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Tetracycline marks visible in Baltic cod *Gadus morhua* otoliths stored for 40 years

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Baltic cod *Gadus morhua* otoliths marked with oxytetracycline (OTC) in 1974–1975 had been sectioned and the sections stored in paper envelopes at room temperature in dry, dark conditions. After 40 years of storage, the historic OTC marks were still clearly visible showing that OTC is suitable for long-term chemical marking of otoliths. They were, however, noticeably paler than marks in recently recaptured and archived Baltic *G. morhua* otoliths, chemically tagged with tetracycline at similar dosage to the historic otoliths.

Key words: artificial tagging; Baltic Sea; *Gadus morhua*; mark-recapture; otolith sections; tetracycline.

Chemical tagging of otoliths is an important tool for studying ecological processes such as habitat connectivity, movement patterns or growth in fishes (Gillanders, 2009). One of the best methods available for age validation in commercially used fish stocks is the mark-recapture of chemically-tagged wild fishes (Campana, 2001).

Oxytetracycline (OTC) hydrochloride is a fluorescent calcium-binding substance, widely used as a chemical marker in fishes (Campana, 1999). It produces clear fluorescent marks in the otolith when viewed with ultraviolet light (Fig. 1). Since first evidence of its usefulness for studying bone growth (Harris, 1960), OTC has been used since the 1960s to mark otoliths (Kobayashi *et al.*, 1964; Jensen & Cumming, 1967; Weber & Ridgway, 1967; Jones & Bedford, 1968). Mark loss over the lifetime of the fish is not reported and unlikely given the chemical stability of otoliths (Campana, 1999). For instance, Beamish & McFarlane (2000) found clear OTC marks in otoliths of chemically-tagged and recaptured sable fish *Anoplopoma fimbria* (Pallas 1814) that had spent up to 20 years in the wild.

OTC is, however, a light-sensitive chemical and it could be expected that the visibility of OTC marks would deteriorate in archived broken or sliced otoliths. This is an

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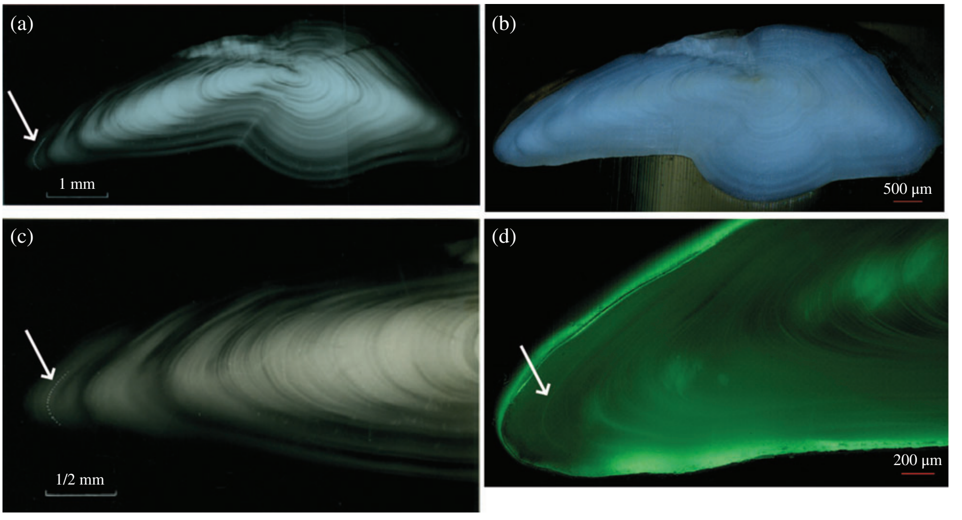

FIG. 1. (a) Reproduced from Fig. 2 of Bingel (1977), slice of a historic Baltic *Gadus morhua* otolith oxytetracycline (OTC)-marked and released on 22 September 1974 in the Arkona/Bornholm Sea, 58 cm total length (L_T), recaptured 21 February 2015, 63 cm L_T. (b) Image of transverse section of the remaining part of the same otolith in 2015. (c) Reproduced from Fig. 2 of Bingel (1977), zoom on the pointed end of the otolith in (a), OTC mark is indicated with white dots and an arrow. (d) Historic otolith section photographed in 2015, arrow indicates OTC mark. Images in (b) and (d) were taken under reflected light.

important issue because mark-recapture studies are costly and each chemically-tagged otolith is unique and of high scientific value when available, *e.g.* when serious discrepancies in ageing require re-evaluation of historic data series (Eero *et al.*, 2015). OTC marks in archived otoliths should be persistent to allow revisiting previously collected material, *e.g.* for use of future new analytical methods. The long-term persistence of OTC marks in stored otoliths had not previously been assessed.

