Otolith Mass Marking Techniques for Aquaculture and Restocking: Benefits and Limitations

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Abstract

The use of farmed and restocked fish to supplement the worldwide human consumption of fish, recreational fishing stocks, and conservation efforts, is growing at a rapid rate. Yet, monitoring the benefits of using hatchery-raised fish for supplementation is lacking, often due to hatcheries not marking or tagging all fish prior to release, despite a range of easy to apply, cost effective and accurate mass-marking methods being available to mark farmed and restocked fish en masse. Here we review otolith marking techniques that have the capability of mass marking millions of hatchery-reared fish that are, or could be, used for monitoring and compliance purposes. The otolith mass marking methods consist of otolith thermal marking and a range of otolith chemical marking methods (tetracyclines, alizarin compounds, calcein, strontium chloride, stable isotopes of Ba and Sr, and rare earth elements). We assessed and compared marking technique in terms of 1) ease of application, 2) cost of application, 3) mark retention and detectability, and 4) fish welfare. In addition, we determine the suitability of different otolith marking techniques for mass marking entire hatchery populations whether it be for restocking purposes, or for identifying and tracing escapees from aquaculture facilities. We conclude that although some techniques have restricted use due to regulations, the majority of otolith mass marking techniques are simple, easy to apply, cost effective and highly suitable for long term monitoring of hatchery produced fish.

Key words: cost effectiveness, detection accuracy, marking efficiency, mark retention, mark longevity

Introduction

Declines in fishery stocks worldwide have incentivised the global expansion of aquaculture (Naylor et al. 2000) and progressed recent efforts to domesticate hundreds of new fish species (Duarte et al. 2007; Teletchea 2015). This has resulted in hatchery facilities now producing millions of larval

and juvenile fish annually for both aquaculture and restocking, but for very different endpoints. As global fisheries have declined, the demand for hatcheries to supply aquaculture facilities with domesticated fish suitable for farm production has grown rapidly. Likewise, fisheries declines have also driven demand for the hatchery production of fish appropriate for restocking wild populations to supplement wild capture fisheries.

The process of restocking wild fish populations with hatchery reared larvae and juveniles is extensive and has been occurring for over 50 years (Ariki and Schmid 2010), for example hatchery facilities in America, Canada, and Japan, release approximately 5×10^9 juvenile salmon (pink, chum, coho, chinook, sockeye, steelhead, and masu) annually into the North Pacific Ocean (<http://www.npafc.org>). In Sweden, 2.5 million European glass eels, *Anguilla anguilla* (L.) are released each year into fresh and saltwater locations across northern Europe (Wickström and Sjöberg 2014). In southern Australia, between 1995 and 2003, hatcheries released over 4 million salmonid fingerlings (*O. mykiss*, *S. trutta* and *Oncorhynchus tshawytscha*) into regional rivers and lakes (DEPI 2005). Furthermore, in last 25 years over 40 million native fish, (Mainly *Bidyanus bidyanus*, *Maccullochella peelii* and *Macquaria ambigua*) have been released into the Murray-Darling Basin (Gillanders et al. 2006). Despite the huge investment into restocking fisheries, how beneficial, or harmful hatchery driven restocking programmes are, is still highly debatable (Madeira et al. 2005; Araki and Schmid 2010) and can be problematic to assess. This is often due to the lack of commitment to appropriate monitoring of re-stocked fish post release (Cowx 1994), for example, not marking or tagging all fish prior to release, despite a range of mass-marking methods being available to use.

Hatcheries that produce for fish farm aquaculture face a different set of assessment issues to that of restocked fish, particularly for marine fish farming in waters cohabited by wild conspecifics. Aquaculture in marine environments has expanded rapidly with 65 species of marine fish now producing a total of 2.2 million tons (Teletchea 2015), in addition to the 2.5 million tons of anadromous salmonids now farmed in marine waters (FAO 2015). Levels of domestication are increasing, with many species undergoing selective breeding programs that optimise traits suitable for

domestication, which shifts genotypes away from wild populations (Madeira et al. 2005). A consequence of domestication, and one of the major environmental problems of marine fish farming, is escapes of hatchery-bred domestic strain individuals into wild populations. Escapes can result in hybridisation between farmed and wild strains which can lead to poorer performance and survival in the wild (Hindar et al. 2006; Hutchings and Fraser 2008); competition for food resources (Fleming et al. 2000; McGinnity et al. 2003); and the transfer of disease from cultured to wild populations (Toledo-Guedes et al. 2012). The number of farm fish escapees is alarming, with documented reports of 9 million escapees in Europe between 2007 and 2009, which were mainly sea bream escapees in the Mediterranean (Jackson et al. 2015) and 3 million salmonid escapees in Norway from 2000-2009 (Jensen et al. 2010). The core challenge in assessing the effects of escapees is differentiating farmed fish from wild fish. Identification of the source of escaped farmed fish is often possible using DNA-based methods (Glover 2010), but the method does not always detect the farm origin. Recent advances in mass genome sequencing (Campbell et al. 2015) and identification through DNA parentage of hatchery brood stock (Beacham et al. 2017) may improve genetic identification methods in near future. However, using DNA parentage requires hatcheries to have a very high standard of brood stock management and record keeping. In addition, DNA records may not be freely available from commercial hatcheries that supply fish farms, due to intellectual property (IP) rights related to specialised breeding programs (for example attempts to breed sea lice resistant fish for the Norwegian salmon industry). These restrictions may limit the use of DNA parentage identification techniques. In contrast, there are no IP restrictions with any of the otolith mass marking methods discussed in this review.

