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OTOLITH FLUORESCENT MARKING OF PIKE (*ESOX LUCIUS* L.) LARVAE

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K e y w o r d s: otoliths, pike, *Artemia salina*, alizarin red S, tetracycline hydrochloride.

A b s t r a c t

The aim of the present study was to verify the feasibility of using live *Artemia salina* nauplii embedded with fluorochromes for the mass marking of pike *Esox lucius* (L.) larvae. In the experiment, pike larvae 6 days post hatch were fed *ad libitum* with nauplii dyed with 600 ppm tetracycline hydrochloride (TC) or 200 ppm alizarin red S (ARS) for 3 or 6 days. The highest percentage of marked fish (100%) and the best quality of this marking was found in the groups fed *A. salina* stained with TC for either 3 or 6 days. In groups fed *A. salina* stained with ARS for 3 or 6 days exhibited a lower percentage of marked fish (ranging from 76.7–88.3%). No significant differences between experimental groups were noted regarding survival rate, final body weight and length of the reared pike larvae.

FLUORESCENCYJNE ZNAKOWANIE OTOLITÓW LARW SZCZUPAKA (*ESOX LUCIUS* L.)

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S ł o w a k l u c z o w e: otolity, szczupak, *Artemia salina*, alizaryna red S, chlorowodorek tetracykliny.

A b s t r a k t

Celem badań było sprawdzenie możliwości wykorzystania żywych naupliusów *Artemia salina* barwionych uprzednio w dwóch fluorochromach do masowego znakowania larw szczupaka *Esox lucius* (L.). W eksperymencie sześciodniowe larwy szczupaka karmiono naupliusami solowca bar-

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wionymi w chlorowodorku tetracykliny (TC) w stężeniu 600 ppm oraz alizarynie red S (ARS) w stężeniu 200 ppm, odpowiednio przez 3 lub 6 dni. Najwyższy procent znakowanych otolitów/ryb (równy 100%) i wysoką jakość znaczków uzyskano w grupach, w których podawano naupliusy barwione TC zarówno przez 3, jak i 6 dni. Z kolei w grupach otrzymujących pokarm barwiony ARS przez 3 lub 6 dni odnotowano niższy odsetek znakowanych osobników (w zakresie 76,7–88,3%). Nie stwierdzono jednocześnie istotnych różnic statystycznych między wartościami przeżywalności oraz końcową masą lub długością ciała larw szczupaka w poszczególnych grupach doświadczalnych.

Introduction

Pike (*Esox lucius* L.) as a piscivorous fish species at the top of the trophic food web that is an attractive prey for fishermen and anglers. It is also a potential fish for use in aquaculture. Pike is a desirable and protected species in many reservoirs due to its significant contribution to the management of fish communities by effectively eliminating planktivorous fish. Stocking of pike are used in bio-manipulation experiments as an indirect tool to reduce the eutrophication process (PREJS et al. 1997). According to data from MICKIEWICZ (2015), pike stocking was practiced in 91% of the fishery farms in Poland. In the year 2014, it was ranked first with regard to the amount of material it released into open waters as a result of stocking actions.

The production of juvenile stages of fish in hatcheries and stocking freshwater reservoirs using these artificially propagated fish is common and provides the opportunity for larvae or fry mass marking. Marked fish can be used to gain an unbiased evaluation of the potential success/failure of stocking actions and for the determination of the effectiveness of natural reproduction. The choice of marking technique for juvenile stages must be able to be applied to a huge number of fish at the same time, be minimally stressful and ensure that the marks are easy to read after several months or even years (BROWN and HARRIS 1995).

In the case of juvenile stages, chemical marking of otoliths and bones is a commonly used and well established method (BROTHERS 1990). The first studies of this topic were initiated in the 1960s (BROTHERS 1990). Firstly, strontium chloride was used as an analog of calcium and incorporated in bony fish structures (OPHELL and JUDD 1968, BAGENAL et al. 1973). The otoliths marked by this method were analyzed using atomic mass spectroscopy and atomic absorption spectroscopy. The second, more popular method of juvenile stage mass marking was based on the use of fluorochromes (BROTHERS 1990). These compounds form chelation complexes with calcium ions and are embedded in the skeletal structures of fish (bones and otoliths). In this case, the presence of the mark (i.e. illuminating calcium-fluorochrome complexes) can be confirmed by observing these structures under ultraviolet light (BEVERANDER and GOSS 1962, THOMAS et al. 1995). Commonly used chemical markers