Therefore, the life span of OTC marks archived in the mid-1970s was assessed to test their persistence on a time scale of decades and to compare them with tetracycline marks of recently archived otoliths.

In 1974 and 1975 OTC (stabilized solution, Farbwerke Hoechst AG Frankfurt/M; www.sanofi.co.uk/l/gb/en/index.jsp) was injected into the abdominal cavity of western Baltic cod *Gadus morhua* L. 1758 from Kiel Bight ($n = 56$; size range: 12–40 cm total length, L_T) for laboratory experiments (Bingel, 1977) and in wild eastern *G. morhua* from the Bornholm Basin in a mark-recapture experiment (size range of 42 recaptures: 31–81 cm L_T; Bingel, 1981*a*). In the laboratory experiments, OTC was injected up to three times. The solution was applied at a mean concentration of 56·7 mg OTC kg^{-1} fresh mass in the first injection, and 99 mg OTC kg^{-1} fresh mass in the second and third injections. In the mark-recapture experiment, a mean concentration of 100 mg OTC kg^{-1} fresh mass of fish was applied; the '1000 μl g^{-1} fish weight' given in Bingel (1981*a*) should read '1000 μl kg^{-1} fish weight'. External marks were used to identify individuals (Carlin tags for smaller and spaghetti tags for larger specimens).

The sagittal otoliths were removed, embedded in polyester and sliced at the level of the nucleus with a saw (thickness: 0·2 cm). For this study, however, only the remaining otolith sections (not the slices) were available. The sections had been stored in paper envelopes, which were put in a plastic box. The box was enclosed in a plastic bag and sealed with adhesive tape. Storage conditions were: dry at room temperature and air humidity, in darkness. The otoliths were stored in Kiel, Germany, before they were transported to Erdemli, Turkey, in the 1980s.

In 2014, stored *G. morhua* otolith sections were re-discovered from 1974 to 1975 and they were sent to Rostock. In total, 32 sections of different otoliths from 1974 to 1975 were re-discovered. Unfortunately, the historic otoliths could not be assigned to individual fish data anymore, but two individual otoliths, due to their unique characteristics, could be assigned to pictures available in Bingel (1977). An Olympus U-MF2 tetracycline filter set was used (excitation filter: 387/11 nm; dichroic filter: 405 nm; barrier filter: 525/50 nm) to make the OTC marks visible under a fluorescence light microscope (Olympus BX60; www.olympus.co.uk). Photographs were taken under reflecting light using the Cell∧D programme of Olympus.

For comparison between the quality of the OTC mark in historic and recent otoliths, wild western Baltic *G. morhua* that were chemically-marked with 100 mg tetracycline-hydrochloride (TET) (Carl Roth, www.carlroth.com/de/en; dissolved in physiological salt-solution) kg^{-1} fresh mass and released at Fehmarn (western Baltic Sea) in the fourth quarter of 2014 were used. Sagittal otoliths were removed from 40 recaptured *G. morhua* and the whole right otolith was embedded using GTS Polyester casting resin (Voss Chemie; www.vc-24.de/styrol.html; 35–40% Styrol) and a MEKP hardener. Then the otoliths were sliced (thickness: 0·5 mm) along their nucleus using an ATM Brilliant 250 bone saw (www.atm-m.com/products/cut-off-machines/benchtop/brillant-250) and the remaining otolith sections were archived for a few weeks like the historic otoliths. The settings for making photographs were the same as mentioned above. Four age readers independently assessed the 32 historic otoliths in terms of (1) the presence or absence of OTC rings, (2) the number of rings and (3) the ring quality of historic and recent marks.

