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Optimizing the mass marking of fish with alizarin red S: an example with glass eels

Janek Simon

Potsdam Institute of Inland Fisheries, Potsdam, Germany

Correspondence
Janek Simon, Potsdam Institute of Inland Fisheries, Im Königswald 2, D-14469 Potsdam, Germany.
Email: janek.simon@ifb-potsdam.de

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Abstract

Fish marking is an essential tool for fisheries management, especially for evaluating the stocking of endangered fish species to support conservation and sustainable use of fish stocks. Batch marking of young European eels *Anguilla anguilla* (L.) prior to stocking is recommended as the benefits of stocking for the spawning stock can be evaluated by recapturing marked fish over time, therefore mass marking of young eels with substances such as alizarin red S (ARS) is becoming increasingly important. To improve the marking method and reduce marking costs when immersing glass eels in an ARS solution, eight laboratory experiments under varying conditions (e.g., temperature, ARS concentration, immersion time, osmotic induction, fish density) and with ARS from different suppliers were carried out. The results show that optimal marking of glass eels can be carried out in the field or during transport by putting approximately 50 g of glass eels per liter in 150 mg L^{-1} ARS solution for 3 h at 10–15°C. Lower concentrations did not result in reliable marking. Water temperatures of 5°C and below can have a stunning effect on the eels and increase mortality significantly, regardless of the concentration of ARS. Glass eel densities below 50 g L^{-1} in the marking bath increase marking costs unnecessarily, while a higher density of 100 g L^{-1} resulted in significantly higher mortality and lower marking success. A somewhat more difficult but less expensive alternative is to bathe the fish in a saline solution of 1% (10 PSU) of 80 mg L^{-1} ARS for 3 h at 10°C. Costs can also be significantly reduced by choice of supplier for ARS, but care should be taken as the quality of the powder appears to vary (mean percentage of sufficiently marked eels ranged from 59% to 91% among suppliers in the present study) and can lead to marking failure. The optimal marking conditions can help ensure that stocked glass eels can be reliably identified in future studies to assess stocking benefits while reducing costs.

KEYWORDS
Anguilla anguilla, marking cost, marking success, stocking, survival

1 | INTRODUCTION

Marking and tagging of fishes are essential tools with growing importance in aquaculture and in field studies for fisheries management. In aquaculture, for example, they are needed to identify parental fishes during breeding programs and to identify and trace farmed fish escapees in the wild. In field studies marking fishes can be used for age validation, to study fish behavior (e.g., migration), and to estimate predation, individual growth, stock size, and survival through mark–recapture experiments (e.g., Nielsen, 1992; Parker et al., 1990; Warren-Myers et al., 2018). Provided that sufficient numbers of individuals can be marked and recaptured in subsequent years, marking stocked fish is a key part of evaluating the success of stocking programs. Successful marking of large numbers of fish is thus especially important for evaluating the success of stocking programs focused on the conservation or reintroduction of endangered and threatened fish species like the catadromous European eel (*Anguilla anguilla*).

In the last decade, natural recruitment and commercial landings of the European eel stock have reached a historical minimum (ICES, 2021). One major limiting factor for eel recovery is the regulation and fragmentation of waterways, which hinder or prevent glass eels (the young and transparent life stage of the eel with c. 7 cm body length and c. 0.3 g body mass) arriving at the European coast from immigrating upstream in rivers and settling in inland waters (e.g., Belletti et al., 2020; Dekker & Beaulaton, 2016; Simon, 2023). For over 100 years, therefore, glass eels were caught on the coast, transported to certain inland waters in Europe, and released there to maintain eel populations in inland waters (e.g., Dekker & Beaulaton, 2016; Moriarty & McCarthy, 1982; Wickström, 1984). This practice of transport and release is called restocking and recently has gained in importance and become more common, partially as a result of European Union (EU) Council Regulation (EC) No. 1100/2007 (EU, 2007) focused on the recovery of the European eel. Eel restocking is one option listed in the regulation as a measure to help stock recovery and increase the number of mature European silver eels. Furthermore, according to this regulation, 60% of eels <12 cm total length caught annually in EU member states by fisheries have to be made available for restocking operations of freshwater bodies in European countries. Currently 15 EU Member States and the United Kingdom (UK) have reported restocking activities, and over 48 million glass eels were restocked yearly in 2018 and 2019 (ICES, 2021). The benefit of eel restocking for the spawning stock, however, is under discussion (ICES, 2011). Within this context, ICES has recommended the batch marking of all eels prior to restocking for distinguish between groups (restocked versus natural recruits) recovered in later surveys to evaluate their fate and their contribution to silver eel escapement (ICES, 2016).

