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The use of alizarin complexone for immersion marking of the otoliths of embryos and larvae of the turbot, *Scophthalmus maximus* (L.): dosage and treatment time

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Abstract The purpose of this work was to determine the efficacy of marking turbot, *Scophthalmus maximus* (L.), eggs and larvae with alizarin complexone (ALC) for use in enhancement programmes and in studies of the ecology and behaviour of the species. The main aim was to determine the optimum dosage and treatment time for large batches of larvae destined for release into the sea.

Eggs and larvae were immersed in a range of ALC concentrations (0–60 mg L^{-1}) for various times (3–24 h). On day 8 after hatching, eggs marked with the different ALC dosages had 99% marking success and had mainly good-quality or very-good-quality marks, whereas larvae achieved 100% success and had mainly very-good-quality marks. The best results were obtained after marking larvae with ALC at 60 mg L^{-1} for 6–24 h.

During the first months after marking, the fluorescent rings formed in the otoliths were easily detected when directly examined with a UV microscope, no prior preparation of the otoliths was necessary, but after 7 months the marks become faint as the otoliths get thicker, so it is necessary to cut or polish the otoliths before examination.

KEYWORDS: Alizarin, larvae, otolith marking, *Scophthalmus maximus*, turbot eggs.

Introduction

To enable a rigorous evaluation of stock-enhancement programmes, a means of identifying the released fish upon recapture is required (Støttrup 1996). Many methods are available for marking juvenile fish, but almost all, for example Floy tags (Floy Tag, Inc., Seattle, WA), involve handling individual fish. For very large batches this would be labour intensive and expensive (Blom, Nordeide, Svåsand & Borge 1994). Because the application of chemical marks does not require individual handling of the fish, this technique has considerable potential for the mass-marking of eggs, larval and juvenile fish (Tsukamoto & Umezawa 1988; Secor & Houde 1995).

Immersion in alizarin complexone (ALC) has been used to mark the otoliths of the early life stages of several groups of fish including salmonids (Tsukamoto 1988; Nagata, Nakajima & Okada 1995), sparids (Tsukamoto, Kuwada, Hirokawa, Oya, Sekiya, Fujimoto & Imaisumi 1989; Secor & Houde 1995), Atlantic cod, *Gadus morhua* L. (Blom *et al.*

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1994), red drum, *Sciaenops ocellatus* (L.) (Thomas, Holt & Arnold 1995) and plaice, *Pleuronectes platessa* L. (Støttrup, personal communication).

The objectives of this study were to: (1) adapt the ALC marking methodology to the turbot, *Scophthalmus maximus* (L.); (2) compare marking quality of eggs and newly hatched larvae to determine the optimum dosage (ALC concentration and immersion period); and (3) assess mark durability without prior preparation of the otoliths by keeping marked individuals in the laboratory for 12 months.

Materials and methods

Turbot eggs and larvae were obtained by dry fertilization from a broodstock subjected to artificial photoperiod (16 L : 8 D) (Forés, Iglesias, Olmedo, Sánchez & Peleteiro 1990) at the Spanish Institute of Oceanography in Vigo (NW Spain) in July 1994.

ALC was dissolved in about 100 ml of distilled water before dilution in sea water, and then the pH was adjusted to 7–7.5 with 1 mol L^{-1}. During immersion of the eggs and larvae, water temperature was 16–18°C, salinity 34–35‰ and oxygen concentration 6–8 mg L^{-1}.

In short experiments, groups of 500 eggs (1 day before hatching) and 100–200 larvae (2 days post-hatching) were immersed in solutions of 0, 20, 40 and 60 mg ALC L^{-1} in 1-L plastic beakers for 3, 6, 12, 18 and 24 h. After the treatment, they were transferred to 5-L plastic beakers of sea water (35‰ salinity) and held under aeration until the larvae were 8 days old (hatching of the eggs occurred during this period). Temperature (°C), salinity (S‰), oxygen concentration (mg L^{-1}), light intensity (lux) and pH were measured during the experiments. When the larvae were 3 days old, rotifers enriched with the microalga *Isochrysis galbana* Parke were added to the beakers at a density of 3 ind. mL^{-1}. Dead larvae were removed from the tanks and counted every day, and mortality estimated 48 h after the treatment. Surviving larvae were preserved in 96% ethanol with Tris.

In longer-term experiments, nine batches of 2-day-old larvae were immersed in solutions of 20–60 mg ALC L^{-1} in 15-L aerated incubation tanks and in 15-L aerated plastic bags, at densities of 2700–8200 larvae L^{-1}, for 6–24 h. After the treatments, the larvae were kept in tanks and fed live food (enriched rotifer and *Artemia*) at the beginning and formulated food after weaning (from day 35). Samples of 3–6 larvae and juveniles were taken every 15 days during the first three months and every month subsequently.