A simple way to mass mark with a permanent tag or coded mark to guarantee correct identification of farmed fish would greatly improve assessments of the impacts of escapees from aquaculture. Past assessments have been made of the relative costs of mass marking techniques (Hammer and Blankenship 2001); the compatibility between marking methods and mark recapture designs (Skalski et al. 2009); and otolith chemical and natural marking techniques for assessing biological interactions in marine systems (Gillanders et al. 2009). However, these assessments do not consider mass marking

entire hatchery populations or fish farms to allow for population monitoring and industry scale compliance as to date, marking all farmed fish has not been considered a feasible option or a necessary requirement.

Here, we review the suitability of potential otolith mass marking methods currently available for hatchery-reared fish that may enable a low cost, simple way of monitoring of all fish produced for aquaculture production or restocking purposes. Marks created in the otoliths of fish, whether they are created via a chemical signature, or thermal signature, are considered to be permanent (Campana 1999) making them ideal for large-scale, long-term mass marking programmes. Here we selected techniques to compare based on their feasibility to batch mark tens to hundreds of thousands of individuals at a time. Methods selected comprise of otolith thermal marking and a range of chemical methods, calcein; tetracycline; alizarin complexone and alizarin red S; strontium chloride (SrCl_2), enriched stable isotopes of barium (Ba) and strontium (Sr); and rare earth elements. Where applicable, each method for is assessed for marking hatchery-reared fish in terms of: 1) ease of application; 2) cost of application; 3) mark retention and detectability; and 4) fish welfare issues. Finally, we recommend techniques based on their relevance to specific applications, such as identifying and tracing farmed fish escapees in the wild, or assessing the benefits of restocking programs.

Marking methods

Otolith thermal marking

Otolith thermal marking is a process that uses short-term temperature fluctuations of 2 to 5 degrees Celsius to induce distinctive light and dark bands in the otoliths of hatchery incubating fish to create simple (single band) and complex (multiple band) marking codes (Review by Volk et al. 1999).

Identification of marks requires the extraction and polishing of otoliths, yet the marks are permanent making them suitable for long term studies of hatchery bred fish destined for restocking purposes, for example in salmonids (Volk et al. 1994; Courtney et al. 2000; Morita et al. 2013). Otolith thermal marking has the potential to correctly mark 100% of fish (Munk et al. 1993) although correct

identification of marked fish does vary depending on the person reading marks (60 to 100%, Hagen et al. 1995). Furthermore, false positives in unmarked wild fish are a potential issue due to the light and dark bands formed in otoliths being strongly linked to environmental conditions (Volk et al. 1994).

Thermal marking is widely used for mass marking salmonids, as billions of fish have been marked using this technique (e.g. Hagen et al. 1995; Courtney et al. 2000; Morita et al. 2013). It is most suitable for marking large batches of distinct groups, with a relatively low number of marker combinations, in hatcheries that are already set up for controlled manipulation of hatchery water temperatures as equipment set up cost can be expensive (>20,000 \$USD, Munk et al. 1993), plus there is ongoing additional costs of heating large water volumes. The main drawback of otolith thermal marking is that complex marks can take several weeks to apply (Munk 1999) meaning close monitoring during the temperature manipulation period is paramount, as any unplanned temperature fluctuation will result in incorrectly marked fish.

Chemical markers

Calcein

Immersing of larvae or juvenile fish in a calcein solution can produce bright green marks in the fins, scales, vertebrae, and otoliths when viewed under ultraviolet light (Mohler 1997, Mohler 2003).

Calcein can be 100% effective, with little effect on mortality or growth of fish if applied in one of two ways, either immersion for periods of 24-48 hours at low concentrations (e.g. 125 mg.L⁻¹, Mohler 1997), or for periods of 3.5-10 minutes at higher concentrations ranging between 500-1000 mg.L⁻¹ if combined with osmotic induction (Mohler 1997; Crook et al. 2009; Smith et al. 2010) (Table 1). For some species, calcein concentrations higher than 250 mg. L⁻¹ can be toxic for fish if immersed for long periods (≥ 6 hours) (Brooks et al. 1994; Bumguardner and King 1996). Calcein marking has also been applied via food, but mark success results vary depending on the species marked (Honeyfield et al. 2006) and can require several weeks of feeding with calcein spiked food to achieve clear marks (Honeyfield et al. 2011).

Internal marks with calcein in the otoliths should be permanent, yet external marks may not be and can depend on the age of the fish when marked, for example with marking salmonid larvae, mark detection rates can drop rapidly after the first year (Negus and Tureson 2004; Stubbing and Moss 2007). However, if fish are marked at a later stage (i.e. juveniles) fluorescent external marks are often visible in fin rays and scales through to adulthood (e.g., Mohler 2003; Negus and Tureson 2004, Crook et al 2012). Photo-degradation of external marks in salmonids within 12 months of marking is a major issue (Honeyfield et al. 2008; Hill and Quesada 2010; Richard et al. 2014), but this should not affect internal marks in the otoliths.

Calcein marking is most effective if applied through short-term immersion (3-10 minutes) combined with osmotic induction, rather than via feed intake or immersion for several days, mainly because it allows hatcheries to mark quickly with full control over concentration and exposure time. If applied correctly, calcein marking is a useful method for batch marking large numbers of juvenile or adult fish with one or two fluorescent marks (e.g., Mohler 2003). It is less suitable for marking larvae due to potential issues with mortality and photodegradation of external marks (Elle et al. 2010). Estimated material cost to mark using short immersion times is approximately 0.016 \$USD per fish (Crook et al. 2010).

