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ORIGINAL ARTICLE

Micro-X-ray fluorescence image analysis of otoliths to distinguish between wild-born and stocked river-spawning whitefish captured in the Baltic Sea

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Abstract

Strontium concentrations are low in fresh waters compared to seawaters. Therefore, wild-born river-spawning and stocked freshwater-reared whitefish *Coregonus lavaretus* L. display regions with low concentrations of strontium in the centre of their otoliths. Strontium in otoliths from wild-born river-spawning whitefish ascending the River Tornionjoki, river-spawning whitefish stocked as one-summer-old fingerlings caught ascending the River Kemijoki, and sea-spawning whitefish caught near the Åland Islands was mapped using µ-XRF. The strontium-depleted regions in the centre of the whitefish otoliths, measured using ImageJ, had mean sizes of 0.18 ± 0.2 mm^2 (River Tornionjoki) and 2.3 ± 0.3 mm^2 (River Kemijoki), whereas the otoliths from whitefish caught at sea lacked a strontium-depleted region altogether. Measurement of the area of the strontium-depleted region in whitefish otoliths provides a convenient method with which to differentiate between whitefish of different provenance and to determine the origins of whitefish in mixed catches.

KEYWORDS
Gulf of Bothnia, River Kemijoki, River Tornionjoki

1 | INTRODUCTION

Whitefish *Coregonus lavaretus* L. in the Gulf of Bothnia, northern Baltic Sea (Figure 1) is composed of two sympatric ecotypes: anadromous river-spawning and sea-spawning whitefish. Besides their choice of spawning grounds, the ecotypes differ in migratory behaviour and the number of gill rakers. River-spawning whitefish migrate up the rivers from the sea during the summer–autumn period to spawn. After hatching in the spring, the larvae are flushed to the sea, normally within a few weeks (Jokikokko et al., 2012). After reaching the sea, many of these larvae initiate a southward migration towards their feeding grounds (Leskelä et al., 2002). The

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[Correction added on 19 January 2022, after first online publication: The copyright line was changed.]’

FIGURE 1 Map showing the location of the River Tornionjoki with the Kukkolankoski rapids (■), River Kemijoki, and the Yxskär Island (●) northeast of the Åland Islands in the Gulf of Bothnia, Baltic Sea

migration can occur over long distances (>700 km), for example from the northernmost part of the Gulf of Bothnia to the Åland Islands in the Archipelago Sea (Lehtonen, 1981). After reaching the feeding grounds, the whitefish may remain there for several years until reaching maturity after which they return to their natal rivers for spawning, although a small proportion of return spawners stray to non-natal rivers (Lehtonen, 1981; Leskelä et al., 2009). At the feeding grounds, river-spawning whitefish mix with sea-spawning whitefish in largely equal proportions (Linden et al., 2019). The sea-spawning ecotype spawns along the seashore in October–November and is found all along the Finnish coast. In contrast to the river-spawning ecotype, sea-spawning whitefish are relatively stationary and rarely migrate more than 25 km from the spawning grounds (Lehtonen, 1981; Leskelä, 2008; Wikgren, 1962). The two ecotypes have traditionally been distinguished by their differing number of gill rakers on the outermost gill arch with the river-spawning ecotype having on average 30 gill rakers and the sea-spawning ecotype 27 or fewer gill rakers (Himberg et al., 2015; Lehtonen, 1981; Svärdson, 1957). The number of gill rakers for the ecotypes form Gaussian-like curves with some overlapping, rendering this characteristic problematic to differentiate at the individual level.

The river-spawning stock of whitefish is listed as endangered (EN) by the HELCOM Red List of Baltic Sea species (HELCOM, 2013), as it has steadily declined since the 1990s. This decline has mainly been attributed to the loss of spawning grounds as a consequence of dam construction and eutrophication, and to overfishing (HELCOM, 2013; Urho, 2011). To reinforce the whitefish stocks, millions of one-summer-old fingerlings and tens of millions of free-embryos are stocked annually along the Finnish coast, with a major proportion stocked into the mouths of dammed rivers in the northern Gulf of

Bothnia (Jokikokko & Huhmarniemi, 1998). Stocking of one-summer-old fingerlings is considered to be more effective than stocking of free-embryos, an assumption supported by Finnäs et al. (2020). To obtain the whitefish used for stocking, mature female and male whitefish are caught during the spawning season to be stripped of their eggs and milt, respectively. The embryos hatch in the spring and are either released into the wild soon thereafter as free-embryos or are reared in freshwater ponds during the summer (≈6 months) to be released as fingerlings in the autumn. Stocked river-spawning whitefish are thought to display a similar migratory behaviour as its wild-born counterpart, undertaking the same foraging migration southward and subsequently return to the natal river upon maturation (Leskelä et al., 2004).