The total length (TL) of all larvae was measured to the nearest 0.1 mm before otolith extraction; juveniles were also weighed to the nearest 0.1 g. The otoliths (sagittae and lapilli in larvae and only sagittae in juveniles) were dissected out and mounted directly on glass slides with Eukitt (O. Kinder GmbH & Co.). For the short experiments, the otoliths from 20 larvae in each lot were extracted, while from the longer-term experiments the otoliths from all the larvae and juveniles taken in the samples were examined. These otoliths were not prepared before analysis of mark quality, no cutting or polishing was performed; they were directly observed with a UV-light microscope using a Zeiss 487715 epi-fluorescence filter with a 546 nm excitation filter. Mark quality was subjectively classified as absent, faint, good or very good with respective values of 0, 1, 2 and 3 (Figs 1 and 2). The mean mark quality per

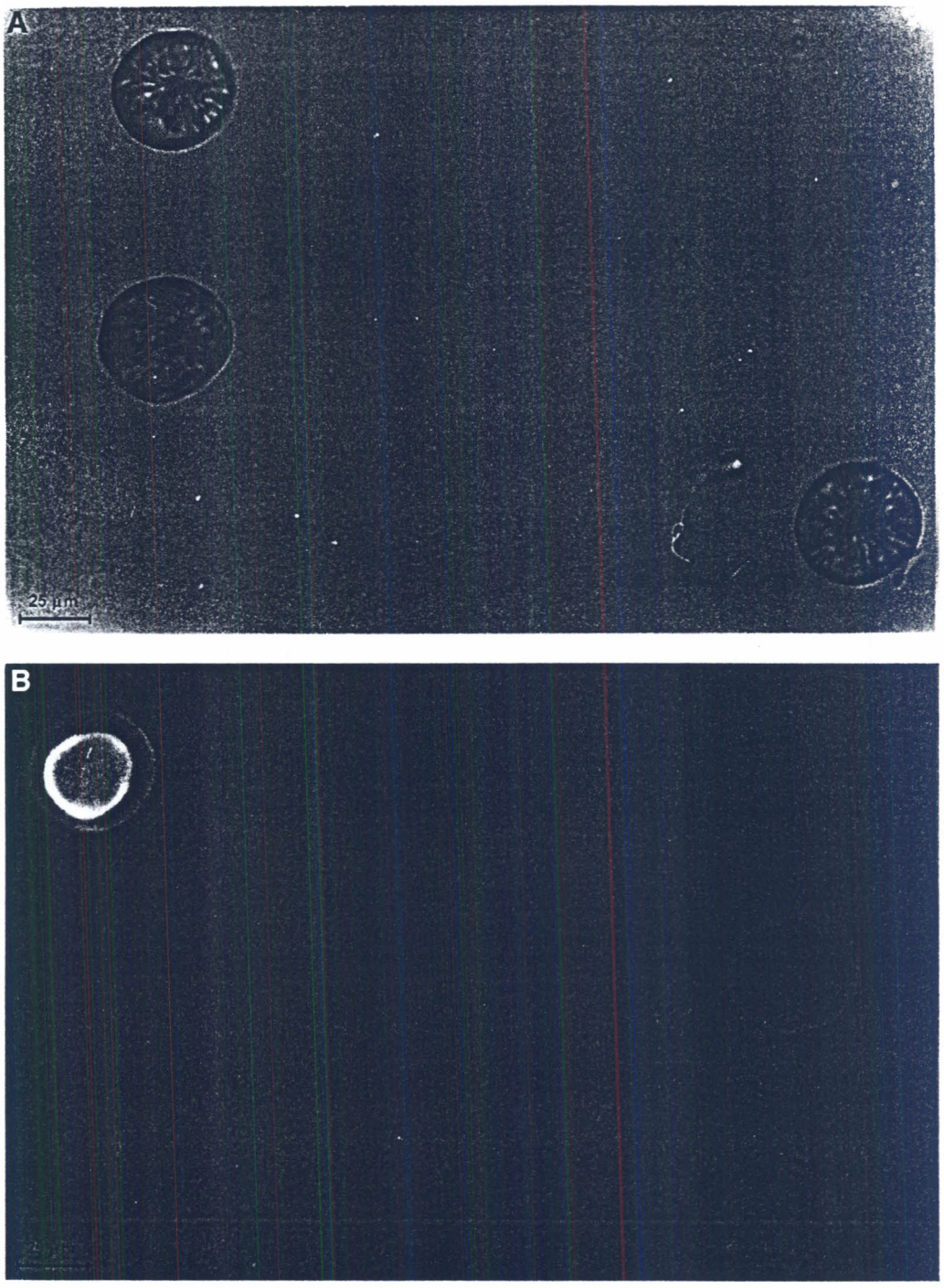

Figure 1. Legend on page 410.

