Handbook for Allis shad marking

The re-introduction of Allis shad (*Alosa alosa*) in the Rhine system

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Résumé
La réintroduction de la grande alose (*Alosa alosa*) dans le bassin du Rhin bénéficie du soutien de la Commission Européenne au travers d'un programme Life Nature. Ce programme est le fruit d'une collaboration entre l'Allemagne (Lander de Rhénanie du Nord – Westphalie et Hesse), les Pays-Bas et la France. Le Cemagref est partenaire du projet et bénéficie du soutien financier de la Région Aquitaine au titre de la coopération interrégionale Aquitaine – Hesse. Ce rapport présente la mise au point d'une méthode de marquage de masse des larves de grande alose.

Abstract:
The Allis shad re-introduction in the Rhine basin is granted by the European Commission by the mean of a Life Nature programme. This programme is the result of a collaboration between Germany (Lander NorthRhine Westphalia and Hessen), Netherlands and France. Cemagref is partner of this programme and is granted by Region Aquitaine in the frame of the Aquitaine – Hesse Interregional Cooperation. This report presents the development of a chemical mass marking method for Allis shad larvae.

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1. Introduction

The last century has seen dramatic declines in numerous diadromous fish species, (Limburg and Waldman 2009). Stocking programmes are commonly used to rehabilitate endangered fish species (Brown and Day 2002 ; St-Pierre 2003). For many restoration or reintroduction programmes success of stocking programmes are rarely assessed (Schneider 2001). They can be evaluated in terms of survival rates at different stages (Cote and Pomerleau 1985), return rates (Aprahamian et al. 2003 ; Salminen et al. 2007), growth or ecological interactions (Cowx 1994).
To evaluate the success of such programmes, and following recommendations of the IUCN Re-introduction Specialist Group (1998), hatchery-reared individuals have to be discriminated from wild ones (Champigneulle and Cachera 2003 ; Baer and Rösch 2008). At a finer scale, discriminating groups of stocked individuals can be used to improve stocking practices, by assessing the relative survival of specimens released at different ages and sites for example (Caudron and Champigneulle 2009).
The identification of stocked individuals constitutes an important methodological bottleneck for the reintroduction actions. These problems are recurring in anadromous migrating fish, where the strategy consists in marking very young individuals, and to obtain a detectable mark several years later on adults at the time of their spawning migration.
Fish tagging has been widely used for more than a century for scientific purposes (Thorsteinsson 2002). The need to identify individuals or group of fish, led to develop a huge variety of tags (see McFarlane et al. 1990 for an extensive review).
Thorsteinsson (2002) defined tag as “man made objects attached to the fish”, that this author differentiated from mark defined as “identifiable characteristics either natural or applied to a fish”. Therefore, are considered as tag, all physical objects, inserted or attached, externally or internally to a fish.

2. Choice of a method aiming at identifying fish

Determination of an appropriate marking method must match several criteria and constraints. The first concern is linked to the number of fish which have to be marked. Basically in restoration programmes, the goal is to tag several ten of thousands fish.
Externally visible tag such as anchor or dart tag, can only be used in fish of a minimum size (Morgan and Walsh 1993), allowing to insert the tag without damage for the fish (Nakashima and Winters 1984) and to minimize negative impact on locomotion. Visible Implant Elastomer (VIE) doesn’t impair behaviour and can be used on small individuals (Imbert et al. 2007). Externally non visible tags such as Passive Integrated Transponder (PIT) tag or Coded Wire Tag (CWT) have been widely used in restoration programmes (Smith and Clugston 1997 ; St-Pierre 1999 ; Secor et al. 2000). The insertion of all these external or internal tags requires individual handling which doesn’t match with the yearly number of fish involved in a restoration programmes.
In Allis shad the choice of mass marking method is limited by the high sensitivity and the small size of individuals (less than 20 mm) at the time of marking. Therefore any tagging methods are not appropriated.

3. Overview of mass marking methods

Chemical mass marking is a good way to mark fish at a very young stage, without individual handling (Tsukamoto 1988 ; Secor et al. 1991 ; Taylor et al. 2005). With this technique, fish are immersed into a bath containing chemicals that fluoresce under UV light and bind to hard tissues like otoliths, fin rays, and scales. That way, a large number of fish can be marked simultaneously. Marks have been successfully produced in young stages of fish using chemicals like oxytetracycline (Hendricks et al. 1991 ; Reinert et al. 1998), tetracycline (Dabrowski and Tsukamoto 1986), alizarine complexone (Van der Walt and Faragher 2003), alizarine RedS (Lagardère et al. 2000) and calcein (Mohler 1997).
Marking efficiency is assessed at 2 levels. Survival and behaviour are the first criteria which can be immediately assessed. Survival indicates which chemical and which concentration can be withstood by the fish, results being able to vary according to species and/or age (Brooks et al. 1994 ; Rojas-Beltran et al. 1995 ; Beckman and Schulz 1996).
Mark quality and its persistence with time (Reinert, et al. 1998 ; Jenkins et al. 2002) is the main criteria to select the marking protocol. Mark quality is generally observed in otoliths which are considered as a reference structure as they appear early in life, grow through the entire fish life and does not resorb (Campana 1999).
Mass marking operations have been implemented in North American shad restoration programmes in the Chesapeake Bay. Different methods with a 4 or 6-hours immersion in 200 mg.l-1 oxytetracycline (OTC) or tetracycline hydrochloride (TC) have been developed (Lorson and Mudrak 1987 ; Hendricks, et al. 1991 ; Minkkinen et al. 2001). Millions of shad larvae have been marked following these methods and marks have been detected in otoliths of returning adults demonstrating the method efficiency (St-Pierre 2003).