The River Tornionjoki is >500 km long remaining in its natural state located in the northernmost part of the Gulf of Bothnia at the border between Finland and Sweden (Figure 1). The Tornionjoki has not been subjected to any regular supplementary stocking in the 21st Century, and it is the main river in Finland for natural reproduction of river-spawning whitefish (Jokikokko et al., 2018). By contrast, River Kemijoki is the longest river in Finland (≈550 km) entering the sea ≈20 km southeast of the River Tornionjoki (Figure 1), and has a hydroelectric dam located 5 km from the river mouth, acting as a barrier to spawning grounds further upstream. To counter the strong decline in whitefish spawning in the River Kemijoki, annual supplementary stockings of fingerlings (≈3.1 millions) and a larger amount of surplus free-embryos occur below the lowest dam (Finnäs et al., 2020; Jokikokko & Huhmarniemi, 1998). As a consequence, whitefish ascending the River Kemijoki were dominated by fish stocked as free-embryos and fingerlings, the latter contributing 65% to whitefish in catches (Finnäs et al., 2020). Around the Åland Islands, the southern foraging grounds for the river-spawning ecotype, supplementary stocking of freshwater-reared free-embryos and fingerlings, mainly of the sea-spawning ecotype, is also undertaken to increase the local catches (Leskelä, 2008; Linden et al., 2019).

Stocked whitefish display a low concentration of strontium (≈200–2300 μg/g) in the central part of the otolith, reflecting the low, ambient concentration found in the freshwater ponds where the fingerlings were reared before stocking (Finnäs et al., 2020; Lill et al., 2018). This can be contrasted with otoliths from sea-spawning whitefish in the Gulf of Bothnia, which display a strontium concentration of ≈2600–4400 μg/g, reflecting the salinity-range of ≈1–7 PSU in the north–south direction in this area (Finnäs et al., 2020; Lill et al., 2018). Finnäs et al. (2020) use a combination of μ-XRF mapping of otolith strontium distributions and spot analysis using LA-ICP-MS to estimate the proportion of whitefish stocked as one-summer-old fingerlings in catches of adult fish ascending the River Kemijoki to spawn. The low-strontium concentration in the central region of the otoliths, corresponding to the time the stocked fish were reared in freshwater ponds, was displayed as a darker region in the μ-XRF maps, and proved a good identifier of fish stocked as fingerlings.

In the present work, the somewhat subjective visual inspection of the μ-XRF maps was replaced with image analysis. The areas of the entire otoliths, as well as the areas of the dark, strontium-depleted region in the centre of the otoliths, were measured. The sharp edges of the areas allow for accurate measurements. To demonstrate the usefulness of image analysis of μ-XRF maps to distinguish whitefish of different provenance, the approach was applied to whitefish caught ascending the River Tornionjoki hypothesised to represent wild river-spawning whitefish, to a subsample of whitefish from the River Kemijoki previously determined to be stocked as one-summer-old fingerlings (Finnäs et al., 2020), and to a sample of whitefish caught during the spawning season at Yxskär Island, northeast of the main Åland Islands, representing sea-spawning whitefish.

2 | METHODS

Whitefish from the River Tornionjoki (n = 10) were sampled using dip nets in July 2013 at the Kukkolankoski rapids located 20 km from the river mouth (Figure 1). The fish were ascending the river from the sea to spawn. A further sample of whitefish (n = 20) ascending the lowest reach of the River Kemijoki was caught with gillnets (45–50-mm knot distance) during the spawning season in October 2015. A subsample of these fish, consisting of 13 individuals that previously were identified as being stocked as one-summer-old fingerlings (Finnäs et al., 2020), was chosen for the present study. Whitefish (n = 20) from Yxskär Island were sampled from the spawning grounds in the sea during October–November 2018 using bottom gillnets (45-mm knot to knot mesh size) (Figure 1). Specimens were killed by a percussive blow to the head according to Directive 2010/63/EU, Annex IV. Total length, weight and sex of each sampled fish was recorded, gill rakers were counted and the sagittal otoliths were removed for age reading by counting of the annuli, and for elemental analysis using μ-XRF.