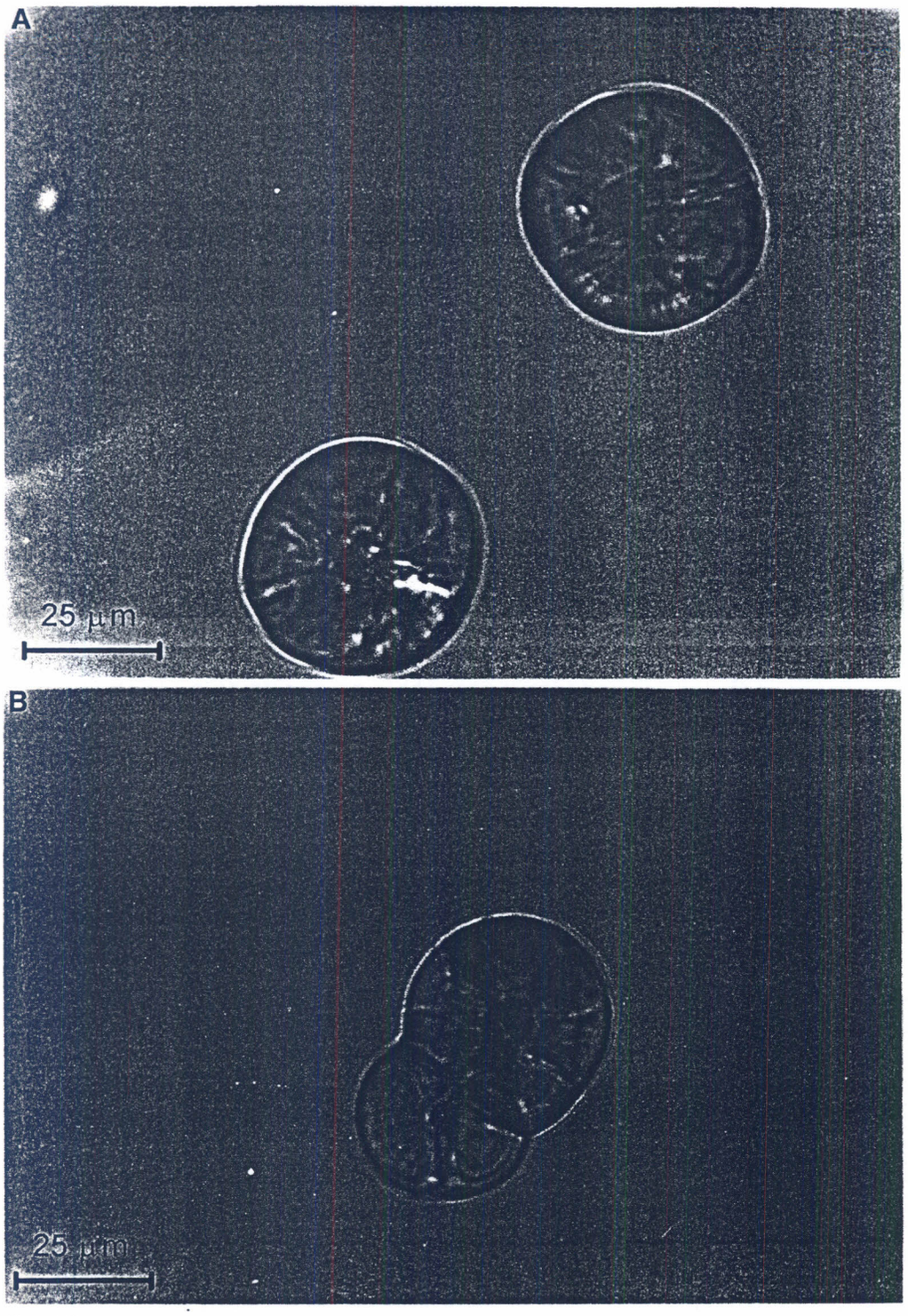

Figure 2.

