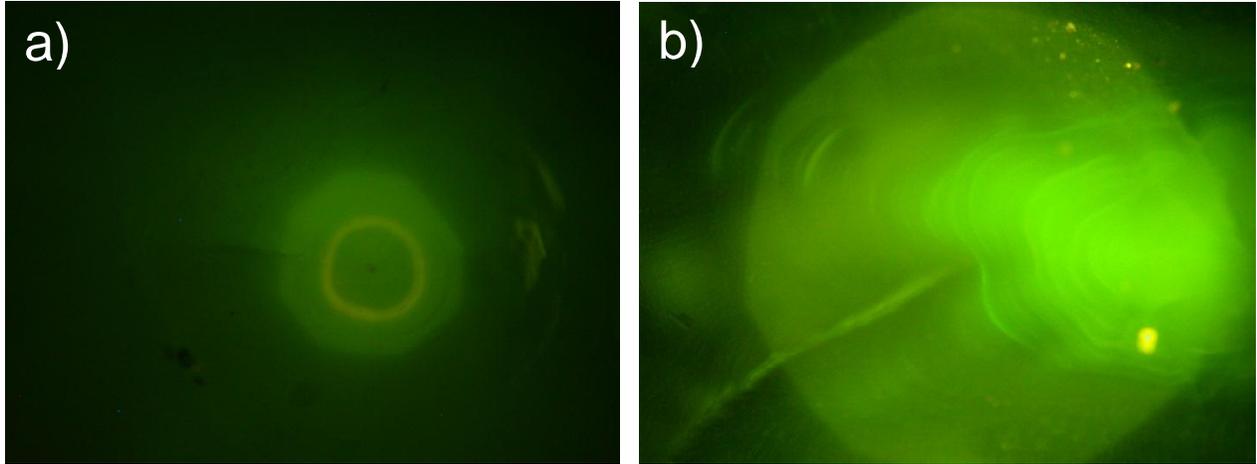


Figure 5. Fluorescent marks on the otoliths of walleye fingerlings after harvest from rearing ponds in the October, 2008. Photograph (a) shows the yellow-gold ring of a walleye marked as a newly hatched fry with OTC, whereas (b) is an off-centered photograph showing apple-green stained center of an otolith of a walleye treated with calcein as a fryling. Both photographs were taken at 200 X through an epifluorescent microscope.

calcein marks were visible under 100 X magnification without any type of processing of the otoliths. Removing some of the overlying material through polishing with fine sandpaper, however, enhanced the visibility of the mark and often revealed a brighter ring on the edge of the mark. The enhanced marks of the polished otoliths had similar appearance under the microscope as those on the scales except that the edges of the marks on the scales were much brighter.

Discussion

This study demonstrated that immersion of walleyes in a concentrated NaCl solution followed by immersion in either OTC or calcein can produce fluorescent marks at shorter immersion periods than those typically used to mark fish by immersion in OTC or calcein alone. However, this study also demonstrated that the tolerance of the walleyes to the treatment process, the intensity of the marks, and the retention of the marks can be inconsistent.

The utility of a marking method can be negated if the marking process adversely affects the survival or growth of the fish being marked.

Researchers have generally reported little mortality associated with immersion marking of walleye fry in OTC solutions (Younk and Cook 1991; Peterson and Carline 1996), but the effects of OTC immersion preceded by immersion in a concentrated NaCl solution has not been evaluated. Walleye fry treated during the current study obviously became stressed when immersed in the NaCl solution but appeared to recover almost immediately upon transfer to the OTC solution. The high mortality that followed the first treatment episode was likely exacerbated by stress caused by the additional handling that accompanied my learning how to efficiently conduct the osmotic induction process. Mortality varied among treatments, but not consistently with NaCl immersion durations. Subsequent treatment episodes also failed to result in higher mortality of the NaCl immersed walleyes even though they were conducted at a higher NaCl concentration. The return of marked walleyes from Pond 3 in the same ratio that they were stocked and the similarity in total length between the two groups also indicates that the walleye fry tolerate the osmotic/OTC marking procedure conducted under production level conditions at the Waterville State Fish Hatchery.