Tetracycline

Tetracycline antibiotics produce fluorescent marks in the bony parts of fish (Weber and Ridgway 1967). The most common form used is oxytetracycline hydrochloride (OTC), which produces yellow/gold fluorescent marks in otoliths, fin spines and vertebral centrum (Odense and Logan 1974; Brooks et al. 1994; Wells et al. 2013) and is most visible when viewed under ultraviolet light. Marks are created by immersing fish in OTC, combining OTC with feed, or by direct injection of OTC (Table 2). Marking via immersion is potentially the most feasible way to mark with OTC with the optimal time to mark being the larval stage, as 100% successful marks can be achieved with larvae immersed for as little as 2 to 6 h (Secor et al. 1991; Hendricks et al. 1991) or embryos for 1 h (Jones et al. 1999). Although marking via feed is possible, it requires a longer time to complete the marking process (3 days to 2 weeks; Weber and Ridgway 1967; Odense and Logan 1974; Hendricks et al.

1991) making it less optimal for application in fish hatcheries. Marking via direct injection with OTC is only suitable if fish are already destined to be injected (e.g. during routine vaccination).

OTC marking can have some minor health issues for some species of fish. Immersion in OTC has been found to be toxic for striped bass *Morone saxatilis* if administered at doses > 500 mg for periods longer than 6 h (Bumgardner and King 1996) and marking with feed with OTC has been reported to increase the rate of spinal deformities in Atlantic salmon *Salmo salar* (Toften and Jobling 1996). This suggests there is the potential for species-specific side effects when marking with OTC, yet the majority of studies (Table 2) report no effect.

Externally visible marks with OTC are often visible for up to 1 year. However, long term, OTC has been demonstrated to be a useful technique for marking otoliths with a check mark for assessing growth rate and aging validation studies, for example in American shad, *Alosa sapidissima* (Duffy et al. 2012).

Alizarin complexone (AC) and Alizarin red S (ARS)

Fluorochrome labelling dyes, Alizarin complexone (AC), and Alizarin red S (ARS) are feasible alternative mass marking chemicals to OTC and calcein (Bashey 2004; Simon et al. 2009) and produce fluorescent marks ranging in colour from yellow to red to violet, depending on the light source used (Beckman and Schulz 1996; Lagardère et al. 2000; Liu et al. 2009). External marks are retained in fins and scales for several months post marking (Crook et al. 2007; Liu et al. 2009) and internally in otoliths for several years (Simon et al. 2009). Mark success rates of 100% are achievable with little to no effect on mortality in juvenile fish if the optimal concentration and immersion times are used (Table 3). However, eggs, or young larvae, of some species appear to be vulnerable to possible effects on hatch rate, growth, or survival if exposed to higher concentrations, or for long immersion periods (Beckman and Schulz 1996; Van der Walt and Faragher 2003; Crook et al 2007; Meyer et al. 2012).

Mass marking with ARS has been demonstrated to be highly successful, for example marking larval turbot, *Scophthalmus maximus* (Iglesias and Rodriguez-Ojea 1997) and glass eels *Anguilla anguilla* (Caraguel et al. 2015). Osmotic induction can reduce immersion times and improve the quality of marks (Crook et al. 2007) and the addition of NaHCO₃ or NaOH to ensure a neutral pH greatly improves survival rates when marking larvae (Van der Walt and Faragher 2003; Baer and Rösch 2008).

Application of AC and ARS as a mass marking technique is limited by the number of possible marks, with only 1 external mark realistically feasible, due to poor mark retention after a few months. However, 1 or 2 permanent internal marks in the otoliths is possible through the immersion of fish in alizarin twice, if immersions are separated by a period of time. Alizarin marking is a more cost effective marking method compared to marking with OTC or calcein mainly due to lower raw material costs. Estimate material cost for chemicals for batch marking fish with AC range from 0.003 to 0.008 \$USD per fish (Iglesias and Rodriguez-Ojea 1997; Van der Walt and Faragher 2003) and for ARS 0.002 \$USD per fish (Caraguel et al. 2015).

Strontium chloride

A variety of different delivery methods using strontium chloride (SrCl₂) as a mass marking tool have been tested, for example via feed (Ophel and Judd 1968; Behrens-Yamada et al. 1979), immersion (Schroder et al. 1995; Wickström and Sjöberg 2014), and transgenerational marking (Kuroki et al. 2010; Shippentower et al. 2011). Yet, the success of delivery techniques has had variable results with some finding poor mark success (Shippentower et al. 2011), or poor long-term retention (Behrens Yamada et al. 1979), while others achieved 100% mark success (Ophel and Judd 1968; Schroder et al. 1995; Kuroki et al. 2010). The most effective results have been achieved with marking salmonid fry (Behrens-Yamada & Mulligan 1987; Schroder et al. 1995) and glass eels, *Anguilla anguilla* (Wickström and Sjöberg 2014) via immersion for a period of 24 hours. Marking via immersion produces a visible ring that is detectable in otoliths that have been sectioned, polished, and then analysed using backscatter electron microscopy (BEM) (e.g. Schroder et al. 1995; Wickström and Sjöberg 2014). Successful immersion concentrations used to mark freshwater fish have ranged

between 120 to 1000 mg. L⁻¹. For saltwater fish, higher concentrations may be required as natural Sr:Ca levels in water are positively correlated with salinity (Kraus and Secor 2004).

As a mass-marking tool, marking with SrCl₂ is most suitable for creating a single mark in the otolith of fish, such as when only a single group of fish (i.e., hatchery verses wild) requires identification. Although, one study has shown the SrCl₂ marks were still detectable in the scales of gold fish *Carassius auratus* after one year after marking (Ophel and Judd 1968) and another has shown that double marking can be used for experimental age validation studies using otolith daily increments in captive juvenile sablefish *Anoplopoma fimbria* (Courtney and Severin 2007). Cost to detect strontium chloride marks in otoliths have been estimated at 50 \$USD per otolith (Hammer and Blankenship 2001).