are tetracycline group antibiotics (i.e. tetracycline, tetracycline hydrochloride (TC), oxytetracycline (OTC), and oxytetracycline hydrochloride) and calcein (BABALUK and CRAIG 1990, THOMAS et al. 1995, STAŃCZAK et al. 2015). There are four techniques for fluorochrome application: intraperitoneal, intramuscular, intravenous or subcutaneous injections (BEVELANDER and GOSS 1962, BABALUK and CRAIG 1990); spraying dye onto the external surface of fish bodies (PITCHERA and KENNEDY 1977, LESKELÄ et al. 2004); immersing fish in a dye solution (HETTLER 1984, BAER and RÖSCH 2008); and finally feeding fish for several days with artificial or live feed supplemented with the fluorochrome (THOMAS et al. 1995, STAŃCZAK et al. 2015).

Fluorochromes are widely applied in aquaculture today. Besides their applicability in marking procedures, tetracycline and its derivatives are also used as antibacterial drugs (SCHNICK et al. 1986). Therefore, labelling protocols should take into account species specificity, because an overdose of some fluorochromes may have unfavorable and ultimately lethal effects on the fish (HETTLER, 1984, TSUKAMOTO 1985). Therefore, it is necessary to adjust the dose that would enable the most effective marking of fish to ensure minimal mortality rates for the fish stock, which is especially significant for endangered/protected or commercially valuable fish species (MACFARLANE and BEAMISH 1987).

Therefore, the goal of this study was to verify the feasibility of using live *A. salina* nauplii stained with either TC or alizarin red S (ARS) for the mass marking of pike *E. lucius* (L.) larvae.

Material and Metods

For our experiment, we used pike larvae 6 days post hatch (DPH), just before the end of yolk sac resorption, that had an average total length of 13.38 ± 0.64 mm and an average body weight of 12.43 ± 1.4 mg. Fish were obtained from controlled reproduction of spawners originating from the Fish Farm "Czarci Jar" near Olsztyn (NE Poland). During experimental rearing, the fish were administered the live feed *A. salina* nauplii (Ocean Nutrition Ltd., USA), incubated following the producer's instructions, and then immersed in one of two fluorescent dyes, either TC at 600 ppm or ARS at 200 ppm (Sigma-Aldrich Ltd.), according to the method published by STAŃCZAK et al. (2015).

The pike larvae were divided into five experimental groups (each experiment was carried out in duplicate). The fish were placed in separate aquaria (100 fish each) with a total volume of ca. 2 dm3 and coupled to a recirculation system. Each experimental group was fed *ad libitum* manually four times a day. Administration of stained nauplii was conducted for 3 or 6 days for both

fluorescent dyes (TC-3 and TC-6 or ARS-3 and ARS-6 groups, respectively). After treatment, the pike larvae were fed exclusively dye-free (pure, plain) nauplii for the next 7 days. The control group (C) was fed only dye-free *A. salina* nauplii. Throughout the experiment, the aquaria were cleaned of food remains and fish waste once a day in the morning before the first feeding.

Survivors of the experimental rearing were counted and 30 larvae per variant were sacrificed with an overdose of anesthetic (MS-222; 5 g dm^{-3}), individually weighed (to the nearest 0.1 mg) and measured (to the nearest 0.01 cm). After measurements were made, larvae were preserved in 70% ethyl alcohol and then otoliths were dissected from each specimen, placed on microscope slides, embedded in Entellan (Sigma-Aldrich Ltd.), and the intensity of the fluorescence TC in a spectrum with a UV wavelengths from 450 to 490 nm; ARS wavelengths from 510 to 560 nm, with a Nikon Eclipse 90i fluorescent microscope equipped with a Lumen 200 UV lamp (Prior Scientific) in order to detect the fluorescent band within the daily increments of the otolith. The identification and evaluation of the mark quality was carried out using a three grade scale from 0 to 2: 0 – no visible mark, 1 – a noticeable mark and 2 – a conspicuous mark. The mark quality was separately graded twice, and the marks were assessed a third time when the two scores were not consistent. In each group, a mean value of the mark assessment for the 30 studied fish was calculated.

Growth parameters and survival rates were compared using one-way ANOVA. The fit of parameters to a normal distribution was tested using Cochran's C test. The data, expressed in percentages, were arcsin-transformed prior to statistical analysis. Significant differences between groups were estimated using a post hoc LSD Fisher test ($p < 0.05$). Analyses were performed using Statistica software (StatSoft).