4. Development of a mass marking method for Allis shad larvae

Following the method developed in American shad, we tested a marking method using tetracycline as marker.
Marking experiment was carried out on 5 days old larvae. They were reared in 250 l cylindrical tanks, and fed with artemia nauplii and dry food.
One control and 6 different combinations (concentration – duration) were tested:

- 3 marker concentrations: 200, 250 and 300 $mg.l^{-1}$
- 2 bath durations: 4 and 6 hours

4.1 Materials and method

4.1.1 Products used

Tétracycline hydrochloride (TC) :
- Supplier: Sigma – reference : CAS 64-75-5
- Formula: $C_{22}H_{24}N_2O_8$, HCl (95%)
- Storage: -20°C

Sodium Phosphate dibasic heptahydrate (NaP) :
- Supplier: Sigma – reference : CAS 7782-85-6
- Formula: Na_2HPO_4, 7 H_2O (99%)
- Storage: -20°C

Potassium Phosphate dibasic trihydrate (KP) :

Supplier: Sigma – référence: CAS 16788-57-1

Formula: K_2HPO_4, $3H_2O$ (99%)

Storage : -20°C

4.1.2 Preparations

Marker:

Following conditions defined above, 250 l volume and marker of 95% purity, the needed quantities are as follows:

200 mg/l (or 200 ppm): 52,6 g of product (50 g of pure matter)

250 mg/l (or 250 ppm): 65,8 g of product (62,5 g of pure matter)

300 mg/l (or 300 ppm): 78,5 g of product (75 g of pure matter).

Buffer:

Owing to the drop of the pH after dissolution of TC, the solution has to be buffered.
The buffer was obtained by mixing 2 parts of NaP and 1 part of KP. The mixing was conserved dry.

Preparation of high concentrated solutions

The products are not mixed directly in the rearing tank, to prevent mass mortality of larvae (pH and thermal shocks).
We thus prepared 6 high concentrated solutions containing the desired quantity of marker. These solutions were buffered by progressively adding the buffer mixing, until neutrality was reached.

4.1.3 Marking bath

The high buffered concentrated solutions were poured in each corresponding tank (Figure 1).

Figure 1: A rearing tank with the device creating the water current during the marking bath.

During the marking bath, it is necessary to:

STOP WATER RENEWAL;
stop feeding;
control the pH;
control dissolved oxygen ;
bring pure O_2 if necessary;
ensure a sufficient mixing of the bath by creating a water current in the tank. .
During the marking bath, tanks must be maintained in dim light to prevent UV from causing damages to the marker molecule.

At the end of the bath, it is necessary to:

Turn on water renewal;
Turn on food distribution;
A special collector must be used to collect outflow.

4.1.4 Effluent treatment

Effluent with high level of TC should not be diffused in the sewage network or in the natural environment to prevent emergence of resistant bacterial strains. The simplest and cheapest solution is to store highly contaminated water in a concrete raceway. TC is naturally degraded in non dangerous by-products under sunlight and hydrolysis actions (Doi and Stoskopf 2000). Between 3 to 5 weeks are needed to obtain a total process. Controls (by spectrophotometry) could be carried out to evaluate the kinetics and the effectiveness of degradation. However, the effluents can also be treated by a specialized firm.

4.2 Results

4.2.1 Growth and survival

4.2.1.1 Direct marking effect on mortality

On Day 6, the day after the marking, mortality was checked (Table I). Whatever the marking treatment, no difference was detected compared to the control batch, except for the 300/6 batch where the higher mortality rate was recorded (Pearson Chi-square p=0).

Table I: Direct mortality after marking according to treatments

Batches	**200/4**	**250/4**	**300/4**	**200/6**	**250/6**	**300/6**	**Control**
Number of dead larvae	44	49	61	48	41	486	48
Added mortality	0.4%	0.7%	0.7%	0.5%	0.5%	5.5%	0.6%

4.2.1.2 Mortality on Day 10

The best survival was recorded for 300 ppm/4 hours and 200 ppm/4 hours (Table II). Mortality was significantly lower in batches exposed during 4 hours compared to the control (Pearson Chi-square, $p<0.05$). No difference was recorded between 200 and 250/6 batches and the control, whereas 300/6 treatment with the highest mortality was significantly different from all the other treatments (Pearson Chi-square, p=0).
A grouping analysis with respect to duration demonstrated a significant difference between the 2 durations, even if the 300/6 batch was excluded from analysis (Pearson Chi-square, p=0). A 6 hours bath elicited a higher mortality rate than the 4 hours bath.

Table II: Mortality on Day 10 (5 days after marking) according to treatments

Batches	**200/4**	**250/4**	**300/4**	**200/6**	**250/6**	**300/6**	**Control**
Added mortality from marking	5.8%	6.9%	4.1%	7.7%	8.2%	13.6%	8%

4.2.1.3 Growth

Length growth was quite similar among batches (Figures 2). No difference could be detected among treatments or compared to the control, at the end of the experiment (Anova, $p>0.05$).