Stable isotopes

The use of isotopes as a mass-marking tool for fish was initially suggested by Trefethen and Novotnoy (1963), yet was only recently validated as a technique (Thorrold et al. 2006). Typically, stable isotopes are used to mark the otoliths of fish by the introduction of an enriched form of either the common isotope of an element (e.g., ¹³⁸Ba, Woodcock et al. 2011b), or the less common isotopes of an element (e.g., ¹³⁷Ba, ¹³⁵Ba (Almany et al. 2007); ¹³⁶Ba, ¹³⁴Ba, (Warren-Myers et al. 2015a,c). Isotope marks occur when the successful uptake of an enriched isotope into the calcified structures of fish results in an increase in its abundance relative to other isotopes of the same element (i.e. a ratio higher than the naturally occurring isotope ratio). Marks can be detected using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) (Thorrold et al. 2006).

To date, enriched stable isotopes of Ba and Sr have been the most successful and marking with isotopes has been validated using a range of delivery techniques, for example, transgenerational (Table 4), immersion of eggs and larvae (Table 5), during vaccination (Warren-Myers et al. 2014, 2015a), or food supplementation (Woodcock et al. 2013). Mark retention and detection rates with stable isotopes can be 100%, but it depends on delivery method, concentration of isotope used, length of exposure, and life-history stage of the fish when marked. The main limitation with stable isotope

marking is that it requires initial testing and optimisation for each species. Once these initial requirements have been met, the raw material cost to mark can be less than 0.0002 \$USD per fish (e.g. Warren-Myers et al. 2015a, 2015b,c). Detecting marks has some limitations as it requires the use of LA-ICP-MS technology, at a cost per fish to detect marks of approximately 20 \$USD per otolith (Warren-Myers et al. 2015a).

Rare earth elements

Rare earth elements that naturally occur in the environment in low concentrations, for example lanthanum, samarium and cerium, have been trialled as chemical markers for fish. For example, immersion of coho salmon fry *Oncorhynchus kisutch* in lanthanum and samarium at a concentration of 100 µg L⁻¹ for 6 weeks resulted in detectable marks in the bony structures and otoliths (Ennevor and Bearnes 1993). The addition of samarium at 66 mg per gram of feed for 30 days produced detectable marks in the branchial arch and scales in goldfish (Michibata 1981). Injecting rainbow trout *Oncorhynchus mykiss*, with dysprosium, europium and samarium (Giles and Attas 1993) found mark retention in the gut tissue was successful. More recently, attempts to mark farmed Atlantic salmon *Salmo salar* with lanthanum, cerium, praseodymium, neodymium and dysprosium via feed for 10 weeks produced detectable marks in scales for up to 4 months post feeding (Pérez de Nanclares et al. 2016), but detectable marks in the otolith were not reported. More research is required to test whether marks are detectable in the otoliths of hatchery reared fish before the use of rare earth elements can be considered a viable otolith mass marking method, in terms of ease of application, mark retention and detectability.

Discussion

Limitations of methods

Every otolith mass marking technique has limitations, for example: 1) the developmental stages at which fish can be marked; 2) how many unique mark combinations are available; 3) the associated material cost per fish to mark; and 4) the method required to detect marks (Table 6). The cost to

detect marks in otoliths is also a potential limitation if thousands of fish required identification. Of the techniques reviewed here within, the cost of detection ranges from approximately 15 to 50 \$USD per otolith depending on the marking method (Hammer and Blankenship 2001; Warren-Myers et al 2015b). In addition, some techniques generate fish welfare issues either during or after marking (Table 7). Although it is unrealistic to expect a marking method to have no limitations, an ideal method for marking millions of fish to later identify stocked or escaped farmed fish in the wild should meet four minimum criteria: 1) high ease of application; 2) low cost of application; 3) high mark retention and detectability; 4) no associated fish welfare issues.

Comparing methods

Ease of application

Otolith marking methods that are easy to apply and complement standard hatchery procedures are necessary to minimise additional labour or loss in production time due to marking for hatchery facilities. All of the otolith marking methods discussed here can potentially be used to mark large batches at once. However, only the otolith chemical marking methods (except rare earth elements) can mark fish in periods of less than a day (e.g. Van der Walt and Faragher 2003; Crook et al. 2009; Wickström and Sjöberg 2014; Caraguel et al. 2015; Warren-Myers et al. 2015a,b) whereas otolith thermal marking takes several days to weeks to create marks. Furthermore, chemical marking with stable isotopes can produce a range of unique mark combinations for different batches of fish in less than a day (de-Braux et al. 2014; Warren-Myers et al 2015b). Multiple marks can be created using otolith thermal marking, but this then further increases the time to create marks from days to weeks, meaning the logistical ease of applying multiple mark combinations is lower for otolith thermal marking compared to stable isotope marking.

Cost of application

Material cost and the number of producible unique codes are elements in determining the cost of applying a mark to each fish. The time and technician hours required to apply marks is an additional cost of marking (e.g. Hammer and Blankenship 2001) for methods that cannot be easily incorporated

into the routine hatchery production cycle. To produce a single mark in an otolith, thermal marking, alizarin, SrCl_2 and stable isotopes of Ba are the most cost effective for materials (Table 6). However, alizarin and SrCl_2 can only produce between 1 and 4 unique codes, through multiple exposures at different times (e.g. Mohler 2003; Wickström & Sjöberg 2014). While stable isotope marking can potentially produce up to 15 unique combinations using isotopes Ba (Woodcock et al. 2011a; Warren-Myers et al. 2015b,c) and otolith thermal marking, hundreds of unique codes (Volk et al. 1999). Furthermore, the ability to apply multiple codes in one application suggests that Ba stable isotope marking is the most cost effective otolith mass marking method to apply, especially for deliver methods that can be incorporated in to standard hatchery production (e.g. Warren-Myers 2015a,b).