Results

Marked otoliths were observed in all experimental groups where the pike larvae were fed live *A. salina* nauplii stained with fluorochromes (Table 1). The highest percentage of marked fish (100%) and highest quality of the mark (1.9–2.0) were obtained from the groups TC-3 and TC-6 (Table 1, Figure 1*a*, *b*). The group ARS-3 had the lowest percentage of marked fish (76.7%) and the average quality of the mark was 1.6 (Table 1, Figure 1*c*). In the ARS-6 group, the percentage of marked fish was insignificantly higher (88.6%), as well as the quality of the marks (1.7) – Table 1, Figure 1*d*. No marked fish were found in the control group (*C*) – Figure 1*e*, *f*. During the experiment the survival rate of pike larvae depended on the group and varied between 90.0 and 93.5% of the

initial stocking. At the end of the experiment, the mean total length ranged from 20.8–21.7 mm and the mean weight of the fish ranged from 38.92–40.37 mg (Table 1). The values of final body weight and length of pike larvae did not differ significantly between experimental groups (Table 1).

Table 1

Results of pike (*Esox lucius* L.) larvae experimental rearing and marking used live *Artemia salina* nauplii immersed with tetracycline hydrochloride (TC) or alizarin red S (ARS)

Group	TL [mm]	BW [mg]	Survival [%]	Marked otoliths [%] $n = 60$	Range and (mean value) of marks quality*
TC-3	21.7 ± 1.0	39.22 ± 6.03	93.5 ± 2.1	100.0	1–2 (1.9)
TC-6	20.8 ± 0.9	38.92 ± 5.36	92.5 ± 0.7	100.0	2 (2)
ARS-3	21.0 ± 0.6	40.37 ± 4.78	92.0 ± 2.8	76.7	1–2 (1.6)
ARS-6	21.2 ± 0.8	39.96 ± 5.34	90.0 ± 1.4	88.3	1–2 (1.7)
C	21.0 ± 0.7	40.15 ± 5.25	93.0 ± 1.5	0	0

* marks quality assigned according to adopted three grade scale: 0 – the lack of fluorescent band on otolith (no mark); 1 – the noticeable mark; 2 – conspicuous mark

No significant differences were found among treatments (One-way ANOVA, LSD Fisher post-hoc test, $p > 0.05$).

Discussion

This paper demonstrates a novel and effective method for mass marking of pike larvae by feeding them live *A. salina* nauplii stained with TC or ARS. Until now, most marking procedures of pike eggs or juveniles have involved immersing them in solutions of chemical markers. CZERKIES (1998) successfully marked fertilized embryos by immersing them in ARS at 200 ppm for 4–6 h or by keeping them in a TC bath of 800 ppm for 6 h. In the case of pike larvae, a 3 h bath in a 150–200 ppm ARS solution or in an 800 ppm TC solution was equally effective. Another method of fluorochrome administration was applied by WAHL and STEIN (1987) to muskellunge (hybrid *Esox masquinongy* and *Esox lucius*). They fed the fish fry with pelleted feed saturated with OTC at a dose of 500 mg/kg body weight and achieved a marking success of 80–100%. BABALUK and CRAIG (1990) also marked pike with OTC using intraperitoneal injection at doses from 25–50 mg kg^{-1} body weight which resulted in a 100% marking rate with marks being visible on all analyzed bony structures as early as 24 hours after injection.

RHOTEN et al. (2014) demonstrated that during immersion marking of northern pike with OTC, success was dependent upon the age of the fish subjected to the marking procedure. In the case of 7 DPH larvae, successful