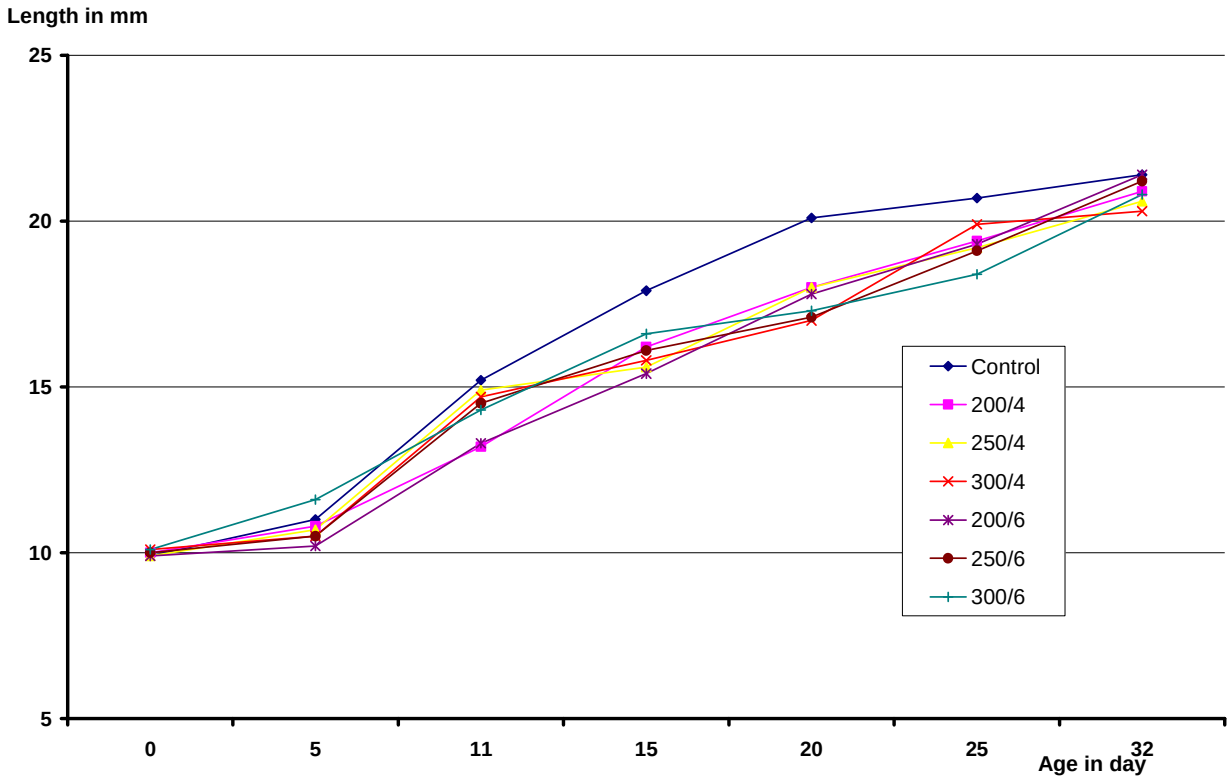

Figure 2: Length growth according to batches

4.2.2 Marking efficiency

For each couple, marker concentration – marking duration, otoliths from 12 larvae were dissected, prepared and observed under a microscope for mark quality. Two groups of fish were sampled to check on mark quality: one group of larvae was sampled at 15-day old and another at 32-day old. The larvae were kept in alcohol 60° small dark containers until analysis.

4.2.2.1 Otolith preparation

A total of 168 larvae were analysed. More precisely, 12 larvae per treatment were dissected under a binocular microscope (Olympus® SZX 12). Sagittal otoliths were removed and cleaned from extraneous tissues. A sagittal section of otoliths was prepared. To facilitate their preparation, otoliths were not mounted directly on a glass slide but rather, on a flat drop of Araldite resin. Each otolith was embedded sulcus side down in Araldite resin and ground with wet sand paper (grit 1200 and 4000) until reaching the primordium. Final polishing was performed on a polishing cloth with a mix of diamante suspension 1 µ (Struers®) and colloidal silica suspension (Struers®) (Figure 5).

Equipment description (Figure 4)

Otoliths were observed under ultra-violet light (source: halogen lamp 1000W) using a microscope Eclipse 90i (Nikon ®). The microscope was equipped with a Nikon B-2A filter cube to identify tetracycline marks. Briefly, the characteristics of this filter cube are: Excitation filter: 450-490 nm / Dichromatic mirror cut-on wavelength: 500 nm / Barrier filter wavelengths: 515 nm cut-on.

An image of each otolith observed under UV light was captured using the Nikon digital camera DXM1200C. To standardize all images, they were all taken at the same magnification (x20), using the same camera parameters, especially exposure time (210 ms) and gamma (0.45). The utilities on NIS-Elements D software (version 2.3) were then used to check on mark quality.

Figure 4: Equipment used for tetracycline mark detection on allis shad larvae otoliths