The otoliths were prepared as described in Finnäs et al. (2020). Briefly, the otoliths were embedded in epoxy and ground on the anti-sulcus side with stepwise finer sandpaper (800p–2500p) and finally polished using a 1-micron polish suspension (Kemet, Liquid Diamond Type WXXStr) until the core was clearly visible. The prepared otoliths were mounted on glass slides. Primarily, the left side otoliths were prepared but in instances where they were damaged or the grinding was unsuccessful, they were replaced with the right side otoliths.

Two-dimensional, semi-quantitative elemental distribution maps of the polished otoliths were acquired using a M4 Tornado μ-XRF spectrometer. The spectrometer was equipped with an Rh X-ray tube and two silicon drift detectors manufactured by Bruker Nano. The X-ray beam was focussed to 20 μm for Mo(Kα) using polycapillary X-ray optics. Multi-elemental maps were obtained with an X-ray tube voltage of 50 kV and a current of 300 μA. The pixel step size was 18 μm. Acquisition time of each spectrum was set to 10 ms per pixel. All measurements were carried out in a 20-mbar vacuum. Prior to XRF element mapping both detectors were calibrated by measuring a pure Zr standard and tuning the spectrum according to the Zr(Kα) peak at 15.775 keV. The measuring time of a glass slide with 20 otoliths was 12 h. The size of the scanned area was 61.5 × 20.2 mm and the total number of data pixels was ≈3.8 million. A map of a single otolith contains 30,000–60,000 pixels. A distinct Kα energy peak of strontium was observed from the sum spectrum. The distribution map of strontium in the otoliths was based on the $K\alpha_1$ energy peak area and a three points averaging filter for background removal. As the results were not verified by measuring certified reference materials, the analysis can only be seen as semi-quantitative. The information depth, the depth range within the otolith from which the detectable X-rays were emitted, was calculated to be 50% from 0 to 104 μm and 90% from 0 to 344 μm (J-O. Lill, pers. comm.; Lill et al., 2020). The colour scale of the μ-XRF otolith maps is the same for all otoliths, which means that they are comparable. The lightest and darkest areas correspond to the extreme values in strontium concentrations measured in μg/g (dry weight), representing high and low concentrations, respectively (Figure 2).

Total area of the otoliths and the area of the strontium-depleted regions were obtained by image analyses (Figure 2a–c) using the ImageJ software (ImageJ 1.52a, Wayne Rasband, National Institute of Health, USA, bundled with 64-bit Java 1.8.0_112). The images were converted to grayscale (16 bit) and a suitable threshold was chosen to set the border between the darker and lighter parts of the otolith. Finally, the areas to be measured were picked out using the wand-tracing tool. The difference in size of the strontium-depleted regions between whitefish from the rivers Tornionjoki and Kemijoki was tested using a Mann-Whitney U Test (www.statskingdom.com/170median_mann_whitney.html, accessed 31 May 2021) as the data did not follow a normal distribution (Shapiro–Wilk test, $p < 0.05$). Comparative time values indicating the length of stay in fresh water as juveniles, either in rivers or in ponds, were calculated from the area of the strontium-depleted region divided by the entire area of the otolith multiplied by the age of the fish. This is a rather crude estimation of the length of stay but without knowledge on the growth parameters of the otoliths, this is the best that could be achieved.

3 | RESULTS

Whitefish from both the River Tornionjoki and River Kemijoki had a high number of gill rakers typical for river-spawning whitefish (Table 1), in the latter river even matching the mean recorded 1981–1995 by Jokikokko and Huhmarniemi (1998). Fish caught at sea near Yxskär Island had a much lower number of gill rakers (Table 1) typical for sea-spawning whitefish (Himberg et al., 2015; Lehtonen, 1981; Svärdson, 1957).

The central strontium-depleted region in the otoliths from wild-born whitefish caught at River Tornionjoki was measured to have a mean area of 0.18 ± 0.2 mm^2 (Figure 2a, Table 1). The corresponding area in otoliths from River Kemijoki whitefish (stocked as one-summer-old fingerlings) was 2.3 ± 0.3 mm^2 (Figure 2b, Table 1). The sizes of the strontium-depleted regions in whitefish from the River Tornionjoki differed significantly from River Kemijoki whitefish (two-tailed Mann–Whitney U-test, $U = 130$, $z = 4.78$, $p = 0.0000017$), and no overlapping of sizes occurred. Whitefish sampled at the spawning ground near Yxskär Island had no strontium-depleted central region in their otoliths (Figure 2c). The three groups of whitefish investigated here could, therefore, be successfully distinguished by measuring the size of the strontium-depleted region in the centre of the otoliths (Figure 3, Table 1).