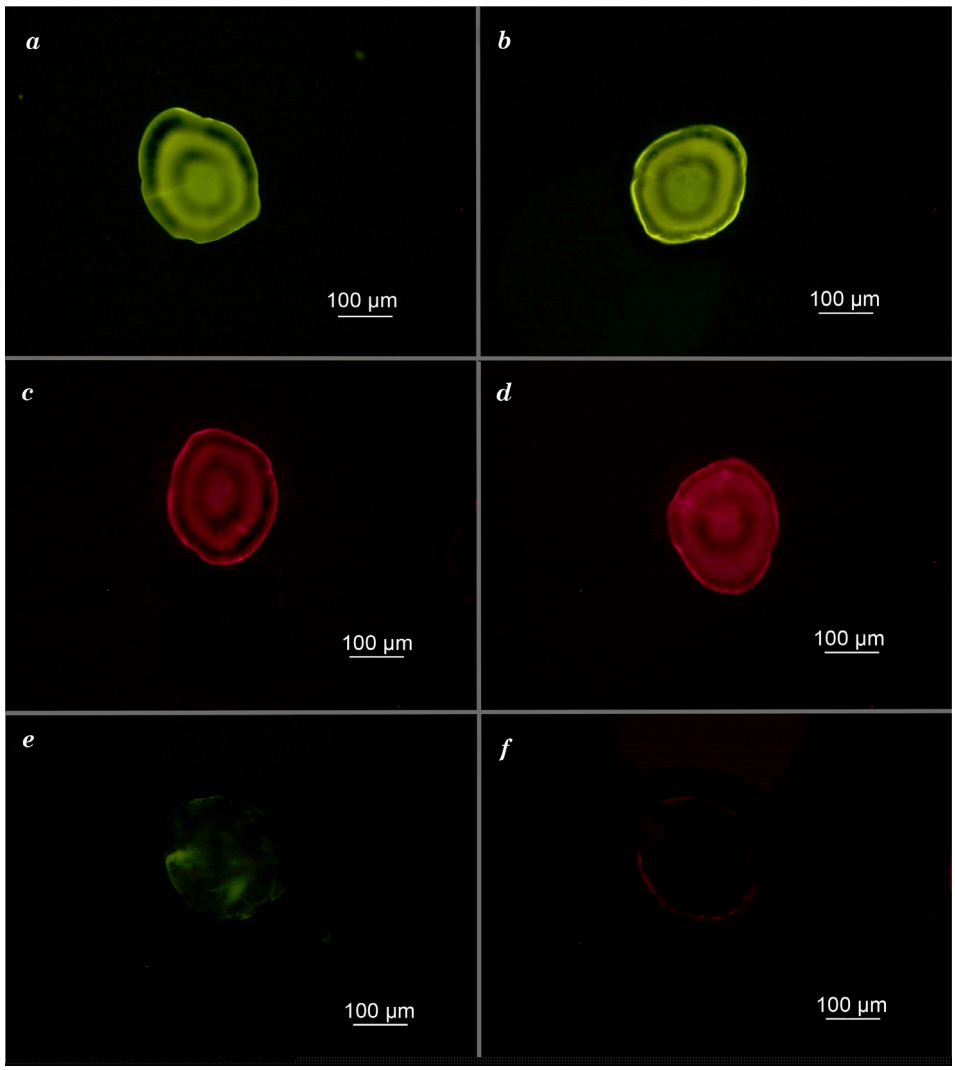

Fig. 1. Otoliths of 18-days old pike larvae after experimental rearing: *a* – group TC-3; *b* – group TC-6; *c* – group ARS-3; *d* – group ARS-6; *e* – control group in the light UV wavelengths from 450 to 490 nm; *f* – control group in the light UV wavelengths from 510 to 560 nm. Bar = 100 µm

marking was noted in only 38% of individuals, whereas in newly hatched larvae (< 1 DPH) the efficiency of marking reached 91% and the quality of the marks was significantly higher. In our study, the marking was conducted using 6 DPH larvae fed live *A. salina* nauplii stained with a derivative of the same fluorochrome. Results were very satisfactory as the marking effectiveness reached 100% when the fish were fed the stained nauplii for both 3 and 6 days.

The described technique of pike larvae mass marking *via* feeding with stained *A. salina* nauplii is much easier to carry out in hatchery conditions, is less expensive as it requires significantly smaller amounts of dyes and is significantly safer for the fish compared to immersion methods. In the case of fluorochrome administration to a fish body using immersion, the marking effectiveness is determined mainly by the physicochemical properties of water. Most of the marker application procedures significantly lower the pH of the water, which reduces the rate of fish survival (HETTLER 1984, TSUKAMOTO 1985). DABROWSKI and TSUKAMOTO (1986) reported a lower efficiency from the immersion method and the formation of a narrower daily growth ridge after using OTC to mark otoliths of peled (*Coregonus peled* L.) larvae reared in water with a temperature of 7.5°C as compared to those reared at 16.8°C. HARRISON and HEIDINGER (1998) concluded that poorly visible marks develop when fish are starved before and after the marking procedure. Additionally, as stated by MEYER et al. (2012), the immersion marking method may induce not only rapid/acute outcomes, but also chronic (sublethal) effects that could result in growth inhibition or increased mortality of the fish.