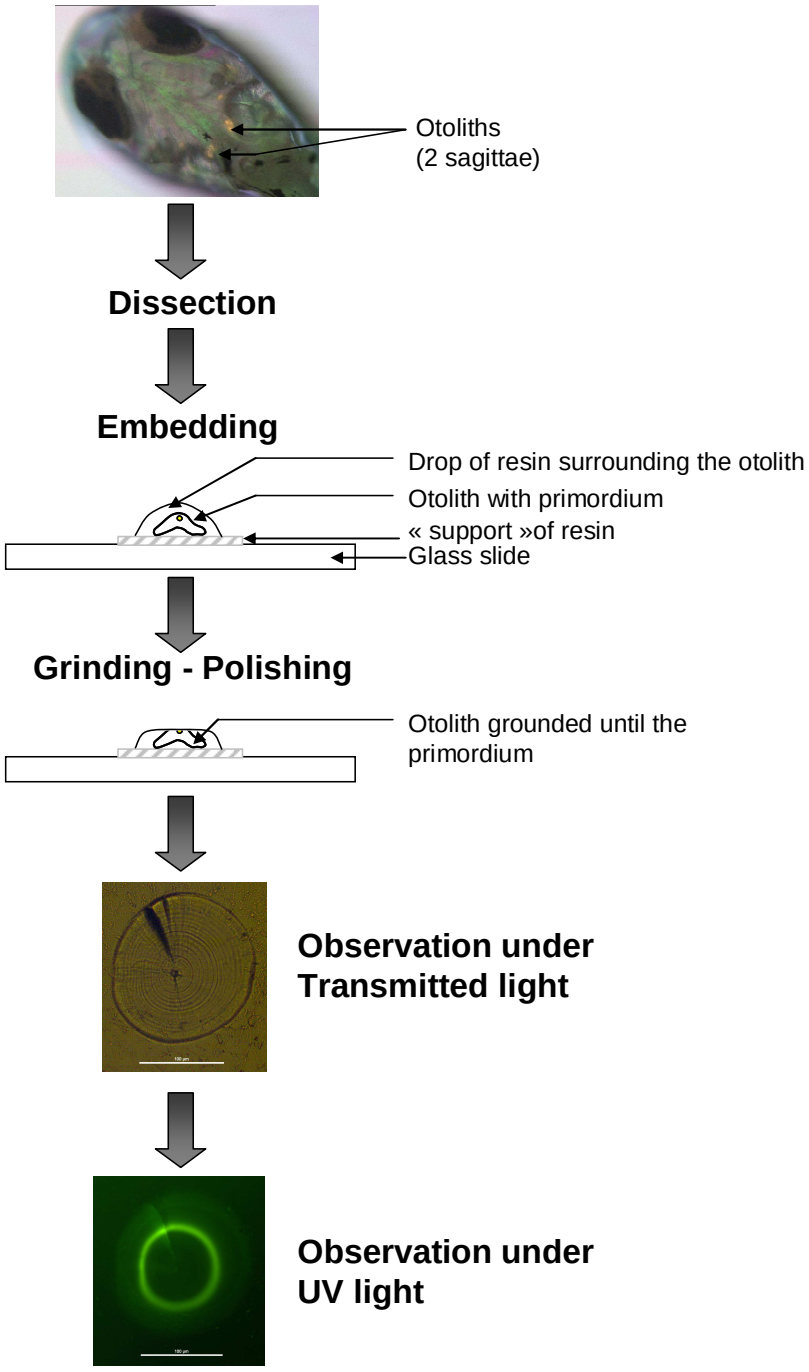

Figure 5 : Protocol of otolith preparation, from dissection to observation under UV light

4.2.2.2 Assessment of mark quality

Using this equipment, tetracycline mark looks like a yellow-green band. Mark quality was expressed by the yellow-green band. This intensity is related to gray scale level. It is a level of greyness or brightness, ranging from completely black to completely white. As the camera used for otolith observations gives 8-bit gray scale images, there are 256 gray levels. A pixel with a value of “0” is completely black whereas one with a value of “255” is completely white or bright. Thus, the higher mark intensity is, the better mark quality is.

The NIS-Elements D software automatically calculate the mean intensity of the area corresponding to the mark.

4.2.2.3 Results on mark quality

Whatever the treatment, all otoliths presented a yellow-green shiny mark, except the controls that did not experience any marking (Figure 6).

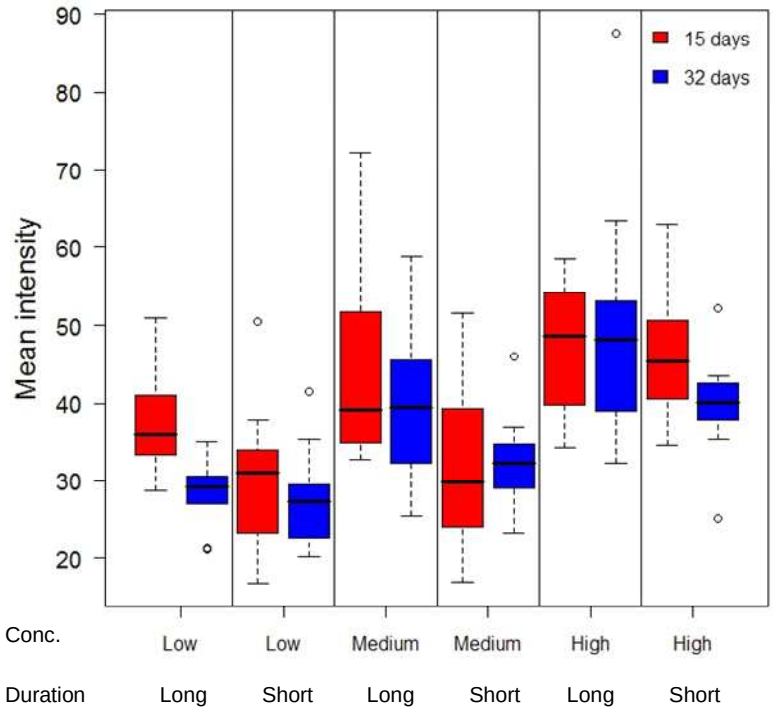

Figure 7: Results on mark quality according to treatments, for 15 and 32 days old larvae