4 | DISCUSSION

The approach of mapping otoliths using μ-XRF and measuring the areas displaying low concentrations of strontium serve as a viable alternative to the more commonly used, transect-based method using LA-ICP-MS (e.g. Rohtla et al., 2017), when it comes to distinguishing the two ecotypes of whitefish found in the Gulf of Bothnia and stocked whitefish. The μ-XRF image of an otolith consists of ≈50,000 pixels, each corresponding to an X-ray spectrum recorded during a very short time (10 ms). The single-spectrum statistics are poor, and the quantification is time-consuming, but considered all together these 50,000 pixels contain a lot of information, and the outcome is more statistically robust. When using transect-based methods a few choices have to be made that are somewhat subjective and hence potentially subject to error. Firstly, the exact location of the centre of the primordium from where to initiate the transect must be pinpointed, a step that often proves to be problematic because the primordium can be quite difficult to define and can consist of several optically

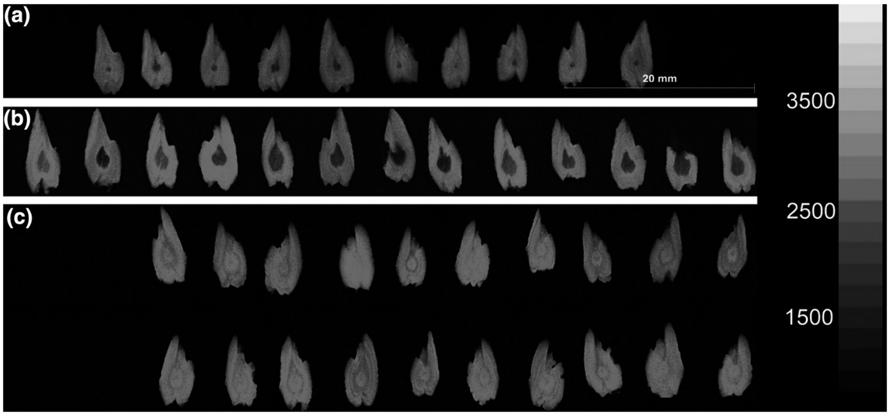

FIGURE 2 Distribution of strontium in whitefish otoliths, from low (dark) to high (light) concentrations measured in μg/g (dry weight). (a) River Tornionjoki whitefish 10 pcs, (b) River Kemijoki whitefish 13 pcs, and (c) whitefish caught at sea near the Yxskär Island northeast of the Åland Islands 20 pcs. The fish were sampled at spawning grounds during the spawning season

TABLE 1 Data for individual whitefish (by number, No.) caught in the River Tornionjoki (July 2013), River Kemijoki (October 2016) and at sea near the Yxskär Island northeast of the Åland Islands (November 2018)