Our feeding method transfer of fluorochromes eliminates such physiological barriers. The marking of a comparable amount of pike larvae with this method requires several times less dye. This result is important economically and also important for the quality of natural environments by minimizing the effects of water contamination and reducing post-production sludge. In addition, application of the feeding method for marking ensures that the theoretically adopted dose of fluorochrome will not be exceeded.

Many reports demonstrate that the quality of marks is affected by fish size, dye concentration and the length of time to which a fish body is exposed to the fluorochrome (PARTRIDGE et al. 2009). However, higher concentrations of dyes may contribute to increased mortality rates of marked fish (UNFER and PINTER 2013). The methods of pike larvae mass marking described in this paper had no negative impact on the survival or growth of fish (weight/total length). Similarly positive results were obtained when marking brown trout (*Salmo trutta* L.) (BAER and RÖSCH 2008) or European glass eel (*Anguilla anguilla* L.) (CARAGUEL et al. 2014). Thus, we conclude that mass marking with chemical substances (fluorochromes) applied in species specific experimentally determined doses will not have any negative effects on fish growth parameters.

An important aspect of this marking technique is also the retention of marks. According to POCZYCZYŃSKI et al. (2011), the marks obtained for vendace (*Coregonus albula* L.) immersed in 200 ppm ARS were detected without any problems in fish at the age of 3 years or older. In a long-term study on restoration of the autochthonous population of common whitefish (*Coregonus lavaretus* f. *lavaretus* L.) from Lake Łebsko (North Poland), the

fluorochrome marks were identified in fish at the age of 5 years or older (MARTYNIAK et al. 2013). KRUMME and BINGEL (2016) confirmed that the marks obtained on cod (*Gadus morhua* L.) otoliths upon injection of OTC were still clearly visible after 40 years of dark-storage at room temperature.

The results of the present study reveal a need to not only adjust marking techniques by species but also to consider the ontogenic development of fish. In conclusion, feeding 6 DPH pike larvae for 3 days with live nauplii of *A. salina* stained in 600 ppm TC solution is recommended for effective and safe mass marking and this method could become commonly used in fisheries.

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References

BABALUK J.A., CRAIG J.F. 1990. *Tetracycline marking studies with pike, Esox lucius* L. Aquaculture and Fishery Management, 21: 307–315.

BAER J., RÖSCH R. 2008. *Mass-marking of brown trout* (*Salmo Trutta* L.) *larvae by alizarin. Method and evaluation of stocking.* J. Appl. Ichthyol., 24: 44–49.

BAGENAL T.B., MACKERETH F.J., HERON J. 1973. *The distinction beetween brown trout and sea trout by the strontium content of thrir scales*. J. Fish Biol., 5: 555–557.

BEVELANDER G., GOSS R.J. 1962. *Influence of tetracycline on calcifaction in normal and regenerating teleost scales*. Nature (London), 193: 1098–1099.

BROTHERS E.B. 1990. *Otolith marking*. American Fisheries Society Symposium, 7: 183–202.

BROWN P., HARRIS J.H. 1995. *Strontium batch-marking in golden perch* (*Macquaris ambigua*, Richardson) *and trout cod* (*Maccullochella macquariensis*, Cuvier). In: *Recent developments in fish otolith research*. Eds. D.H. Secor, J.M. Dean, S.E. Campana. University of South Carolina Press, Columbia, pp. 303–318.

CARAGUEL J.M., CHARRIER F., MAZEL V., FEUNTEUN E. 2014. *Mass marking of stocked European glass eels* (Anguilla anguilla) *with alizarin red S*. Ecol. Freshw. Fish, 24: 435–442.

COWX I.G. 1998. *Stocking strategies: issues and options for future enhancement programmes?* In: *Stocking and Introduction of Fish*. Eds. I.G. Cowx. Fishing News Books. Blackwell Scientific Publications, Oxford, pp. 3–13.

CZERKIES P. 1998. *Selection of the optimal method of mass marking the early stages of development pike* (*Esox lucius* L.). PhD thesis. AR-T Olsztyn.

DABROWSKI K., TSUKAMOTO K. 1986. *Tetracycline tagging in coregonid embryos and larvae*. J. Fish Biol., 29: 69–698.

HARRISON D.R., HEIDINGER R.C. 1998. *Factors determining quality of oxytetracycline marks in fingerling Walleye otoliths*. Proceedings of the Annual Conference of Southeast Association of Fish and Wildlife Agencies, 50: 174–181.

HETTLER W.F. 1984. *Marking otoliths by immersion of marine fish larvae in tetracycline*. Transactions of the American Fisheries Society, 113: 370–373.