Mark retention and detectability

Achieving 100% initial mark success is a must for any mass marking method to be used for compliance purposes and retention of any chemical or physical mark in the otolith of a fish may be considered to be permanently retained (Campana 1999). However, detection of marks varies among methods. Thermal marking has potential issues with detection of false positives as naturally occurring marks in wild fish can be mistaken for hatchery-marked fish (Volk et al. 1994). Ease of detecting chemical marks in the otolith varies as tetracyclines, alizarin compounds, calcein or strontium chloride marks otoliths first have to be sectioned and polished before identifying marks, which can be time consuming. In addition, assessing mark intensity may require a fluorescence dissecting microscope (e.g. for calcein and alizarin marks, Crook et al. 2009). Alternately, in some cases, for example farmed Atlantic salmon, detecting stable isotope marks does not require otoliths of young fish (<1.5 years of age) to be sectioned or polished, as marks can be detected with spot ablation using LA-ICP-MS (Warren-Myers 2015). However, for other species the ease of detecting stable isotope marks in older fish, or large otoliths, still requires further research, and is a knowledge gap that needs to be addressed. The requirement of specialised equipment (LA-ICP-MS) is also a further limitation to ease of detection for stable isotope marking.

Fish welfare

In general, if undertaken correctly, the welfare effects of otolith mass marking methods are low (Table 7). Among the chemical marking methods, calcein, oxytetracycline, and alizarin are all toxic if higher than recommended concentrations or longer than recommended exposure periods are used (Brooks et al. 1994; Beckman and Schulz 1996; Bumgardner and King 1996; Meyer et al. 2012). Otolith thermal, SrCl_2 and stable isotope marking methods are a lower risk to apply and have no reported welfare issues for fish. The main limiting welfare factor of all otolith-marking methods is that the killing of fish is required to extract the otoliths, which is a negative impact for hatcheries working with endangered or rare species, but less likely an issue for farmed species, such as Atlantic salmon. Alizarin and calcein marks are detectable externally for up to 2 years post marking in some cases (Crook et al 2007; 2012) meaning they have an added welfare advantage over other methods, due to the ability to initially non-lethally detect marks in the short term.

Useability of otolith marking

Otolith mass marking fish for aquaculture

Most of the discussed otolith marking methods reviewed here are applicable for mass marking millions of farmed fish for aquaculture as they are in general, cost effective, easy to apply, and have high mark retention and detection rates. Yet, some techniques are more suitable than others, depending on what is required from a marking program. In a scenario where farmed fish were to be marked purely for differentiating between farmed and wild, a single alizarin, SrCl_2 , single Ba isotope, or one otolith thermal mark using one-temperature manipulation, would be the simplest and most cost effective way to mass mark an entire hatchery facility. However, if marking farmed fish for monitoring, compliance, and tracing back to the point of escape, multiple code combinations may be required, for example to differentiate between multiple fish farms in close proximity to each other. Otolith thermal marking (> 1000 codes) and stable isotope marking (> 60 codes) are the techniques that come closest to meeting this requirement. Otolith thermal marking and stable isotopes would also allow for different codes to be allocated each year, to enable estimates of the age distributions of escaped farmed fish in the wild. This is potentially useful for species like *Atlantic salmon* where it has

been demonstrated that the otoliths of farmed fish have a high prevalence of vaterite (Reimer et al. 2016) which makes it difficult to accurately age fish older than 1 year using traditional otolith ageing methods.

Mass marking fish for restocking

Choosing an appropriate marking method for identifying released fish for restocking is more complex in comparison to simply marking farmed fish for aquaculture due to a wider diversity of research questions, mark-recapture programs, and the number, size, and variety of fish species released (see review by Skalski et al. 2009, Gillanders 2009). Furthermore, if native or endangered species are restocked, having to kill fish to collect the marked otoliths greatly limits the practicality of using any otolith marking method. For native fish species, calcein marking is a clear front-runner despite a slightly higher cost per fish to mark (~ \$USD 0.016 per fish, Table 6) as initially marks are externally detectable and in some cases detectable for ~ 2 years post marking in some freshwater species (Crook et al. 2012). Older fish marked at the early larval stage would still require otolith extraction, but the ability to non-lethally detect marked fish in the short term (< 2 years) following release does make calcein marking more fish friendly than other otolith marking methods.

For fish species that are restocked for enhancement where fish are often destined for human consumption (e.g. rainbow and brown trout), or lethal detection is not an issue, the cost to mark and/or the number of code combinations required will still be the main driver of a mass marking programme. Similar, to marking fish for aquaculture, SrCl_2 , stable isotope marking or, otolith thermal marking may be the best otolith marking options to meet these requirements.

Regulation and current restrictions for otolith marking methods

As farming of the world's oceans continues to increase rapidly, marking of farmed and restocked fish may become compulsory for monitoring and compliance purposes in the near future. While there are no regulatory restrictions with the use of otolith thermal marking, there are regulatory restrictions on the use of many otolith chemical marking methods, in particular for fish that will be consumed.

Approval for use of any method is often country specific (Table 8). For example, in the US, calcein

and oxytetracycline have been approved for fish marking, but there are tight restrictions on their use, despite concerns with the overuse of using OTC and broad-spectrum antibiotics in commercial aquaculture (Cabello 2006). In Australia, marking with calcein, alizarin and stable isotopes of Ba and Sr is approved, although testing is still required to show that levels of chemical residues of alizarin and stable isotopes are the same or below natural levels in harvested fish. In Sweden, SrCl_2 is used to mark millions of glass eels (Wickström and Sjöberg 2014). The regular use of any chemical in a hatchery is a potential health and safety issue for hatchery staff, for example in the US there are concerns that alizarin red S is a potential carcinogen (Inouie et al. 2009). MSDS sheets are available for the chemicals used in the otolith marking methods listed here and should be reviewed by all hatchery staff prior to using any chemical for marking (Table 8).