							Otolith areas (mm^2)	
No.	**Total length (mm)**	**Weight (g)**	**Sex**	**Age (y)**	**GRC**	**Whole**	**Sr-depleted region**	**CTV (month)**
River Tornionjoki (2013)								
1	378	360	2	7	32	12.25	0.04	0.3
2	406	600	2	6	29	16.70	0.57	2.4
3	360	375	1	6	31	12.42	0.01	0.0
4	362	360	2	5	31	15.41	0.19	0.7
5	360	310	2	6	28	12.83	0.06	0.3
6	340	280	1	6	30	12.92	0.26	1.4
7	327	280	1	5	29	11.41	0.03	0.2
8	340	300	2	5	30	14.58	0.02	0.1
9	350	365	1	6	30	14.87	0.64	3.1
10	366	340	1	4	31	12.73	0.04	0.2
Mean ± SD	359	357		5.6	30.1	13.61 ± 1.68	0.18	0.9±1.1
River Kemijoki (2017)								
1	467	1017	2	5	28	18.41	2.40	7.8
2	452	869	1	6	29	19.32	2.15	8.0
3	403	619	2	5	28	16.47	2.42	8.8
4	472	916	2	6	29	17.95	2.02	8.1
5	371	456	1	5	29	16.67	3.05	11.0
6	413	653	2	6	30	16.48	2.41	10.5
7	425	720	2	7	29	18.18	2.53	11.7
8	387	552	1	4	28	15.86	2.70	8.2
9	423	574	2	5	31	15.50	2.52	9.7
10	385	480	1	6	26	13.29	1.93	10.4
11	403	507	2	7	27	16.44	2.17	11.1
12	392	527	2	5	31	broken	2.98	
13	427	641	2	6	32	14.15	2.48	12.6
Mean ± SD	417	656		5.6	29.0	16.56 ± 1.75	2.44	9.8±1.6
Yxskär (2018)								
1		741	1	6	27	18.64	0.00	0.0
2		489	2	6	27	16.67	0.00	0.0
3		726	1	5	26	19.10	0.00	0.0
4		557	1	6	28	17.82	0.00	0.0
5		409	1	4	28	13.24	0.00	0.0
6		438	1	5	23	16.79	0.00	0.0
7		394	1	6	27	12.99	0.00	0.0
8		373	1	6	28	12.82	0.00	0.0
9		472	1	6	27	15.05	0.00	0.0
10		470	2	5	28	13.34	0.00	0.0
11		471	1	7	27	18.16	0.00	0.0
12		565	1	6	27	16.17	0.00	0.0
13		522	1	5	27	18.15	0.00	0.0
14		602	1	5	25	19.11	0.00	0.0

(Continues)

TABLE 1 (Continued)

No.	Total length (mm)	Weight (g)	Sex	Age (y)	GRC	Whole	Otolith areas (mm^2) Sr-depleted region	CTV (month)
15		424	1	5	23	12.82	0.00	0.0
16		391	1	5	26	15.48	0.00	0.0
17		517	1	7	27	18.72	0.00	0.0
18		529	1	5	22	17.28	0.00	0.0
19		770	1	7	26	20.57	0.00	0.0
20		463	2	6	27	15.89	0.00	0.0
Mean ± SD		516		5.7	26.3	16.44 ± 2.42	0.00	0.0

Note: The data include body total length, weight, sex (male = 1, female = 2), age, gill raker counts (GRC) and otolith areas. The comparative time values (CTV) indicating the length of stay in fresh water was estimated from the area of the region displaying low concentrations of strontium divided by the whole area of the otolith multiplied by the age of the fish.

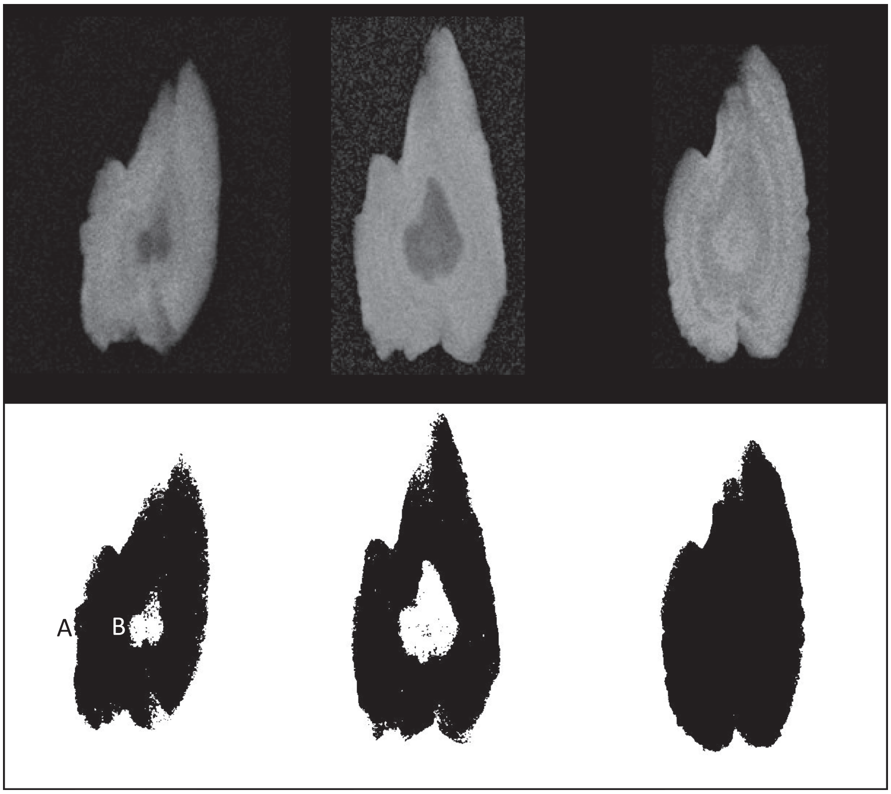

FIGURE 3 Upper images: strontium distribution maps of otoliths from whitefish caught ascending the River Tornionjoki (No. 5 in Table 1) and the River Kemijoki (No. 6), and caught at sea near the Yxskär Island northeast of the Åland Islands (No. 14). Lower images: interpretation of areas – white to black border (a) was used to outline the entire area, black to white border inside the otolith (b) was used to outline the area of the region displaying a low concentration of strontium