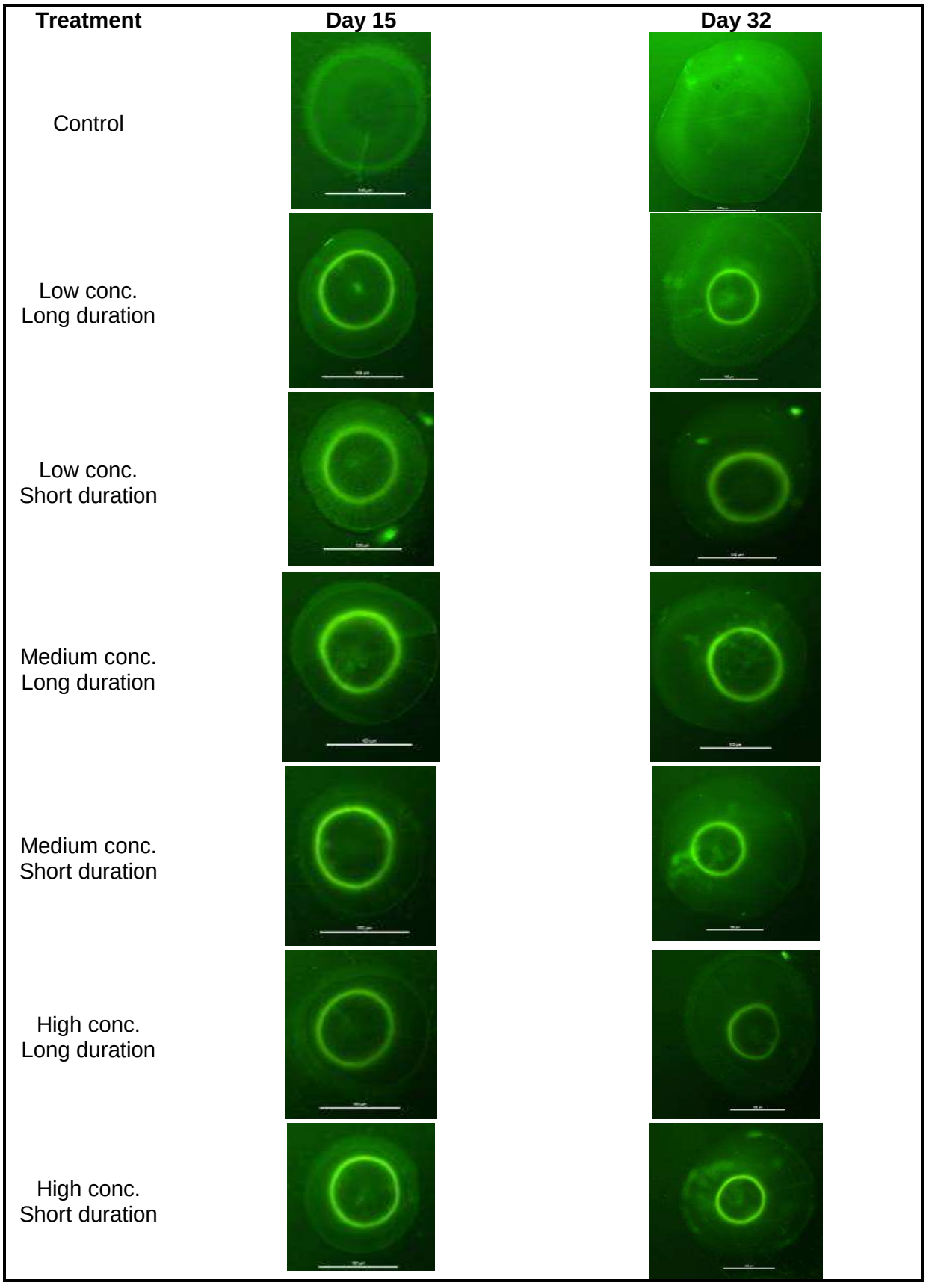

Figure 6 : Images of one representative otolith per treatment, for 15-days old and 32-days old larvae. The shiny yellow-green mark is the tetracycline mark (White scale = 100µm).

At 15 days old, mark intensity depends on both duration and TC concentration (Figure 7). However, there is no interaction between them, which means that, whatever the concentrations, the differences between long and short durations are the same. High concentrations produce a significantly higher intensity mark compared to low or medium concentrations. Long duration produces a significantly higher intensity mark compared to short duration.

At 32 days old, mark intensity also depends on both duration and TC concentration but there is no interaction between them. High concentrations produce a significantly higher intensity mark compared to low or medium concentrations. Long duration produces a significantly higher intensity mark compared to short duration.

5. Conclusion and recommendations for marking operations in Allis shad larvae

Concerning mark quality alone, 3 conclusions could be obviously drawn from the experiment:

- mark quality is better for high concentration
- mark quality is better for long duration
- mark quality decreases with time

Thus with this single criteria, the best choice is the high concentration/long duration (300 ppm/6 hours).
Taking into account results from survival and growth monitoring, conclusions are slightly modified. The batch 300 ppm/6 hours presents the worst survival, thus it is necessary to do a trade-off between the 3 criteria. In these conditions, we recommended the 300 ppm/4 hours treatment.

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Appendix

Publication on Allis shad larvae marking.

Lochet A, Jatteau P, Rochard E (2009) A reliable method to assess mark quality on fish otoliths. Fisheries Manag Ecol 16 (6):508-513. doi:10.1111/j.1365-2400.2009.00691.x

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Management and Ecological Note

A reliable method to assess mark quality on fish otoliths

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Stocking programmes are widely used in fishery conservation to sustain commercial and recreational fisheries (Margenau *et al*. 2008) and to rehabilitate endangered populations (Waldman & Wirgin 1998). Mass marking of fish by immersion in chemical agents is often used to evaluate the success of such programmes to discriminate hatchery-reared fish from wild fish (Champigneulle & Cachera 2003; Baer & Rösch 2006).

Producing a long-lasting mark is challenging as mark retention tends to decrease over time (Lorson & Mudrak 1987; Jenkins *et al*. 2002). When setting a marking protocol, it is crucial to identify the best mark quality among all the protocols tested. However, it can be difficult to agree on common standards for what is considered a good quality mark. Categories are usually arbitrarily defined like absent/faint mark/good mark/very good mark (Iglesias & Rodriguez-Ojea 1997; Sanchez-Lamadrid 2001) and the assignment of one otolith to a particular category is solely based on human visual perception, which is subjective. A more impartial mark assessment method, the pixel luminosity method has been recently applied to caudal fin rays (Frenkel *et al*. 2002) and scales (Honeyfield *et al*. 2006) but never to fish otoliths. In this method, digital images are captured and the average pixel luminosity of the marked area within the calcified structure is calculated and compared between different marking protocols. Mark fluorescence is the combination of marker fluorescence, which is caused by the quantity of marker incorporated into the otolith and background fluorescence, which is due to the otolith itself. Although the importance of background fluorescence is well recognised (Negus & Tureson 2004), its effect on mark quality assessment has never been quantified.