For marking small eels before restocking, several successful applied marking and tagging methods exist, such as tagging with coded wire tags and visible implant elastomer tags (Simon & Dörner, 2011; Thomassen et al., 2000) or immersion marking with chemical markers like alizarin red S (ARS), oxytetracycline hydrochloride (OTC), strontium chloride ($SrCl_2$), and barium chloride ($BaCl_2$) (Simon et al., 2009; Wickström & Sjöberg, 2014). Immersion marking is less time-consuming, less expensive and reduces handling stress in comparison with other marking methods. Immersion marking is therefore most popular for mass-marking procedures and a common tool to evaluate the benefit of large-scale stocking programs with small fishes (Lü et al., 2019; Warren-Myers et al., 2018). The disadvantage of this method is that only batch marking is possible (the single individuals cannot be distinguished) and recaptured fishes must be killed to identify potential marks.

Fluorescent calcium-binding chemicals have distinct lower detection costs compared to identification of $SrCl_2$ and $BaCl_2$ marks and are among the most popular of the chemical markers (Lü et al., 2019; Unkenholz et al., 1997; Warren-Myers et al., 2018; Wickström & Sjöberg, 2014). OTC and ARS are suitable for easy and fast mass-marking of glass eels and fulfill the capture–recapture assumption of the no marking-induced effect on growth and survival of marked fish (Simon et al., 2009; Simon & Dörner, 2005). Mark retention was reported as lasting for up to 4 years in OTC-marked glass eels (Simon & Dörner, 2014) and up to 14 years in ARS-marked glass eels (Simon & Wickström, 2020). ARS is to be preferred over OTC for marking glass eels because ARS solutions do not foam, induce less stress, and do not oxidize over extended immersion times (Simon et al., 2009; Thomas et al., 1995) and therefore ARS has been increasingly used in the last decade. In France alone, for example, 14 t of glass eels were marked with ARS in the period from 2011 to 2021 (Feunteun et al., 2023).

Simon and Dörner (2005) and Simon et al. (2009) adopted the immersion marking method of Eckmann et al. (1998) to mark eleutheroembryos of vendace (*Coregonus albula*) with ARS (150 mg L^{-1}) over 3 h for marking glass eels. Subsequently all other scientists used the same ARS concentration for marking glass eels (Caraguel et al., 2015; Kullmann et al., 2018). Until now, to the author's knowledge, no controlled laboratory experiment has yet been conducted to determine if lower concentrations of ARS can also ensure good marking success while potentially decreasing costs. Furthermore, the success of marking by immersion depends on several factors, such as water temperature, concentration of the marking agent in the bath, and exposure time (e.g., Beckman & Schulz, 1996; Brooks et al., 1994; Iglesias & Rodriguez-Ojea, 1997; Lü et al., 2016; MacFarlane et al., 2002). Until now, it has not been determined if changing marking conditions such as water temperature or the use of osmotic induction (Mohler, 2003) could enable the use of lower ARS concentrations for successful marking of glass eels with lower marking costs.

The osmotic induction technique for marking fish involves exposing fish to saline water during the marking procedure to increase the rate of dye uptake and reduce the immersion time needed to produce good visible marks when compared with direct immersion marking (Crook et al., 2009; Mohler, 2003; Negus & Tureson, 2004). Two different methods of this technique exist. One way is to expose fish to saline water for a short period before immersion in the marking bath (e.g. Mohler, 2003; Negus & Tureson, 2004). The second is to directly increase the salinity of the marking bath (e.g. Baer & Rösch, 2008).