Knowledge gaps and future directions

The use of otolith mass-marking methods as long-term population monitoring tools for farmed and restocked fish is underutilised. Of the methods discussed here, to date otolith thermal marking has had the most uptake as a mass marking technique for restocking, as billions of salmon have been thermal marked and released in the north Pacific (Munk et al. 1999). Now, with the approval of the use of otolith chemical marking methods like calcein and oxytetracycline in the US and calcein, alizarin and stable isotopes in Australia, there are multiple otolith mass marking options available for hatcheries to mark all fish before they are released into the wild.

The possibility of any chemical residues in the flesh of harvest-size fish due to marking larger fish (juveniles and pre-smolts) is still a potential concern that requires further research to guarantee that fish are safe for consumption. Furthermore, although marks created in otoliths are considered permanent (Campana 1999) the ease of detecting some chemical marks, for example Ba stable isotopes in older fish (>1.5 years) is yet to be tested. Sectioning and polishing of otoliths prior to LA-ICP-MS may be required, particularly for species that produce large otoliths as adults and have a stable isotope mark in the embryonic otolith core. This may be difficult to test on restocked fish that

are released, but could be easily tested on farmed fish that are normally grown for >2 years before harvesting.

The numbers of hatchery fish that are otolith marked to monitor restocking efforts continues to increase. However, farmed fish are not being mark, as currently it is not a requirement of the industry. As aquaculture continues to expand, regulation to control and monitor the effects of farm fish escapees may ultimately result in compulsory marking of all farmed fish. With the current selection of otolith mass marking methods now available, the concept of mass marking all farmed fish may be feasible to achieve in the near future.

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Table 1. Comparison of optimal immersion times, concentrations, and use of osmotic induction for calcein marking of fish to achieved 100% mark success.

Species	Stage	Osmotic Induction	Exposure Time	Conc. (mg. L ⁻¹)	Mortality Effect	Reference
<i>Macquaria ambigua</i>	fingerlings	5 min	5 min	5000 - 10000	< 2%	Crook et al. 2009
<i>Macquaria novemaculeata</i>	fry & fingerlings	5 min	10 min	10000	none	Cameron et al. 2012
<i>Morone saxatilis</i>	juveniles	No	6h	125	none	Bumguardner and King 1996
<i>Morone saxatilis</i>	juveniles	No	6 h	160	> 10%	Bumguardner and King 1996
<i>Morone saxatilis</i>	juveniles	No	6 h	240	> 50%	Bumguardner and King 1996
<i>Oncorhynchus mykiss</i> , <i>O. tshawytscha</i>	larvae & juveniles	3.5 min	3.5 min	500	none	Negus and Tureson 2004
<i>Salmo salar</i>	larvae	No	48 h	125	1%	Mohler 1997
<i>Salmo salar</i>	larvae	No	48 h	250	10%	Mohler 1997
<i>Salmo salar</i>	larvae & juveniles	3.5 min	3.5 min	1000	none	Mohler 2003
<i>Stigmatopora argus</i> , <i>S. nigra</i>	adults	10 min	30 min	500	none	Smith et al. 2010
<i>Stigmatopora argus</i> , <i>S. nigra</i>	adults	No	24 h	250-500	none	Smith et al. 2010
<i>Stizostedion vitreum</i>	larvae & juveniles	No	6 h	250-500	> 40%	Brooks et al. 1994

Table 2. Comparison of optimal immersion times, concentrations, and use of osmotic induction for tetracycline marking of fish in studies that have reported 100% mark success.

Species	Stage	Osmotic Induction	Exposure Time	Conc.	Mortality Effect	Reference
<i>Alosa sapidissima</i>	larvae	No	Immersion 6 h	200 mg. L ⁻¹	none	Hendricks et al. 1991
<i>Alosa sapidissima</i>	juvenile	No	Feeding 3 days	88 mg.kg ⁻¹ feed	none	Hendricks et al. 1991
<i>Argyrosomus japonicus</i>	juvenile	Saltwater salinity change 35 to 5 ppm over 3 h before marking	Immersion 24 h (water salinity of 5 ppm)	600 mg. L ⁻¹	< 1%	Taylor et al. 2005
<i>Morone saxatilis</i>	larvae	No	Immersion 3 h	30 mg. L ⁻¹	none	Secor et al. 1991
<i>Morone saxatilis</i>	juvenile	No	Immersion 6 h	447 mg. L ⁻¹	none	Bumgardner and King 1996
<i>Morone saxatilis</i>	juvenile	No	Immersion 6 h	597 mg. L ⁻¹	> 50%	Bumgardner and King 1996
<i>Pomacentrus amboinensis</i>	embryos	No	Immersion 1 h	400 mg. L ⁻¹	none	Jones et al. 1999
<i>Stizostedion vitreum</i>	larvae	No	Immersion 6 h	500 mg. L ⁻¹	> 9%	Brooks et al. 1994

Table 3. Comparison of optimal immersion times and concentrations, for Alizarin complexone (AC) and Alizarin red S (ARS) marking of fish in studies that have reported 100% mark success