All age readers agreed that the same 10 of the 32 otoliths (31%) contained one or more OTC marks. There is no information available anymore on whether or not the other 22 otoliths (lacking an OTC mark when re-inspected in 2015) were OTC-marked before. From the 10 otoliths containing OTC marks, five, three and two otoliths contained one ring (Fig. 1), one or two rings, or two or three rings (Fig. 2), respectively. In the three otoliths with one or two rings, two age readers identified only one ring, while two age readers saw two rings; in the two otoliths with two or three rings, three age readers identified two rings while one age reader saw three rings. The OTC marks in the 10 historic otoliths were continuous and still clearly visible, though all age readers agreed that they were noticeably paler than those from the recently tetracycline marked-recaptured and archived otoliths (Fig. 3). The paleness of the potential first OTC ring in the historic sections with multiple rings was the reason for the variation in OTC ring counts between age readers.

The assignment by all age readers of two historic otolith sections to photographs from Bingel (1977) showed that both the laboratory experiment and the mark-recapture of wild *G. morhua* of Bingel (1977) yielded persistent OTC rings (Figs 1 and 2). The two recognized historic otoliths in Fig. 1(a), (c) and Fig. 2(a) showed that the OTC marks of both the photographs in Bingel (1977) and from the same otoliths in 2015 were paler than the TET rings from a recent recapture (Fig. 3). The OTC rings in Bingel (1977)

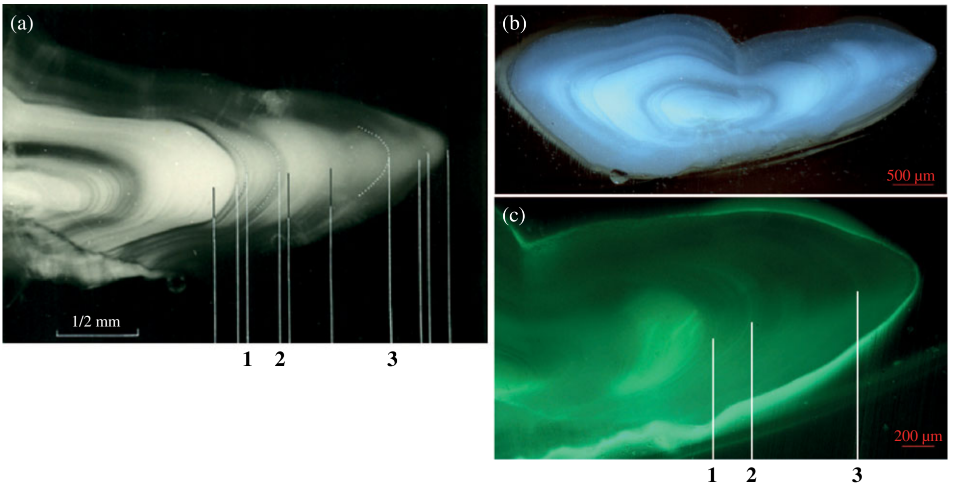

FIG. 2. (a) Reproduced from Fig. 14 of Bingel (1977), slice of a historic Baltic *Gadus morhua* otolith marked three times with oxytetracycline (OTC) during a laboratory experiment in 1974; innermost OTC ring 1 from April, 14 cm total length (L_T) (1); middle OTC ring 2 from May, 18 cm L_T (2); outer OTC ring 3 from October, 27·5 cm L_T (3). (b) Image of transverse section of the remaining part of the same otolith in 2015 showing that form and appearance agree with (a); image was taken under reflected light. (c) Historic otolith section photographed in 2015, lines 1, 2 and 3 indicate same OTC marks as in (a).

were visible mainly due to the dotting indicated on the figures, especially the first ring in Fig. 2(a).