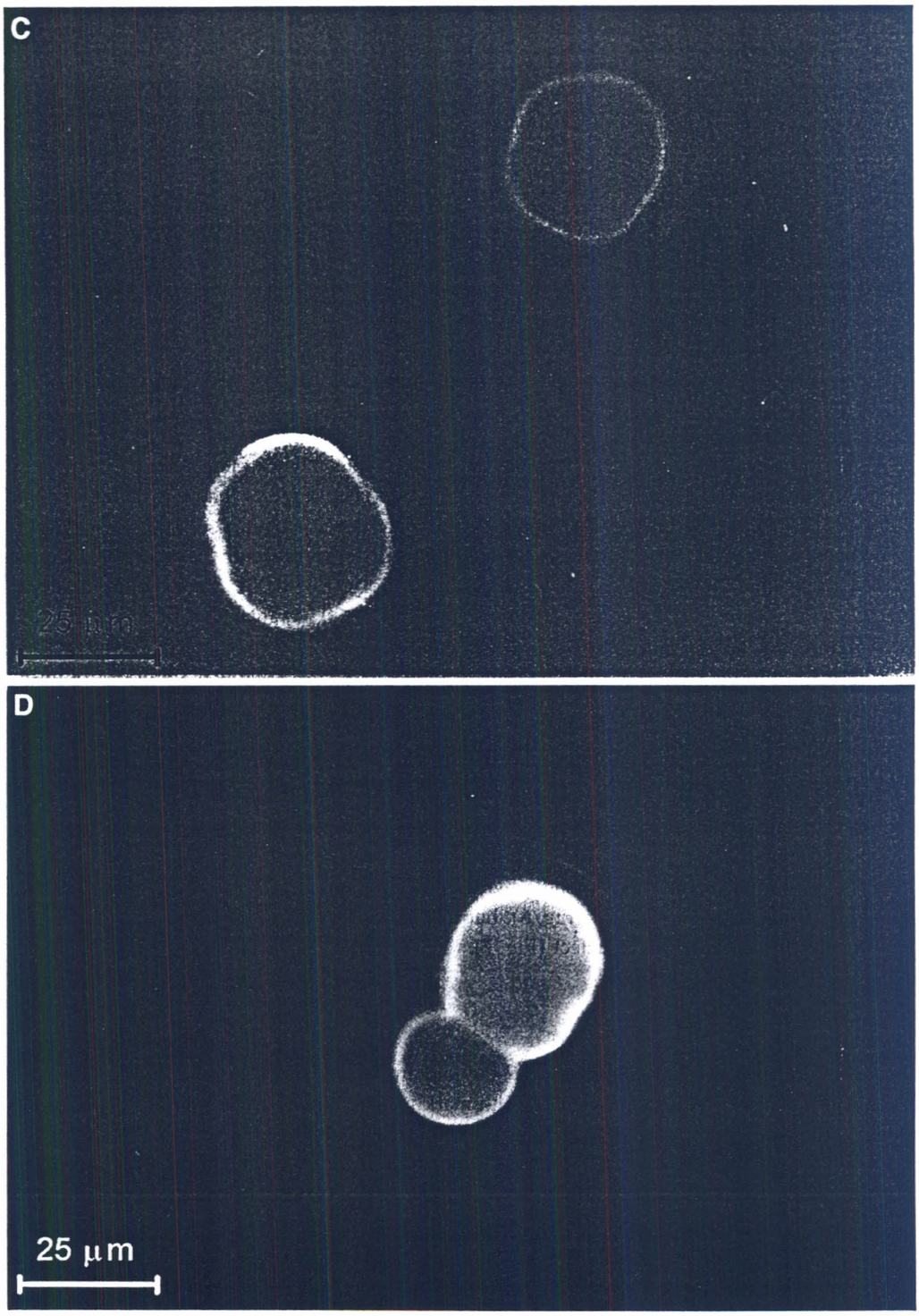

Figure 2. Different marking qualities of sagitta otoliths from 7-day-old larvae of 4.2 mm TL immersed for 12 h in 40 mg L^{-1} ALC solution 2 days after hatching. (A, B) Quality 2. (C, D) Quality 3. (A, C) Normal white light. (B, D) UV light.

treatment (Thomas *et al.* 1995) was used for the statistical comparison of eggs and larvae marks, ALC concentrations and immersion times.

A Wilks–Shapiro test was used to check the normality of the samples. One-way analysis of variance (ANOVA) was used to detect significant differences among treatments. Egg and larval mortality after the treatment was represented by exponential regression, and a slope-comparison *t*-test was used to compare both curves after linear transformation of data.

Results

Eggs marked with ALC concentrations from 20 to 60 mg L^{-1} for 3–24 h had 99% marking success and had mainly good-quality marks, whereas larvae achieved 100% marking success and had mainly very-good-quality marks (Figs 3 and 4).

The quality of otolith marks from larvae marked as eggs was significantly lower (ANOVA, $P < 0.01$) than those marked as larvae (Figs 3 and 4). Furthermore, 48 h after immersion in ALC, mortality of larvae immersed as eggs was significant higher (slope-comparison *t*-test, $P < 0.05$) than that of those marked as larvae (Fig. 5). This suggests that it is better to mark 2-day-old larvae instead of eggs during their embryonic development.

With respect to ALC concentration, mortality did not differ significantly between the different treated groups and the control groups within each category ($P > 0.05$). Mark quality in larvae marked with 20 mg ALC L^{-1} was significantly lower ($P < 0.05$) than that of larvae marked with 60 mg ALC L^{-1}.

With respect to the treatment time, mortality of eggs marked for 3 h was significantly lower than that of eggs marked for $\geq$ 12 h. But eggs and larvae immersed in ALC for 3 h had a significantly lower quality of marks ($P < 0.05$) than those marked for 18 and 24 h.

In the longer-term experiments, tanks proved more suitable than plastic bags for the treatment because of easier handling, a more homogeneous distribution of larvae, and avoidance of a problem related to the bags, that of the production of foam on the surface where there is a higher accumulation of larvae. These marked larvae were reared in the laboratory for 12 months and during the first 2–3 months the fluorescent rings formed in the otoliths were of mainly good or very good quality (Fig. 6), but thereafter the marks became faint and after 7–8 months they were difficult to detect (Fig. 7).

Discussion

It is possible to use ALC for marking 2-day-old turbot larvae with excellent results with respect to the clarity of the fluorescent otolith mark using UV microscopy. The data show that concentrations of 60 mg ALC L^{-1} and immersion periods of 6–24 h are optimal for marking

Figure 1. Marked (right) and unmarked (left) sagitta otoliths extracted from two 7-day-old turbot larvae of 4.85 and 4.55 mm total length (TL), respectively. Both photographs are of the same sample but with normal white light (A) and UV light (B). The marked otolith was extracted from a larva treated for 24 h in 20 mg L^{-1} ALC solution 2 days after hatching. 1, fluorescent mark; 2, edge of otolith.