Species	Stage	Chemical	Habitat	Exposure Time	Conc. (mg. L ⁻¹)	Mortality Effect	Reference
<i>Anguilla anguilla</i>	Glass eel	ARS	Fresh	Immersion 3 h	150	None	Simon et al. 2009
<i>Anguilla anguilla</i>	Glass eel	ARS	Fresh	Immersion 3 h	150	None	Caraguel et al. 2015
<i>Catostomus commersoni</i>	Larvae	ARS	Fresh	Immersion 12-24 h	200-300	0-3%	Beckman and Schulz 1996
<i>Macquaria ambigua</i>	Fingerling	ARS	Fresh	Immersion 5 min	500	None	Crook et al. 2009
<i>Oncorhynchus mykiss</i>	Fry	AC	Fresh	Immersion 18 h	10	< 1%	Van der Walt and Faragher 2003
<i>Paralichthys olivaceus</i>	Juvenile	AC	Salt	Immersion 24 h	300	None	Liu et al. 2009
<i>Paralichthys olivaceus</i>	Juvenile	ARS	Salt	Immersion 24 h	400	None	Liu et al. 2009
<i>Salmo trutta L.</i>	Fry	ARS	Fresh	Immersion 3 h with 10 g NaCl	150	None	Baer and Rösch 2008
<i>Scophthalmus maximus</i>	Larvae	AC	Salt	Immersion 6-24 h	60	None	Iglesias and Rodriguez-Ojea 1997
<i>Scophthalmus maximus</i>	Juvenile	AC	Salt	Immersion 24 h	120	None	Lagardère et al. 2000
<i>Scophthalmus maximus</i>	Juvenile	ARS	Salt	Immersion 24 h	100-400	None	Lagardère et al. 2000

Table 4. Comparison of optimal spawning time and minimum concentrations required for studies that marked fish via transgenerational marking with stable isotopes and achieved 100% mark success.

Species	Isotope	Habitat (Broodfish)	Minimum Conc. ($\mu\text{g. g}^{-1}$) Broodfish	Injection– Spawning Min. days	Reference
<i>Amphiprion melanopus</i>	^{137}Ba	Salt	0.45	3 to 21	Thorrold et al. 2006
<i>Amphiprion melanopus</i>	^{137}Ba	Salt	2	No spawning	Roy et al. 2013
<i>Amphiprion percula</i>	$^{135}\text{Ba} + ^{137}\text{Ba}$	Salt	25-50 (per fish)	field study	Almany et al. 2007
<i>Chaetodon vagabundus</i>	$^{135}\text{Ba} + ^{137}\text{Ba}$	Salt	330 (per fish)	field study	Almany et al. 2007
<i>Centropristis striata</i>	^{137}Ba	Salt	0.8	8	Thorrold et al. 2006
<i>Cyprinus carpio</i>	$^{86}\text{Sr} + ^{84}\text{Sr}$	Fresh	2.28 & 0.45	5	Zitek et al. 2014
<i>Dascyllus aruanus</i>	^{137}Ba	Salt	0.5	2	Cuif et al. 2014
<i>Epinephelus fuscoguttatus</i>	^{135}Ba	Salt	2	13 to 20	Williamson et al. 2009b
<i>Epinephelus fuscoguttatus</i>	^{137}Ba	Salt	0.5	13 to 20	Williamson et al. 2009b
<i>Macquaria ambigua</i>	^{137}Ba	Fresh	20	1	Munro et al. 2009
<i>Melanotaenia splendida</i>	^{137}Ba	Fresh	20	< 30	Starrs et al. 2014b
<i>Melanotaenia splendida</i>	^{87}Sr	Fresh	20	<30	Starrs et al. 2014b
<i>Morgurnda adspersa</i>	^{137}Ba	Fresh	20	7 to 11	Starrs et al. 2014a
<i>Morgurnda adspersa</i>	^{87}Sr	Fresh	20	7 to 11	Starrs et al. 2014a
<i>Morgurnda adspersa</i>	$^{137}\text{Ba} + ^{87}\text{Sr}$	Fresh	20	7 to 11	Starrs et al. 2014a
<i>Plectropomus leopardus</i>	^{138}Ba	Salt	2	No spawning	Williamson et al. 2009a
<i>Salmo trutta</i>	$^{135}\text{Ba} + ^{137}\text{Ba}$	Fresh	0.3	7	Huelga-Suarez et al. 2012
<i>Salmo trutta</i>	^{84}Sr	Fresh	0.0125	14	Zitek et al. 2013
<i>Salmo salar</i>	$^{135}\text{Ba} + ^{137}\text{Ba}$	Diadromous	0.3	31	Huelga-Suarez et al. 2013
<i>Salmo salar</i>	$^{134}\text{Ba} + ^{135}\text{Ba} + ^{136}\text{Ba} + ^{137}\text{Ba} + ^{86}\text{Sr} + ^{87}\text{Sr}$	Diadromous	2	21	Warren-Myers et al. 2015c
<i>Salmo salar</i>	$^{134}\text{Ba} + ^{135}\text{Ba} + ^{136}\text{Ba} + ^{137}\text{Ba}$	Diadromous	0.02	21	Warren-Myers et al. 2015c
<i>Salmo salar</i>	$^{135}\text{Ba} + ^{137}\text{Ba}$	Diadromous	0.02	14	Warren-Myers et al. 2015c

Table 5. Comparison of optimal delivery time and minimum concentrations required for studies that have marked fish via immersion with stable isotopes and achieved 100% mark success. OI = Osmotic Induction.