The ability of the osmotic/OTC procedure to consistently produce detectable marks on walleye fry, however, is much less conclusive. The groups of fry treated with 1 h immersion in OTC, whether they were pre-treated in 1% NaCl or 4.5% NaCl, both had high percentages of individuals with absent or very faint marks. It is not uncommon to have variability in the quality of marks produced by treating walleye fry by immersion in OTC (Brooks et al. 1994; Lucchesi 2002; Logsdon et al. 2004), but the difficulty I experienced detecting marks on such a high percentage of the walleyes would render this technique useless in stocking evaluations because poorly marked fish could be mistaken for naturally reproduced fish.

Increasing the concentrations of NaCl to 2.5% and the OTC immersion period to 3 h increased the quality of the resultant marks. I only had difficulty detecting the marks on two of the 212 walleyes inspected from three treatment episodes across 2 years. Given the exacting nature of the sanding necessary to expose the mark (Logsdon et al. 2004) it is possible that the two difficulties I had observing marks was simply due to irregularities in the preparation of the otoliths.

Pre-immersing the fry in 2.5% for 30 s seemed to be a valid method for reducing the OTC immersion period by half of the 6 h commonly used (Brooks et al. 1994; Fielder 2002; Lucchesi 2002; Logsdon et al. 2004) for marking walleye fry. Perplexingly, replication of the procedure (30 s immersion in 2.5% NaCl followed by 3 h immersion in 700 mg OTC/L) during 2009 left over 75% of the treated walleyes with absent or difficult to detect marks. It is unclear why the treatment failed during 2009 but the appearance (Figure 6) and behavior of the Terramycin 343 that was used as the source of OTC in 2009 differed from that in the past. The powder was coarser and darker in color than the older Terramycin 343. It immediately sank instead of floated and required more sodium phosphate dibasic to raise the pH to 6.8 than previously used. An unusual amount of powdery precipitate was also observed in the bottom of the fry jugs after the end of the immersion period. Its appearance and reaction in the water was more similar to Oxymarine (Alpharma, Bridgewater, New Jersey); a formulation of OTC pow-

der that produced poor mark efficacy when previously used to treat walleye fry by immersion in 700 mg OTC/L for 6 h (unpublished data). Unbeknownst at the time, Pfizer had ceased manufacture of Terramycin 343 and the batch that I used in 2009 was from the last lot that they produced. Whether the observed differences in appearance and behavior are indicative of a difference in the chemical makeup of the formulation and whether these possible changes could affect the efficacy of the treatment can only be speculated; but further evaluation of reduced OTC immersion periods using the osmotic induction procedure should be conducted with the newly FDA approved formulation marketed by PennField Animal Health (Omaha, Nebraska) under the Pennox 343 brand.

The SE-MARK calcein solution, conversely, appeared and reacted consistently across all the treatment episodes. The calcein treatments consistently produced easily-visible marks on many of the external tissues of the fish and the intensity of the marks on the otoliths appeared to increase when the NaCl concentration was increased from 2.5% to 5%. The tolerance of the frylings to the treatment process and the retention of the marks once produced were the issues of most concern regarding the osmotic/calcein treatment experiments. Similar to the osmotic/OTC treatments, the walleyes became obviously stressed when first immersed in the NaCl solution. Unlike the OTC treated fish, which recovered when transferred to the OTC solution, the calcein treated fish did not appear to recover until after they had completed the calcein immersion portion of the treatment and were transferred to freshwater. Similar observations were reported by Negus and Tureson (2004). They described the chinook salmon as simply appearing agitated during the immersion in salt and calcein but noted that some of the rainbow trout, similar to the walleyes treated during the current study, lost equilibrium during immersion in salt and calcein.