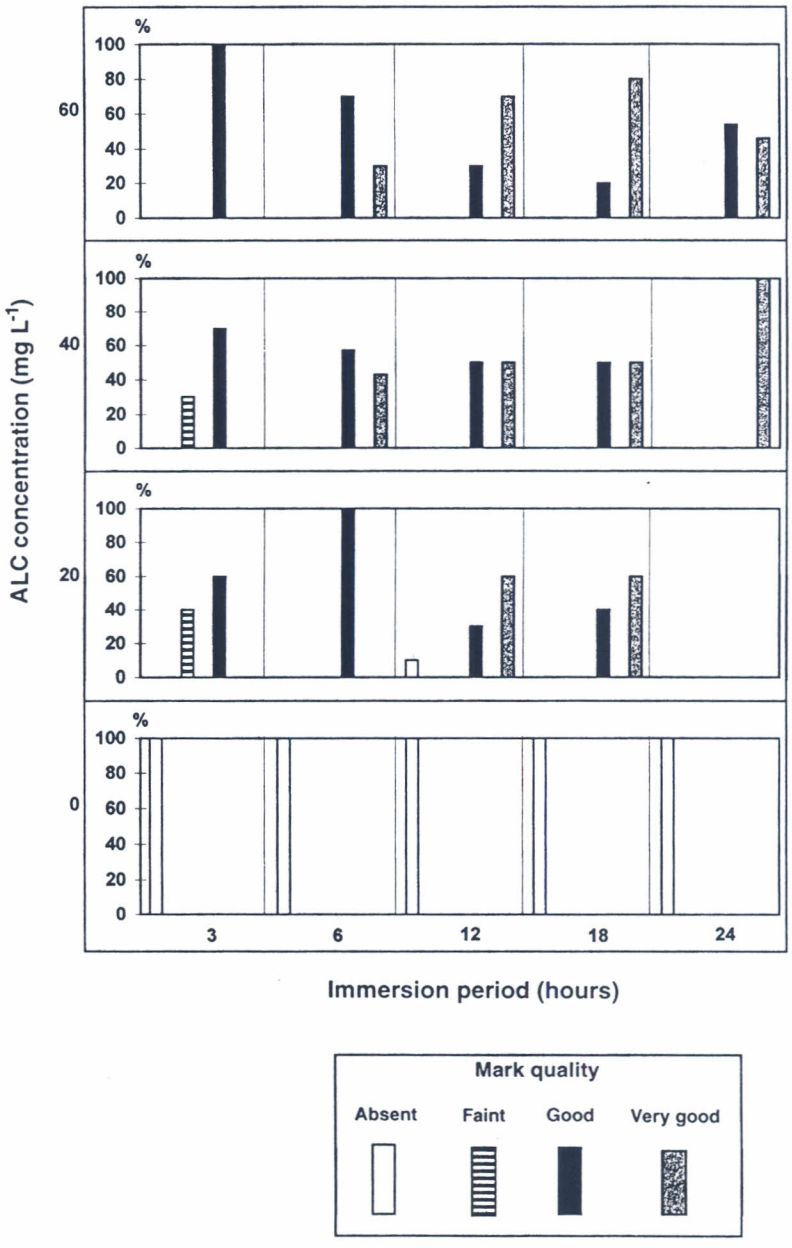

Figure 3. Quality of otolith marks from larvae sampled 9 days after immersion of eggs in 0–60 mg L^{-1} ALC solutions for 3–24 h.

otoliths. Pre-hatching eggs can also be marked, but the mark quality is lower and the mortality higher.

Several authors compared the efficacy of immersion-marking otoliths of some fish species