KRUMME U., BINGEL F. 2016. *Tetracycline marks visible in Baltic cod Gadus morhua otoliths stored for 40 years: tetracycline marks still shine after 40 years.* J. Fish Biol., 89(4): 2189–2194.

LESKELÄ A., JOKIKOKKO E., HUHMARNIEMI A., SIIRA A., SAVOLAINEN H. 2004. *Stocking results of spray-marked one-summer old anadromous European whitefish in the Gulf of Bothnia*. Annales Zoologici Fennici, 41: 171–179.

MARTYNIAK A., STAŃCZAK K., KOZŁOWSKI J., MIERZEJEWSKA K., WZIĄTEK B., LEJK A.M., HLIWA P. 2013. *Alizarin mark retention in the otolith of European whitefish (Coregonus lavaretus f. lavaretus L.) from Lake Łebsko, Poland*. Advances in Limnology, 64: 83–89.

McFarlane G.A., Beamish R.J. 1987. *Selection of dosages of Oxytetracycline for age validation studies*. Canadian Journal of Fisherieas and Aquatic Sciences, 44: 905–909.

Meyer S., Sørensen S.R., Myron A.P., Støttrup J.G. 2012. *Sublethal effects of alizarin complexone marking on Baltic cod (Gadus morhua) eggs and larvae*. Aquaculture, 324–325: 158–164.

Mickiewicz M. 2015. *Restocking of lakes Polish carried out in 2014*. In: *Sustainable use of fishery resources in the background of their state in 2014*. Eds. M. Mickiewicz. IRŚ, Olsztyn.

Ophel I.L., Judd J.M. 1968. *Marking fish with stable strontium*. J. Fish. Res. Board. Can., 25: 1333–1337.

Partridge G.J., Jenkins G.I., Doupé R.G., De Lestang S., Ginbey B.M, French D. 2009. *Factors affecting mark quality of alizarin complexone-stained otoliths in juvenile black bream Acanthopagrus butcheri and a prescription for dosage*. J Fish Biol., 75: 1518–1523.

Pitcher T.J., Kennedy G.J.A. 1977. *The longevity and quality of fin marks made with a jet inoculator*. Fish Manage, 8: 16–18.

Poczyczyński P., Kozłowski K., Kozłowski J., Martyniak A. 2011. *Marking and return method for evaluating the effects of stocking larval vendace, Coregonus albula (L.), into Lake Wigry in 2000–2001*. Arch Pol Fish, 19: 259–265.

Prejs A., Pijanowska J., Koperski P., Martyniak A., Boron S. Hliwa P. 1997. *Food-web manipulation in a small, eutrophic Lake Wirbel, Poland. Long-term changes in fish biomass: a basic measure of water quality. A case study*. Hydrobiologia, 342/343: 383–386.

Rhoten J.C., Fisher S.J., Logsdon D.E., Pittman B.J. 2014. *Effects of age at treatment on oxytetracycline mark formation in larval Northern Pike*. North American Journal of Fisheries Management, 34(4): 735–740.

Schnick R.A., Meyer F.P., Walsh D.F. 1986. *Status of fishery chemicals in 1985*. Progressive Fish-Culturist, 48: 1–17.

Stańczak K., Krejszeff S., Dębowska M., Mierzejewska K., Woźniak M., Hliwa P. 2015. *Mass-marking of Leuciscus idus (Linnaeus 1758) larvae using Artemia salina (Linnaeus 1758) as a vector of fluorescent dyes*. J. Fish Biol., 87: 799–804.

Thomas L.M., Holt S.A., Arnold C.R. 1995. *Chemical marking techniqes in larval and juvenile red drum (Sciaenops ocellatus) otoliths using different fluorescent markers*. In: *Recent developments in fish otolith reserch*, Eds. D.H. Secor, J.M. Dean, S.E. Campana. Univ. South Carolina Press, pp. 703–717.

Tsukamoto K. 1985. *Mass-marking of ayu eggs and larvae by tetracycline tagging of otoliths*. Bulletin of the Japanese Society for the Science of Fish, 51(6): 903–911.

Unfer G., Pinter K. 2013. *Marking otoliths of brown trout* (Salmo trutta L.) *embryos with alizarin red S.* J Appl. Ichthyol., 29: 470–473.

Wahl D.H., Stein R.A. 1987. *Application of liquid oxytetracycline in formulated feeds to mark and treat tiger muskellunge.* Prog. Fish-Cult., 49(4): 312–314.