Species	Stage	Isotope	Habitat	Conc. ($\mu\text{g. L}^{-1}$)	Immersion Hour (h) Days (d)	Reference
<i>Fundulus heteroclitus</i>	Juvenile	^{137}Ba	Salt	5	70 d	Walther and Thorrold 2006
<i>Fundulus heteroclitus</i>	Juvenile	^{86}Sr	Salt	100	70 d	Walther and Thorrold 2006
<i>Maccullochella peelii</i>	Larvae	^{137}Ba	Fresh	100	6 d	Woodcock et al. 2011b
<i>Maccullochella peelii</i>	Larvae	^{138}Ba	Fresh	100	6 d	Woodcock et al. 2011b
<i>Macquaria ambigua</i>	Larvae	^{137}Ba	Fresh	30	1 d	Woodcock et al. 2011a
<i>Macquaria ambigua</i>	Juvenile	^{137}Ba	Fresh	5	24 d	Munro et al. 2008
<i>Macquaria ambigua</i>	Juvenile	^{137}Ba	Fresh	15	8 d	Munro et al. 2008
<i>Macquaria ambigua</i>	Juvenile	^{86}Sr	Fresh	1	24 d	Munro et al. 2008
<i>Salmo salar</i>	eggs	^{136}Ba	Fresh	1000	2 h	Warren-Myers et al. 2015b
<i>Salmo salar</i>	eggs	$^{135}\text{Ba} + ^{137}\text{Ba}$	Fresh	100	2 h	Warren-Myers et al. 2015b
<i>Salmo salar</i>	Larvae	^{137}Ba	Fresh	30	1 d	de-Braux et al. 2014
<i>Salmo salar</i>	Larvae	^{86}Sr	Fresh	75	12 h	de-Braux et al. 2014
<i>Salmo salar</i>	Larvae	^{86}Sr	Fresh	250	3.5 min (OI), 1 h	de-Braux et al. 2014

Table 6. Comparison of marking limitations of otolith mass marking methods by life-history stage.

Unique codes = estimated number of unique codes available. Cost \$USD is the estimated material cost to mark 1000 fish

Method	Stage			Unique codes	~ Material cost \$USD Per 1000 fish	Detection method
	Eggs	Larvae	Juvenile/Adult			
Otolith thermal marking	Y	Y	Y	>1000	1 ^a	Sectioning
Alizarin	N	Y	Y	1	4 ^b	Sectioning Fluorescence microscopy
Oxytetracycline	Y	Y	Y	<4 [†]	110 ^c	Sectioning Fluorescence microscopy
SrCl ₂	Y	Y	Y	2 [†]	3 ^a	Sectioning and BSM
Calcein	N	Y	Y	2 [†]	16 ^d	Sectioning, Fluorescence microscopy
Stable isotopes (Ba) (Sr)	Y	Y	Y	15	2 ^e	LA-ICP-MS
	Y	Y	Y	3	50 ^e	LA-ICP-MS
Sources for estimating cost per individual to mark ^a Hammer and Blankenship 2001 ^b Van der Walt and Faragher 2003; Caraguel et al. 2015 ^c Taylor et al. 2005 ^d Crook et al. 2010 ^e Warren-Myers et al. 2015c [†] Based on multiple exposures (Hendricks et al. 1991; Mohler 2003; Wickström and Sjöberg 2014)						

Table 7. Comparison of potential welfare effects on fish during, or after marking using common mass marking methods when applied in optimal conditions.

Method	Mortality	Growth effect	Other effect	References
Otolith thermal marking	0%	None	None	Volk et al. 1999
Alizarin	< 1 %	None	Toxic at high doses for larvae	Van der Walt and Faragher 2003; Meyer et al. 2012
Oxytetracycline	< 1%	None	Spinal deformities in some species	Bumguardner and King 1996; Toften and Jobling 1996
SrCl ₂	< 1%	None	None reported	Ophel and Judd 1968; Shippentower et al. 2011
Calcein	< 5%	None	Toxic at high doses	Brooks et al. 1994; Bumguardner and King 1996
Stable isotopes, Ba and Sr	0%	None	None	Williamson et al. 2009a; Warren-Myers et al. 2015a,b,c

Table 8. Current regulations and restrictions on the use of chemical marking techniques.

Method	Regulation	Restrictions on use	Handling and Disposal	Information Source's
Alizarin	- Approved for marking in AUS	- Legality questions around food safety outstanding	- See MSDS for Alizarin	AUS ^{3,5}
Oxytetracycline (Terramycin® 200)	- Approved for marking in US	- INAD 9332 study protocol (US) - No withdrawal period for stocked fish "if" not caught or harvested within 70 days of marking - Limit on maximum allowable concentration	- Burial or in landfill -See MSDS for Terramycin® 200	US ^{1,4}
SrCl ₂	- Being assessed in the US - Used in SWE	- Unrestricted use in Sweden. - Under assessment in US	- See MSDS for SrCl ₂	US ^{5,6} SWE ⁷
Calcein (SE-MARK)	- Approved for marking in AUS and US	- INAD -10-987 study protocol (US) - No withdrawal period - Only fish <2 g can be mark (US) - Limit on maximum allowable concentration	- Solidification and disposal in a landfill, or incineration - US specific disposal via shipment to Emerald Services, Inc. - See MSDS for Calcein	US ^{2,4} AUS ³
Stable Isotopes (Ba, Sr)	- Approved for marking in AUS	- Concentrations used must be below allowable drinking water standard, (Australia: 2 mg L per day) Country dependent - Legality questions around food safety outstanding	- See MSDS for SrCl ₂ and BaCl ₂	AUS ^{3,5}
Information sources: ¹ https://www.fws.gov/fisheries/aadap/inads/Oxytetracycline-dihydrate-INAD-9332.html ² https://www.fws.gov/fisheries/aadap/inads/Calcein-Se-Mark-INAD-10-987.html ³ Sanger and Crook 2007 ⁴ MSDS for calcein (SE-MARK) and Terramycin® 200 visit https://www.syndel.com ⁵ MSDS for alizarin, SrCl ₂ and BaCl ₂ visit https://www.fishersci.com ⁶ www.fws.gov/fisheries/aadap/22nd-AADAP-Coordination_meeting/6-MRO-AADAPP-Workshop-2016.pdf ⁷ Wickström and Sjöberg 2014				

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