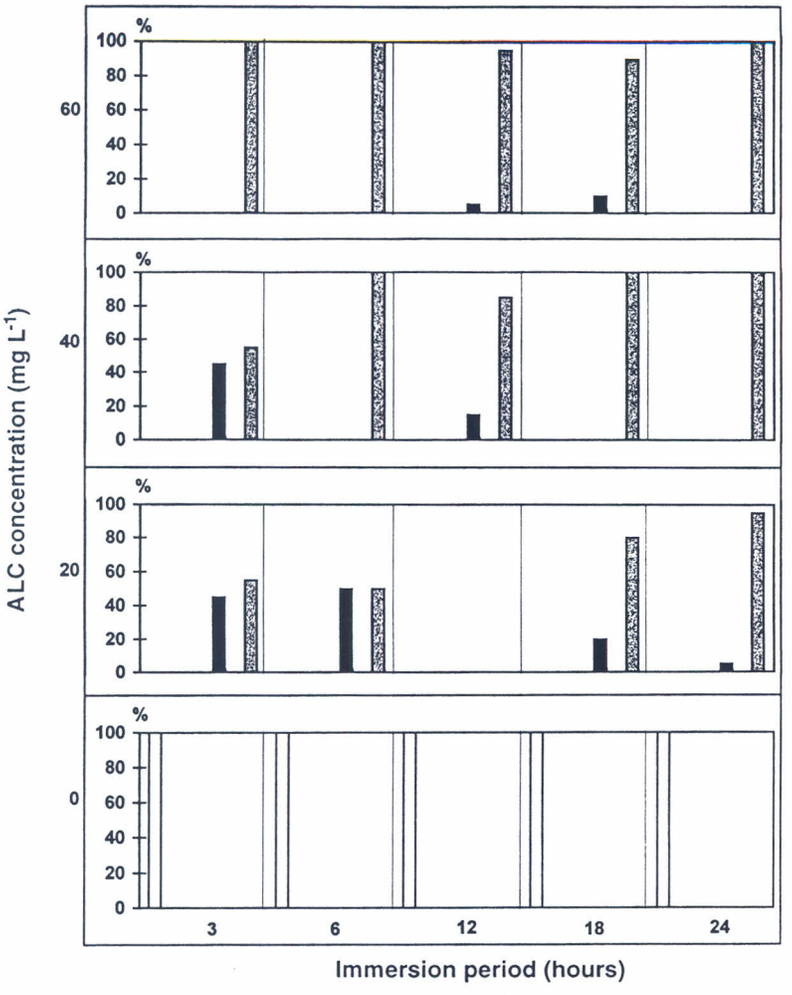

Figure 4. Quality of otolith marks from larvae sampled 6 days after immersion of larvae in 0–60 mg L^{-1} ALC solutions for 3–24 h. Mark quality key as in Fig. 3.

using different ALC concentrations. Optimum conditions for treatment were 50–200 mg L^{-1} for ayu, *Plecoglossus altivelis* T. & S. (Tsukamoto 1988) and Atlantic cod (Blom *et al.* 1994), while Monteleone, Houde, Secor & Morin (unpublished data) achieved the best results with 25–50 mg L^{-1} and J. Støttrup (personal communication) with 50 mg L^{-1}. On the other hand, only a few authors have compared the results obtained using ALC during different immersion periods. For Monteleone *et al.* (1993), mark quality, survival and growth of larvae were best for embryos and larvae immersed for 6 h, while most of the authors (Tsukamoto 1988; Blom *et al.* 1994; Støttrup, personal communication) used only 24 h.

Otolith marks were clearly recognizable during the first months after immersion with no

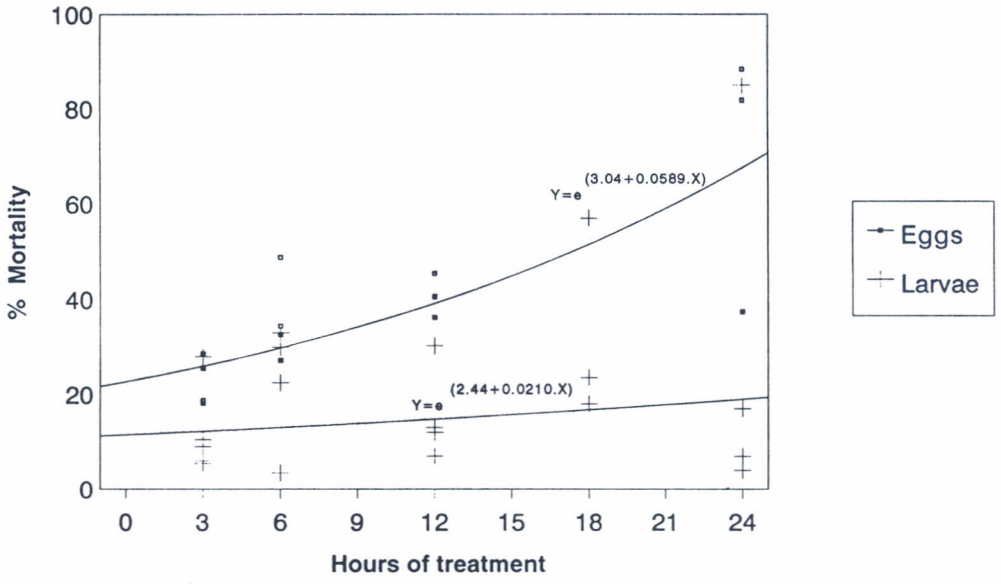

Figure 5. Mortality percentage of turbot eggs and larvae 2 days after immersion in different ALC solutions and for various hours of treatment. The different ALC concentrations are not described on the figure as mortality did not differ significantly ($P > 0.05$) between the treated and control groups within each category with respect to ALC concentration.

pre-treatment of the otoliths required. This suggests that marking with ALC can be very useful in field experiments on larval dynamics and recruitment. It is possible to estimate growth, mortality and distribution patterns of species of commercial interest prior to recruitment through experimental releases of marked larvae. For example, Tsukamoto *et al.* (1989) marked and released red sea bream, *Pagrus major* (Temminck & Schegel), juveniles into a small bay ($< 15\ km^2$), and recaptures permitted the estimation of abundance, dispersal and size-dependent mortality. Secor & Houde (1995), working with anadromous and marine species, evaluated the potential of larval mark–release experiments to make contributions to fisheries research. They concluded that these experiments were feasible for anadromous species, where released larvae could be retained within closed systems, while in marine (open) systems they could be used to test predictions on larval movements and short-term survival and growth.