Allis shad, *Alosa alosa* L., is an anadromous clupeid species. As with many anadromous species, over fishing, dam construction, water quality degradation and deterioration of spawning grounds have led to a contraction of its distribution range (Baglinière 2000; Waldman & Limburg 2003). The use of a reliable method to assess mark quality on allis shad is of great importance because its reintroduction to watersheds from where it disappeared strongly relies on the efficiency of stocking programmes (De Groot 2002).

The first objective of this note was to test the relevance of the pixel luminosity method to assess mark quality in fish otoliths. Mark quality evaluation using the pixel luminosity method and a visual estimation method was assessed for the same set of allis shad otoliths. The second objective was to address the importance of background luminosity on the assessment. Using the pixel luminosity method, mark quality was assessed by comparing the intensities measured for mark fluorescence (marker fluorescence + background fluorescence) and marker fluorescence alone. The third objective was to test the effect of grind quality on these fluorescences.

Five-day-old allis shad larvae were exposed to different marking treatments (concentration and duration) using tetracycline hydrochloride ($C_{22}H_{24}N_2O_8$·HCl; Sigma®, Aldrich Corporation, Saint Quentin Fallavier, France). One control and six different treatments were set combining three marker concentrations (low: 200 ppm, medium: 250 ppm, high: 300 ppm) and two marking durations (short: 4 h, long: 6 h). Mark quality was assessed for 15- and 32-day-old fish that were killed with a high concentration of Eugenol and kept in 60° alcohol in dark containers until otolith dissection and analysis.

A total of 168 larvae (total length: 11.5–20 and 16–24 mm for 15- and 32-day-old larvae respectively) were analysed. Twelve larvae per treatment were

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dissected under a binocular microscope (Olympus® SZX12, Olympus S.A.S, Rungis, France) for both ages. One of the two sagittal otoliths was prepared for each fish. Otoliths were first mounted on a flat drop of Araldite® resin of known depth (333 ± 44 µm, mean ± SD), on top of a glass slide. Otoliths were embedded sulcus side down in Araldite® resin and ground with wet sand paper (grit 1200 and 4000, Dil S.A., Courtaboeuf, France) until reaching the primordium. Final polishing was performed on a polishing cloth with a mix of diamante suspension 1 µm (Struers®, Champigny sur Marne, France) and colloidal silica suspension (Struers®, Champigny sur Marne, France) to remove all scratches. The second otolith for 11 randomly selected fish was prepared and mark, background and marker intensities were measured at different levels of grinding: level 1 (otolith barely ground), level 2 (nucleus reached) and level 3 (beyond the nucleus plan). Results were expressed as percentage of intensity, where level 1 was arbitrarily set at 100%.

Detection of tetracycline was carried out using a compound microscope Eclipse 90i (Nikon®, Champigny sur Marne, France) fitted with an UV light source. The microscope was equipped with a Nikon B-2A filter cube (excitation filter: 450–490 nm/dichromatic mirror cut-on wavelength: 500 nm/barrier filter wavelength: 515 nm cut-on, Champigny sur Marne, France). Digital images of otoliths were recorded at 20× magnification using the Nikon® digital camera DXM1200C. For proper image standardisation, otoliths were observed using the same fluorescent light intensity and all images were recorded using the same camera settings (exposure time: 210 ms; gamma: 0.45).

Mark luminosity was reported on a 256 grey-level scale (0 = black and 255 = white or bright) using the NIS-Elements D software (version 2.3, Champigny sur Marne, France) to assess mark quality with the pixel luminosity method. Mark fluorescence was the average pixel luminosity of the area corresponding to the mark, appearing as a bright yellow-green ring. Background luminosity was calculated by averaging the mean

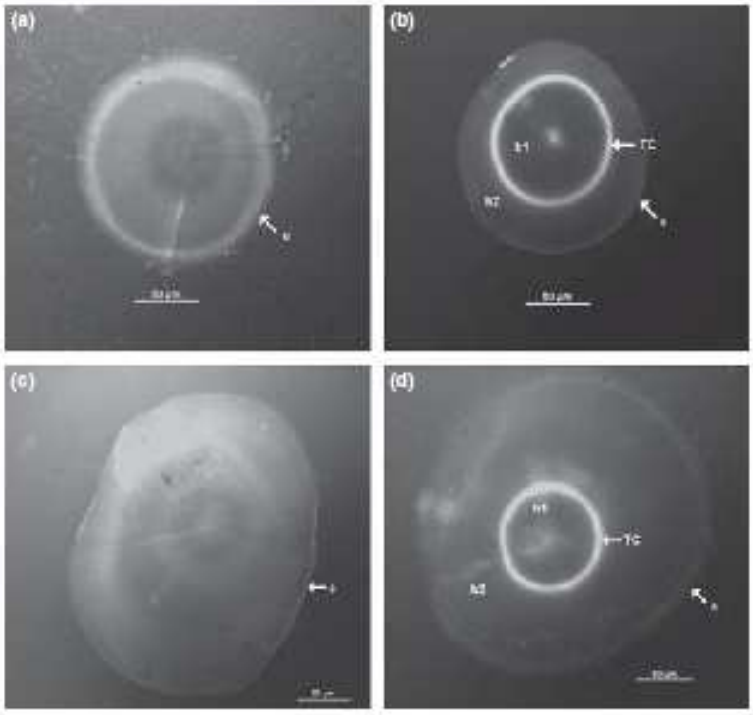

Figure 1. Photographs of control (left) and marked (right) allis shad otoliths. The bright ring corresponds to the tetracycline mark (TC). Luminosity of the background area was measured from the nucleus to the mark (b1) and from the mark to the edge of the otolith (b2). 'e' stands for otolith edge (bar scale: 50 µm). Panels are as follows: (a) Otolith of a 15-day-old larva from the control treatment, (b) Otolith of a 15-day-old larva from the 200-ppm–6-h treatment. (c) Otolith of a 32-day-old larva from the control treatment. (d) Otolith of a 32-day-old larva from the 200-ppm–6-h treatment.