For mass marking of larger batches of glass eels, the biggest cost position is the price for the ARS substance. The price for ARS varies greatly depending on on the supplier (Table 1), but it is possible that

TABLE 1 Comparison of bath costs for different suppliers of alizarin red S (CAS-No.: 130–22-3) in 2018 used for marking 10 kg of glass eels with a fish density 50 g L^{-1} in a 150 mg L^{-1} alizarin red S solution.

Supplier	Packet size (g)	Catalogue price (€)[a]	Price for 1 g (€)	Cost of the marking bath (€)	Source
Sigma-Aldrich, Chemie GmbH, Munich, Germany[b]	25	104.00	4.16	124.80	www.sigmaaldrich.com
Merck KGaA, Darmstadt, Germany	100	217.00	2.17	65.10	www.merckmillipore.com
Carl Roth GmbH + Co. KG, Karlsruhe, Germany	100	85.90	0.86	25.80	www.carlroth.com
Thermo Fisher (Kandel) GmbH, Karlsruhe, Germany	25	41.50	1.66	49.80	www.alfa.com
Waldeck GmbH & Co. KG, Münster, Germany[c]	1000	399.48	0.40	12.00	www.waldeck-ms.de

[a]Possible volume discounts or customer allowance not included.
[b]Product was certified by the Biological Stain Commission.
[c]Supplier for dyestuffs and their solutions, not for chemicals.

marking quality or other negative effects on fish could be seen from some suppliers. Recent studies have shown, however, that a change to a low-priced supplier can result in an anesthetizing effect and a failure of the marking success (Wanke et al., 2016). It is thus important to evaluate if costs can be reduced by using ARS from less expensive sources without diminishing success.

The aims of the present study were therefore to (i) better understand the factors influencing the success of immersion marking glass eels in an ARS solution, (ii) find treatment combinations that reduce the required ARS concentration for successful marking of at least 95% of glass eels, which would be necessary for a successful recapture estimate (Buckley & Blankenship, 1990), and (iii) evaluate the quality and cost associated with using ARS powder from different suppliers.

2 | METHODS

2.1 | Fish marking and rearing

Eight experiments were conducted in this study, and the experimental set-up was identical in all of them, as follows. Glass eels were obtained from a commercial eel-trade company in spring in each year. From each delivery of glass eels, first 100 individuals were sampled randomly to determine their total length (L_T) and weight (W) (Table S1). These eels were not used for the experiment. If a temperature adaptation was necessary for the experiment, the glass eels were kept for 24 h in an aerated 150-L rectangular tank in a recirculation system. The tank was half-filled with water from the nearby Lake Sacrow (water depth 24 cm, actual water volume 89 L) to prevent the eels from escaping.

Before each marking, a random sample of glass eels was separated as a control group. Unless otherwise stated for the experiment, ARS (CAS-No.: 130-22-3) from the supplier Sigma-Aldrich Chemie GmbH was used. A stock solution (10 g L^{-1}) of ARS was produced with deionized water a maximum of 24 h before the start of the experiment and stored in a lightproof plastic bottle until the start of the experiment. Boiling salt from ESCO (www.esco-salt.com) was used to mix the salt bath.

The fish were marked by exposing them in 1 L of the marking bath (mixed with the stock solution and water from the nearby Lake Sacrow) and about 1 L of oxygen (giving an oxygen saturation of >100% in the marking bath) in a solid 10-L plastic bag (32 × 50 cm), which was sealed airtight with a rubber band. The plastic bag was stored in a dark room with diffuse light (10 lux) horizontally in a 150-L tank with temperate water to reduce stress during marking and hold the marking temperature stable during immersion time. The fish density in the plastic bags was 25 or 50 g of glass eels per liter in all treatments (including the control group) of all experiments except for experiment 3 (Table 2). The control group was treated in the same way but the fish were only bathed in lake water. The immersion time was 3 h for all treatments except for experiment 8 (Table 2).