It is possible to mark several million larvae of a species and to release them in a place where they are found naturally, and to follow their drift and dispersal patterns over a number of days. An easy way of identifying and subsequently tracking this patch of larvae at sea is using a free-drifting drogue. At different intervals during the following hours or days, the area surrounding the drogue can be sampled by towing the appropriate nets in horizontal, vertical or oblique trajectories. This will permit measurement of the distribution and the decrease in density within the patch of larvae. For example, Heath & MacLachlan (1987) found a patch of wild herring, *Clupea harengus* L., larvae in the Outer Hebrides and immediately deployed

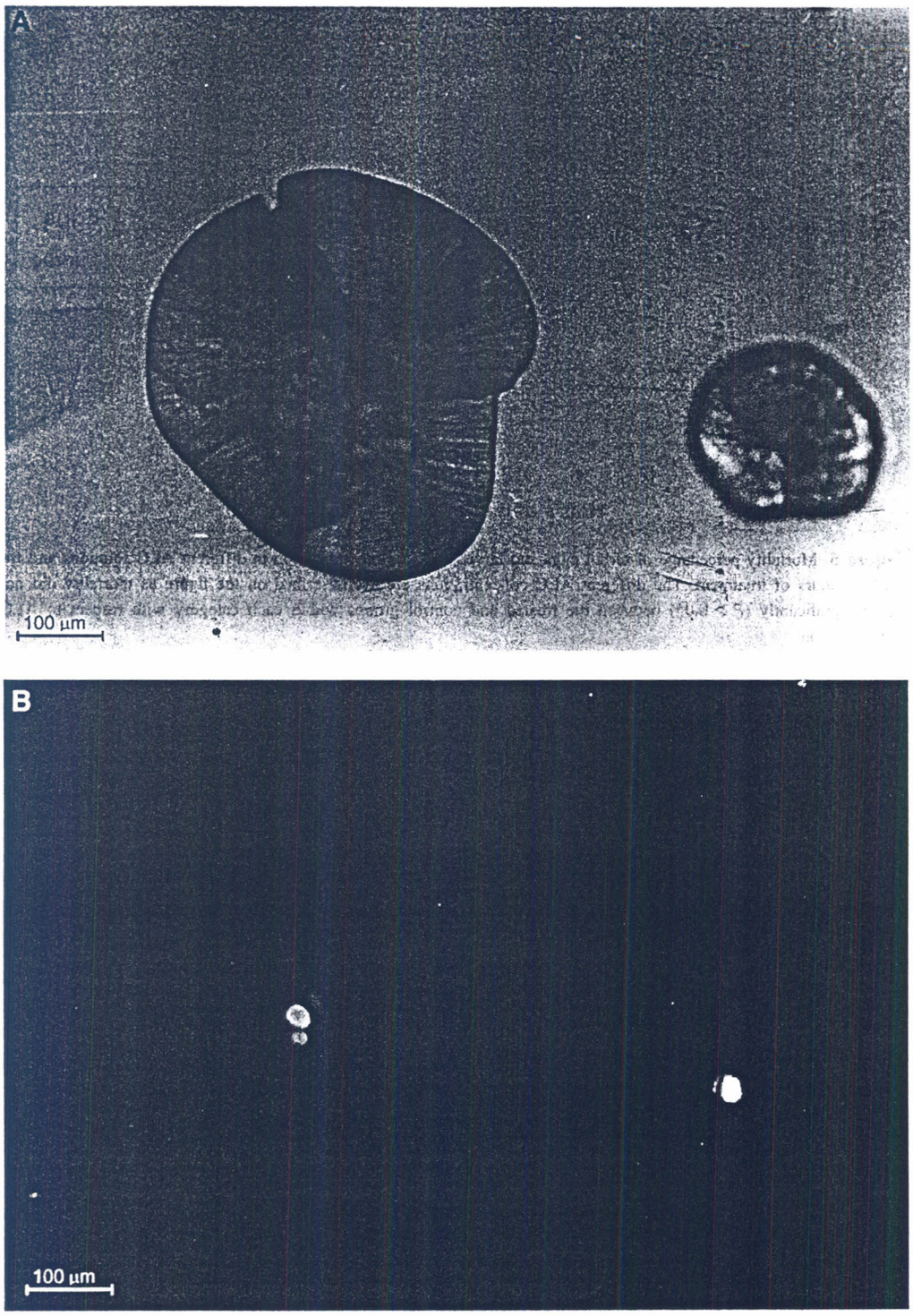

Figure 6.