intensity in the areas from the nucleus to the mark and from the mark to the otolith edge (Fig. 1b,d). Background fluorescence was subtracted from mark fluorescence to quantify marker intensity. For the visual estimation method, one reader was provided with unlabelled and randomly ordered photographs of otoliths. Each otolith was assigned a mark intensity score using the following classification: 0 = no mark; 1 = indistinct mark; 2 = distinct mark but low intensity; 3 = a clear bright mark; 4 = a large bright mark that can include more than one increment. The assignment was done twice and the average was calculated.

Statistical analyses were performed using the R software (R Development Core Team 2008). For the visual estimation, Kruskal–Wallis tests were performed to detect differences in mark quality among treatments, for 15- and 32-day-old larvae. Pairwise comparisons between treatments were performed using Wilcoxon tests, adjusted with a Bonferroni correction. For the pixel luminosity method, a one-way analysis of variance was applied to detect significant differences in mark and marker intensities between treatments, for each age class. Pairwise comparisons were performed using a *post hoc* Tukey test. Data for 32-day-old larvae needed to be log transformed to meet the normality and homogeneity of variances criteria. A Pearson correlation coefficient was used to test the correlation between mark and background intensities. A Friedman test was used to compare mark intensity, expressed as a percentage, with the level of grinding. Pairwise comparisons between the levels of grinding were performed using Wilcoxon tests, adjusted with a Bonferroni correction. The same procedure was applied to background and marker intensities. For all statistical tests, the level of significance was 0.05 (Sokal & Rohlf 1997).

Whatever the marking protocol, 100% of the fish immersed into tetracycline exhibited a bright yellow-green band (Fig. 1b,d). Fluorescent marks were not observed in the otoliths of the control fish (Fig. 1a,c). Twenty-four hours after marking, mortalities were less than 1% for all the treatments except for the 300-ppm–6-h treatment that reached 5.5%.

Mark scoring significantly differed between treatments ($P < 0.001$ for both ages) for the visual estimation method (Fig. 2a). One and two pairwise comparisons were significantly different for 15- and 32-day-old larvae respectively (Table 1). Mark and marker intensities for the pixel luminosity method were significantly different between treatments ($P < 0.001$ for both ages) (Fig. 2b,c). Six and seven pairwise comparisons for mark intensity were significantly different for 15- and 32-day-old larvae respectively.

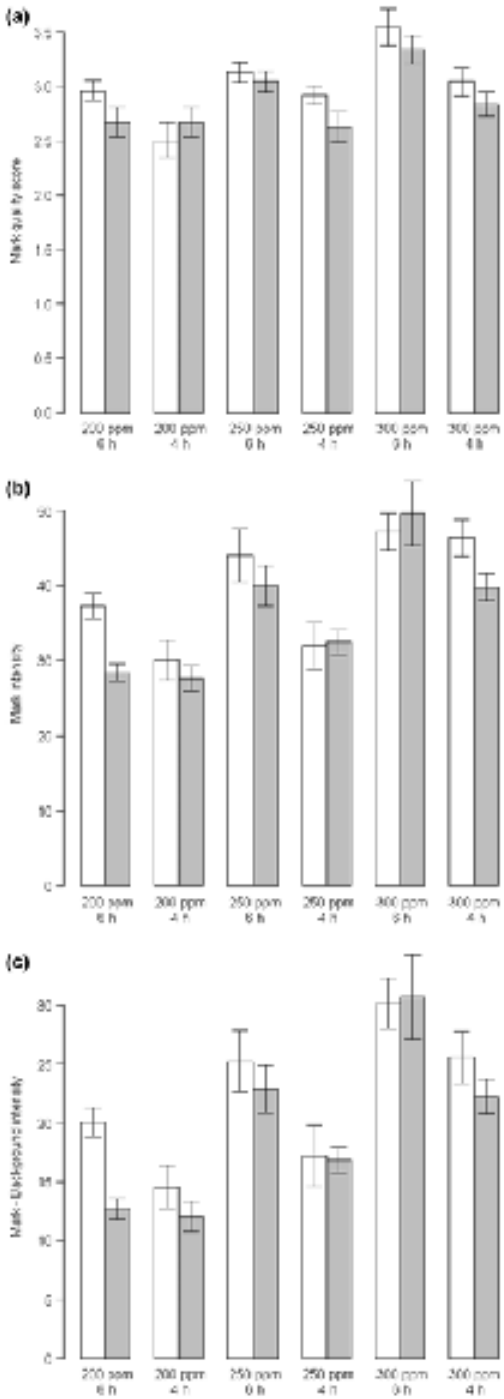

Figure 2. Marking success expressed as mark quality score after visual estimation (a), mark intensity (b) and marker intensity (mark minus background intensity) (c) for all treatments and for 15- (white bar) and 32-day-old larvae (grey bar). Data presented are mean (±SE).