OTC marks of archived embedded Baltic *G. morhua* otolith sections were still visible after 40 years of storage. Hence, OTC provides the same advantageous property (long-term persistence of the mark) for use in an age validation study as for example strontium chloride (Panfili *et al.*, 2002). The historic OTC marks, however, were obviously paler than TET marks in recent otoliths and it cannot be excluded that any of

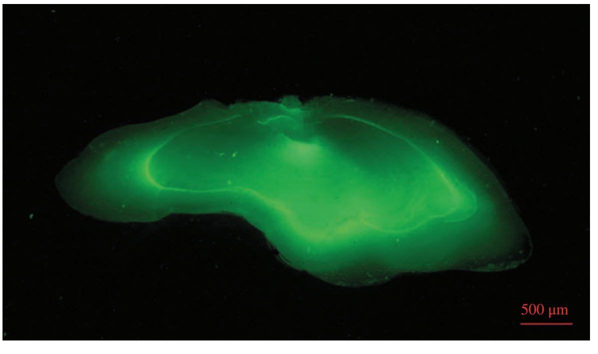

FIG. 3. Image of transverse section of an embedded Baltic *Gadus morhua* otolith (tag number 0623) marked with tetracycline-hydrochloride (TET) (dosage: 100 mg TET kg^{-1} fresh mass) on 29 October 2014 (18 cm total length, L_T) and recaptured on 23 September 2015 (28 cm L_T, female) on the south coast of Fehmarn (western Baltic Sea). Image taken in 2015. Note paler oxytetracycline rings in the historic otoliths of Figs 1 and 2.

the 22 historic otoliths without visible OTC marks (and without additional information about the otolith) had been chemically marked and had lost the OTC mark over time.

Images of both historic and recent otolith sections were taken under equal conditions, suggesting that the greater paleness of the former was real. In contrast, photographs from the historic otoliths in Bingel (1977) were obviously taken under optical settings different from 2015, so that a direct comparison is not possible. It is uncertain how the OTC marks appeared 40 years ago because equal optical settings could not be used. There are, however, reasons for a paler appearance of the historic otoliths. The mean OTC dosage of the first injection of the historic laboratory otoliths was only about half the dosage of the other injections; moreover, mean dosages in Bingel (1977) were given without S.D. In a recent experiment, a dosage of 100 mg TET kg^{-1} fish wet mass also yielded the clearest chemical marks in western Baltic *G. morhua* otoliths compared to TET dosages of 50 and 25 mg TET kg^{-1} fish wet mass (S. Stötera, A. Degen-Smyrek, U. Krumme, D. Stepputtis, B. Limmer & C. Hammer, unpubl. data). Hence, the lower dosage of the first injection in the historic otoliths could explain why some of the historic otoliths were paler. This may concern the five otoliths that contained only one mark (originating either from the laboratory or mark-recapture experiment), and certainly applied for the first mark of the five otoliths with multiple OTC rings. Unfortunately, historic otoliths could not be assigned to the original treatment. Yet, the paler historic OTC marks could also be partly due to the use of different slicing methods. While preparing a thin slice in the 1970s, a fissure in the otolith crystal texture occurred. These areas look like bright patches (F. Bingel, pers. obs.). Therefore, caution is warranted when deciding whether or not these structures are OTC depositions. Different deposition speeds and deposition sizes of the aragonite-crystals in the three-dimensional otolith may also affect visibility of OTC marks (Bingel, 1981*b*). The long-term persistence was ensured by storage without special diligence. Similar or even superior storage conditions may be encountered in the otolith archives of national fisheries laboratories. Storage in total darkness may be an important factor for long-term persistence of OTC/TET marks in otolith sections. Furthermore, the results show that a dosage of 100 mg OTC/TET kg^{-1} fish wet mass produced clear marks in otoliths of western Baltic *G. morhua* (historic and recent marks), eastern Baltic *G. morhua* (historic marks) as well as, *e.g.* in *A. fimbria* (Beamish & McFarlane, 2000). A dosage of 100 mg TET kg^{-1} fish wet mass yielded the best mark quality and highest survival (S. Stötera, A. Degen-Smyrek, U. Krumme, D. Stepputtis, B. Limmer & C. Hammer, unpubl. data).

Consequently, the use of OTC/TET provides a practical option for an age validation study urgently required in Baltic *G. morhua* (Eero *et al.*, 2015); the usefulness of the chemical (OTC/TET) was proven, the appropriate dosage (100 mg OTC/TET kg^{-1} fish wet mass) was determined, and clear, stable and easily recognizable marks in Baltic *G. morhua* otoliths were detected. In addition, the use of a fluorescence microscope for OTC/TET detection may render tagging with OTC a more feasible approach than for example the use of a scanning electron microscope for strontium chloride detection.

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