dense primordial granules (Kalish et al., 1995). Secondly, as the otolith generally does not grow in a perfectly concentric fashion, the direction of the transects has to be standardised, which can be tricky when trying to avoid any major cracks or deformities in the surface of the polished otoliths. Finally, if one wishes to relate changes in otolith elemental composition in a transect to a time scale, then the distance between the primordium and the growth rings (in the present study the first winter increment) in the direction of the transect must to be measured – this again is a potential source of error because the primordium, as previously discussed, can be difficult to pinpoint, and the growth rings not always are uniformly visible and might not be identifiable in the direction of the transect. These issues are circumvented when using the area-based approach because it is not necessary to identify the primordium, standardise the direction of any transect or relate said transect to any time scale. The only subjective choice involved is the selection of the threshold value for the concentration of strontium used to define the area, a decision that can be made more objective by basing it on previous knowledge. Finally, the μ-XRF analysis as applied in the present study is less labour intensive than many other methods because an area of 190 × 160 mm, which is large enough to fit several slides of otoliths, can be analysed at once and the analysis can be set to run without supervision.

Just as the methodology presented here possesses several advantages, there are also some disadvantages. As the complex elemental composition of an otolith is reduced to regions of low- and high-strontium concentrations, as defined by a threshold, much information is lost. In the case of whitefish in the Gulf of Bothnia, the present method can be employed to distinguish between the two ecotypes of whitefish and whitefish stocked as one-summer-old fingerlings, but it is not able to distinguish river-spawning whitefish originating from different rivers. In addition, the method cannot be employed to differentiate between stocked whitefish reared at different locations, or to distinguish fingerling-stocked river-spawning whitefish from fingerling-stocked sea-spawning whitefish. For that reason, the method described here serves as a complement, rather than a direct substitute, to transect-based methods. For studies aiming to follow the migration of the fish on a finer resolution, the transect-based method should still be the first option. However, because the µ-XRF analysis is non-destructive, the researcher is always left with the option of supplementing it with other analytical methods.

The simple model used to calculate comparative time values, which indicates the time spent in fresh waters in the juvenile phase based on the area of the region displaying low concentrations of strontium, needs to be improved. As it stands now, the results can only be seen as suggestive, such as in the mean duration spent in fresh waters for whitefish stocked into the River Kemijoki; this was estimated to be 9.8 months, although it is known that the fish were reared in freshwater ponds for only around five months (Voimalohi OY, 2017). In the River Tornionjoki, the migration to the sea by wild-born whitefish occurs mainly in the few weeks after hatching, but a minor proportion of the fish stay in the river for a longer period, and juvenile whitefish can be found migrating downstream during the whole summer (Jokikokko et al., 2012). This is in accordance with the varied stay in fresh water estimated for whitefish caught in the River Tornionjoki (Table 1). By further investigating the growth rate of otoliths for whitefish, the model could be refined and its accuracy increased, opening up possibilities for other applications and new lines of research.

In conclusion, image analysis of strontium distribution maps, obtained by µ-XRF analysis of polished otoliths, is a viable method with which to determine whether individual whitefish have spent time in fresh water as juveniles and for how long. The method can be employed to distinguish between wild-born river-spawning whitefish, wild-born sea-spawning whitefish and whitefish stocked as one-summer-old fingerlings in mixed catches. The µ-XRF analysis itself is less labour intensive than most other methods as several slides of otoliths can be analysed simultaneously with little to no supervision. The subsequent image analysis of the µ-XRF maps is also a quick, uncomplicated process and can be accomplished using open source software such as ImageJ. The method is especially suitable for analysing large sample sizes and has potential for being used to evaluate the efficiency of supplementary stocking and to examine the composition of whitefish in mixed catches, both important issues for fisheries management.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data supporting the findings of the study are available upon request.

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