Overall, osmotic/calcein treatments have been reported to have no long lasting effects on the treated fish. Mohler (2003), Negus and Tureson (2004), and Hill and Quesada (2010) all reported no additional mortality or reduction in growth of salmonid fry treated by osmotic induction. Mohler (2003), and Negus and



Figure 6. Photograph showing the appearance of the Terramycin 343 used as a source of OTC during 2009 ("New" Terramycin 343) compared to the appearance of the Terramycin 343 used previously ("Old" Terramycin 343) and OxyMarine.

Tureson (2004) also treated fish that were closer in size to the frylings treated during the current study without indication of any increased mortality associated with the treatment. They treated relatively few fish, however, and did so under controlled conditions, thus their conditions were similar to those of mortality tests I conducted following treatment episode one.

Increasing the number of treated frylings to a level that would be useful for management purposes resulted in more crowded conditions preceding and immediately following treatments, more sequential dips in the same solutions, more time out of the water during the process, and higher apparent mortality. The most problematic part of the treatment process was rinsing the calcein from the fish after treatment. The restrictions of INAD Number 10-987 prevented the discharge of any of the calcein solution into the environment. This meant that any unused calcein leftover from the treatment and any of the rinse-water contaminated with calcein be collected in leak proof containers and subsequently shipped to Tacoma, Washington for proper disposal (USFWS 2003). This requirement was simple to comply with when treating the few fish during episode one but treatment of

the larger numbers of frylings in subsequent episodes had the potential to quickly generate more contaminated rinse-water than was practical to collect and ship. To reduce the amount of contaminated rinse-water produced, a low pressure sprayer was used to spray freshwater across the treated frylings until all the visible solution was removed from the fish (Jerry Mohler, USFWS, personal communication). The oily consistency of the solution, however, made the calcein adhere to the fish and slowed down the rate at which it drained from both the fish and the perforated buckets. This substantially increased the amount of time that the fish were out of the water. Abandoning the low pressure sprayer in favor of sequential dips in buckets of freshwater improved the process but still resulted in the fish being handled more, and out of the water for longer time periods, than I would have preferred.

The unhealthy appearance and apparently high mortality of many of the walleyes following treatment was likely exacerbated by elevated water temperatures. Brooks et al. (1994) reported higher mortality of walleye fry immersed in calcein than those immersed in either OTC or calcein blue when treated at 10°C and elevated mortality of all groups when treated

at 15°C. The walleyes treated with calcein during this study were all treated at water temperatures that exceeded 20°C.

Mohler (2003) and Negus and Tureson (2004) reported good retention of osmotically induced marks on fish held indoors. Subsequent studies by Bashey (2004), Honeyfield et al. (2008), Hill and Quesada (2010), and Honeyfield et al. (2011), however, confirmed the photolysis of the calcein marks does occur in fish exposed to sunlight. Hill and Quesada (2010) marked steelhead and chinook fry using a similar osmotic induction procedure as treatment episodes five and six in the current study (immersion for 3.5 min in 5% NaCl followed by immersion for 3.5 min in 0.5% calcein), whereas Bashey (2004) marked guppies *Poecilia reticulata* by immersion for 24 h in 250 mg calcein/L, and Honeyfield et al. (2008; 2011) marked either lake trout *Salvelinus namaycush* or shovelnose sturgeon *Scaphirhynchus platyrhynchus* through dietary supplementation. Hill and Quesada (2010) reported that the calcein marks had faded beyond recognition on all of the fish held in direct sunlight for 9 weeks and 23% of those held in indirect sunlight for 9 weeks. Walleyes pre-immersed in 2.5% NaCl during the current study also lost their marks by the time that they were re-inspected 17 weeks post-treatment. The marks on walleyes treated by pre-immersion in 5% NaCl (episode five) also began to fade quickly but marks were detected on at least some of the structures of all of the walleyes inspected at 15 weeks post-treatment. Although the marking procedure used by Hill and Quesada (2010) was very similar to that used during treatment episode five, the fish they marked were substantially smaller. Therefore, the better mark retention observed during treatment episode five was likely due to the larger size of the frylings and the concomitant increase in the surface area of the walleyes that was available to absorb the calcein at the time of treatment (Negus and Tureson 2004).