Table 1. Probability of pairwise comparisons between marking treatments for 15- and 32-day-old larvae

	15-day-old larvae					32-day-old larvae					
Treatment	200 ppm 6 h	200 ppm 4 h	250 ppm 6 h	250 ppm 4 h	300 ppm 6 h	200 ppm 6 h	200 ppm 4 h	250 ppm 6 h	250 ppm 4 h	300 ppm 6 h	Method
200 ppm	0.24	–	–	–	–	1	–	–	–	–	1
4 h	0.43	–	–	–	–	0.99	–	–	–	–	2
	0.46	–	–	–	–	0.98	–	–	–	–	3
250 ppm	1	0.06	–	–	–	0.66	0.66	–	–	–	1
6 h	0.5	**0**	–	–	–	**0.003**	**< 0.001**	–	–	–	2
	0.53	**0.01**	–	–	–	**< 0.001**	**< 0.001**	–	–	–	3
250 ppm	1	0.29	0.58	–	–	1	1	0.4	–	–	1
4 h	0.74	0.99	**0.03**	–	–	0.64	0.34	0.19	–	–	2
	0.93	0.94	0.1	–	–	0.16	**0.03**	0.16	–	–	3
300 ppm	0.2	**0.02**	0.45	0.12	–	**0.04**	**0.04**	0.51	**0.03**	–	1
6 h	0.12	**< 0.001**	0.96	**0.002**	–	**< 0.001**	**< 0.001**	0.15	**< 0.001**	–	2
	0.01	**< 0.001**	0.6	**0.001**	–	**< 0.001**	**< 0.001**	0.2	**< 0.001**	–	3
300 ppm	1	0.2	1	1	0.36	1	1	1	1	0.08	1
4 h	0.18	**0.001**	0.99	**0.005**	0.99	**0.002**	**< 0.001**	0.99	0.16	0.18	2
	0.46	**0.007**	0.99	0.08	0.66	**< 0.001**	**< 0.001**	0.99	0.19	0.17	3

Probabilities were computed for each method of mark quality evaluation: (1) visual estimation, (2) mark intensity, (3) marker intensity, which is mark minus background intensity. For visual estimation, pairwise comparisons were performed using Wilcoxon tests adjusted with a Bonferroni correction. For mark and marker intensities, pairwise comparisons were performed using a *post hoc* Tukey test. Level of significance = 0.05

Table 2. Mark, background and marker intensities for otoliths ground at different levels: level 1 (otolith barely ground), level 2 (nucleus reached), level 3 (beyond the nucleus plan)

	Mark intensity	Background intensity	Marker intensity
Level 1	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Level 2	74.7 ± 9.7	63.5 ± 11.8	88.5 ± 17.1
Level 3	50.1 ± 10.0	48.3 ± 12.5	52.3 ± 17.2

Intensities are expressed in percentage, where level 1 was arbitrarily set as 100%. Values are mean ± SD.

The significance of the pairwise comparisons remained the same for marker intensity as for mark intensity, except for three of 30 cases (Table 1). A significant positive relationship was found between background intensity and mark intensity ($P < 0.001$), revealing that the higher the background intensity, the higher the mark quality. Finally, mark, background and marker intensities decreased with the level of grinding ($P < 0.001$) (Table 2). Level 1 mark and background intensities was significantly higher than that of level 2 ($P < 0.001$), which was significantly higher than that of level 3 ($P < 0.01$). Level 1 and level 2 marker intensities were not significantly different ($P = 0.75$), but they were higher than that of level 3 ($P < 0.005$).

The present note reveals the much higher discriminating power of the pixel luminosity method than the visual estimation method. Indeed, the number of significant pairwise comparisons between treatments was much higher for the former method than for the latter. The pixel luminosity method was capable of identifying gradual differences between treatments, while the visual estimation method was only capable of discriminating mark quality between treatments that were distinctly different (e.g. low concentration/short duration vs high concentration/long duration). Other elements in addition to mark quality are required for a marking protocol to be efficient, including minimal fish mortality and lack of behavioural change (Bumguardner & King 1996; Van der Walt & Faragher 2003). Based on the visual estimation method, the 300-ppm–6-h treatment appeared to be the most appropriate marking protocol, but it would be unsuitable for mass marking because it induced the highest mortality. Using the visual estimation method, the other treatments provided similar results in terms of mark quality, implying that treatments are equally suitable. On the contrary, the improved discriminating power of the pixel luminosity method optimises the choice for the best marking protocol.

The results also showed that mark luminosity greatly depends on background luminosity. Background luminosity depends on the amount of material in the optical pathway, including otolith thickness, the thickness of the microscope slide and the amount of resin used to embed the otoliths. Thus, it can be difficult to control background luminosity. In the present study, the preparations were carefully standar-

dised using the same kind of slides and a comparable quantity of resin to mount the otoliths and by using otoliths of different but close age (and thus width). Such precautions probably explain the similar significance of the pairwise comparisons between marker and mark intensities. However, such cautiousness may not be possible in a routine check of mark quality as these preparations are time consuming. Thus, it is strongly recommended that the background intensity is subtracted when reporting mark intensity to remove any background effect and reveal the true success of the marker incorporation.

The decrease in mark and background intensities with the level of grinding shown in the present study highlighted the importance of standardising the level of grinding for each otolith. As reported in other studies, over-grinding otoliths can alter mark intensity as too much material is removed (Reinert *et al*. 1998). At the same time, an otolith that is not sufficiently ground can emit too much auto-fluorescence for the detection of the mark to be reliable (Hernaman *et al*. 2000). The pixel luminosity method also has to be applied on standardised images; the magnification at which otoliths are observed, UV light intensity and the camera parameters must be the same otherwise, the intensities reported would be biased. Consequently, the method would not be applicable for structures showing such a high level of fluorescence that they require an adjustment of the fluorescent light intensity for each sample. Such phenomenon has been reported, for example, when detecting the fluorescence on juvenile mulloway, *Argyrosomus japonicus* (Temminck & Schlegel), anal fin spines (Taylor, Fielder & Suthers 2005).

Provided good standardisation, the pixel luminosity method offers a good alternative for handling the subjectivity and the limits of human eye luminosity discrimination. In addition, it can be used by anybody, even non-experienced assessors of mark quality. Thus, assessing mark quality by quantifying its intensity on a grey-level scale is an impartial and reliable criterion.

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