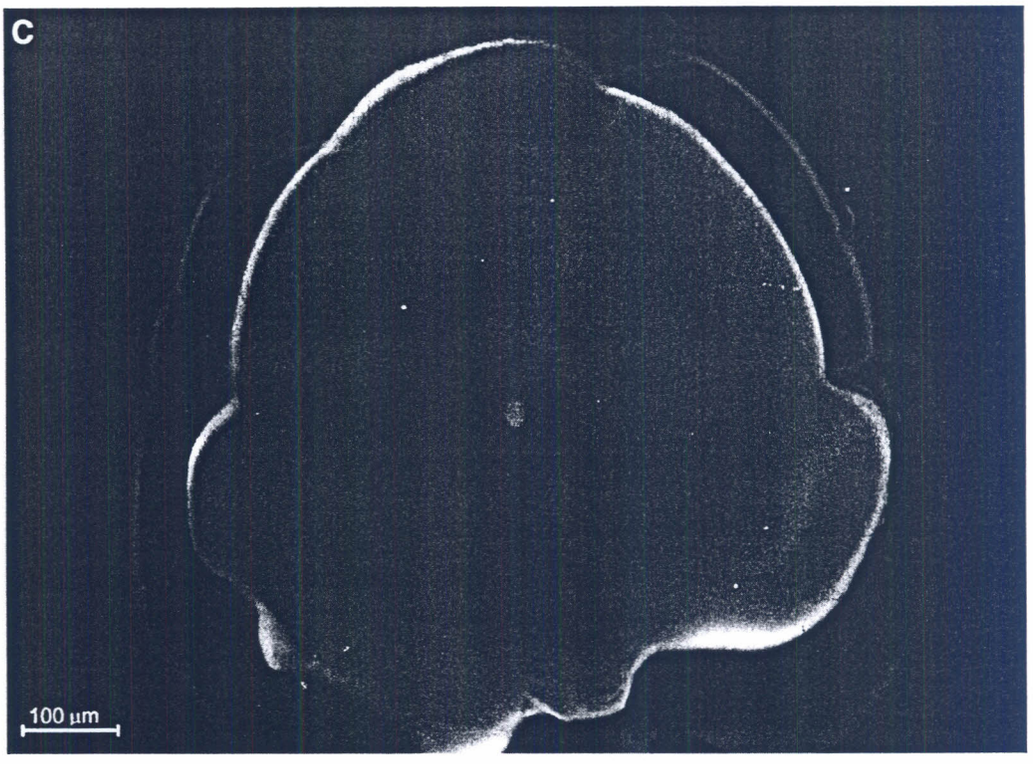

Figure 6. Otoliths from 30-day-old (A, B) and 58-day-old (C) fish treated for 12 h in 60 mg L^{-1} ALC solution. Total length was 1.5 and 2.7 cm, respectively. (A, B) Sagitta and lapilli otoliths marked 2 days after hatching; normal white light (A) and UV light (B); arrows are as in Fig. 1. (C) Sagitta otolith marked 1 and 50 days after hatching; 1, first fluorescent mark (day 1); 2, second fluorescent mark (day 50); 3, edge of otolith.

a parachute drogue buoy at the centre of the patch. During the following 7 days they sampled the area surrounding the drogue, thus estimating the mortality and dispersal of larvae. A similar study was carried out by Fortier & Leggett (1985) with capelin, *Mallotus villosus* (Müller).

The release of larvae previously marked with ALC eliminates some of the problems found in the studies cited above. One of the most important is the assumption that the patch represents the product of an instantaneous point release of larvae. This may not be true where larvae emerge from a spawning locality, or when it is possible that the larvae found in different samples throughout experiments have not originated from the original cohort located in the first coverage. Finally, if it is possible to account for dispersal of the larvae, this will permit a more precise determination of mortality rate by subtracting the contribution of dispersion from the decline in larval density recorded around the drogue. These studies may also provide a useful means of investigating growth, feeding, etc. of fish larvae in nature.

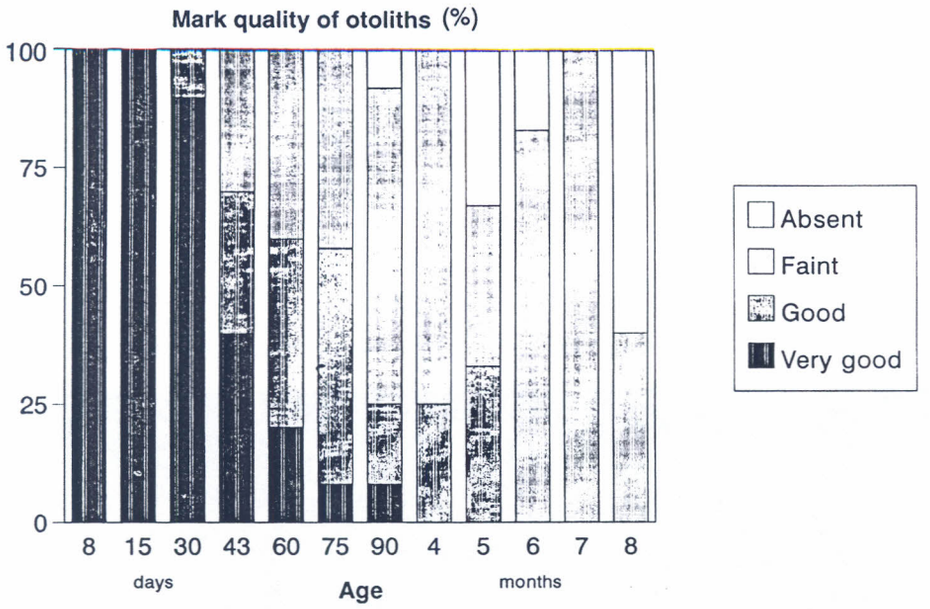

Figure 7. Quality of otolith marks from larvae and juveniles sampled during the first months of life. Fish had been treated for 12 h in 60 mg L^{-1} ALC solution 2 days after hatching. No pre-treatment of the otoliths was performed before examination.

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