Weekly examination of the treated walleyes following treatment episode five allowed identification of patterns in mark degradation and facilitated recognition of marked fish at the end of the experiment. The treated walleyes exhibited distinctive patterns in mark degradation based on the likely exposure of the struc-

tures to sunlight. Consequently, internal surfaces that could be inspected non-lethally such as the roof of the mouth and the gill arches also became targeted for mark inspection. The scales between the pelvic insertions reside in a region of the body surface most protected from the sunlight and proved to be an ideal location for mark inspection. The marks on the pelvic scales produced a distinctive arched pattern when viewed on the whole fish though the SE-MARK detector. Additionally, removed scales could be inspected immediately under the epi-fluorescent microscope without further preparation or easily stored for later inspection.

The current restrictions of INAD Number 10-987 limit the usefulness of osmotically induced calcein marking for evaluating walleye stocking in Minnesota. The MNDNR stocks walleyes at four different life stages: 1) newly hatched fry, 2) frylings, 3) fall fingerlings, and 4) “carry-over” walleyes that have evaded harvest as fall fingerlings and are later captured as older fish. Only the newly hatched fry and frylings meet the 2 g weight restriction of INAD Number 10-987 (USFWS 2003). Even though osmotically induced calcein marking of newly hatched fry was not evaluated during this project, the likelihood of its success is limited by the paucity of calcified structures present on walleyes at this life stage. The jaw and teeth of walleye fry begin to calcify within a few days of hatch (McElman and Balon 1979) but only the otoliths actually form prior to hatching. Otoliths of walleyes treated with calcein as newly-hatched fry could be extracted for mark detection as is done with walleyes treated with OTC as fry (Logsdon et al. 2004), but the green color of the calcein marks is less distinguishable from autofluorescence than OTC marks, calcein is much more expensive than OTC, and the fish would still have to be sacrificed for otolith removal. Alternatively, fry could be held in the hatcheries for a few days to allow development of the jaws and teeth prior to treatment, but this would require additional fry tanks and disruptions to current hatchery procedures for the benefit of marks that could be ephemeral due to the limited amount of tissue available to absorb the calcein during treatment.

The current study shows that calcein marks produced on juvenile walleyes through

osmotic induction can remain visible for at least 15 weeks. This level of mark retention is suitable for short-term management evaluations such as Petersen mark-recapture population estimates, but the high mortality following the treatment negates its utility. The walleyes exhibited an obviously negative reaction to immersion in both the salt and the calcein, but the additional handling at the temperatures prevalent at the time of walleye harvest likely also contributed to elevated mortality. Mohler and Bradley (2008) described an activated charcoal filtration system that could facilitate recovery of calcein from large volumes of waste-water and subsequently lead to reduced handling of the fish during treatment. This filtration system, if approved by all appropriate regulatory agencies, would allow a more practical application of the osmotic induction procedure on large numbers of fish.

Perhaps the greatest potential for the use in osmotically induced calcein marking in Minnesota is for marking fall fingerlings. The INAD under which OTC evaluations were conducted (INAD Number 9033) also included a 2 g maximum size restriction which was lifted when OTC immersion marking met final approval. A similar removal of the size restriction upon approval of calcein would allow marking of walleyes large enough (900-1500 mm) to accept a mark at a time of the year when cooler water temperatures allow more safe handling of the fish. The benefits of batch marking and in-hand mark detection with the SE-MARK detector that are associated with calcein marking would have to be weighed against the benefits of other fish marking techniques available to fish of that size such as coded wire tagging, passive integrated transponder (PIT) tagging, visible implant tagging, and immersion marking with OTC (Guy et al. 1996). Of particular interest would be its comparison to OTC immersion marking which, when used on percids with well-developed spines at the time of treatment, can be detected non-lethally by removal of a spine and later examination under an epi-fluorescent microscope (Brown et al. 2002).

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