After marking, the fish were kept in an aquarium system with 80-L tanks as described by Simon and Dörner (2011). The total capacity of the recirculated system was 120 g of dry feed per day or an efficiency of degradation of 5 g of NH_4-N per day. Water temperature was maintained at least at 10°C with an 800-W water heater (CVB Albert Carl GmbH and Co KG) and at most 20°C with a heat exchanger made of corrugated stainless-steel pipe (DN20/75; Potz-Blitz). From each marking bath a random sample of 12 g of glass eels was kept in a tank (four marking bath and four tanks per group). The 16 tanks were randomly stocked with the different groups (unmarked control group and/or groups marked with ARS). In each tank a hiding place (gutter foliage deflector grid curved to a pipe and fixed with a stainless-steel bolt and nut) was place on the ground to reduce the stress of the eels. The light regime was maintained at 24-h diffuse lighting from outside light and other lamps in the hall (4–40 lux).

In experiment 1 a temperature adaptation to 10°C within 24 h was done before feeding started. In the other experiments feeding started the day after marking. The glass eels were fed with living brine shrimp nauplii (*Artemia salina*) (Sanders®), approximately 20 000

TABLE 2 Experimental conditions during marking of glass eels with alizarin red S (ARS).

Experiment no.	Temperature (°C)	Osmotic induction	Supplier for ARS	ARS concentration (mg L^{-1})	Immersion time (h)	Fish density (g L^{-1})
1	5	No	Sigma-Aldrich	50, 100, 150	3	25
2	10	No	Sigma-Aldrich	50, 100, 150	3	50
3	10	No	Sigma-Aldrich	150	3	25, 50, 100
4	15	No	Sigma-Aldrich	50, 100, 150	3	25
5	10	5 min 1% NaCl -L^{-1} before	Sigma-Aldrich	60, 80, 100	3	25
6	10	1% NaCl -L^{-1} in parallel	Sigma-Aldrich	60, 80, 100	3	50
7	10	5 min 1% NaCl -L^{-1} before	Sigma-Aldrich, Carl Roth, Thermo Fisher, Waldeck	80	3	50
8	10	5 min 1% NaCl -L^{-1} before	Waldeck	100	3, 4, 6	50

nauplii per tank, daily for 2 weeks. After this time living or frozen white chaoborid larvae (*Chaoborus flavicans* Meigen) were fed 5 days per week. During the experiment, the amount of chaoborid larvae fed was increased from 0.5 to 1.2 g. Tanks were cleaned and checked for dead fish on the feeding days. Water temperature and oxygen during the time of experiment were measured on the feeding days with a portable digital two-channel multimeter kit (HQ40D; Hach Lange GmbH).

2.2 | Mark detection

After a clear visible increase in length and pigmentation of most of the glass eels (after 4–7 weeks) all eels were killed with sodium hydrogen carbonate ($NaHCO_3$, 0.030% aqueous solution) buffered ethyl 3-aminobenzoate methanesulfonate (MS-222, 0.015% aqueous solution). Fish were not fed for 2 days prior to killing. After killing, L_T (nearest mm) and M (nearest 0.01 g) were determined for each fish from each tank. To identify ARS marks at least one sagittal otolith per eel was extracted under a binocular microscope (Wild M32 Typ S; Wild Heerbrugg AG) and embedded lying flat in wax (Mounting Wax Crystalbond 590 Amber; Buehler GmbH) on a microscope slide. Finally, the otoliths were observed under a light microscope (Leitz Laborlux S; Ernst Leitz Wetzlar GmbH) equipped with an Hg lamp and the appropriate epi-fluorescence filter pack (excitation filter 515–560 nm, dichromatic mirror 580 nm, barrier filter 590 nm wavelength) at 125-fold magnification. The following score was assigned to evaluate the mark quality: 1, brilliant: 2, sufficient; 3, questionable; 4, absent (Figure 1). Only qualities 1 and 2 were considered as marking success.

2.3 | Experiments overview

In experiments 1, 2, and 4, the eels were marked with different concentrations of the ARS baths at different water temperatures

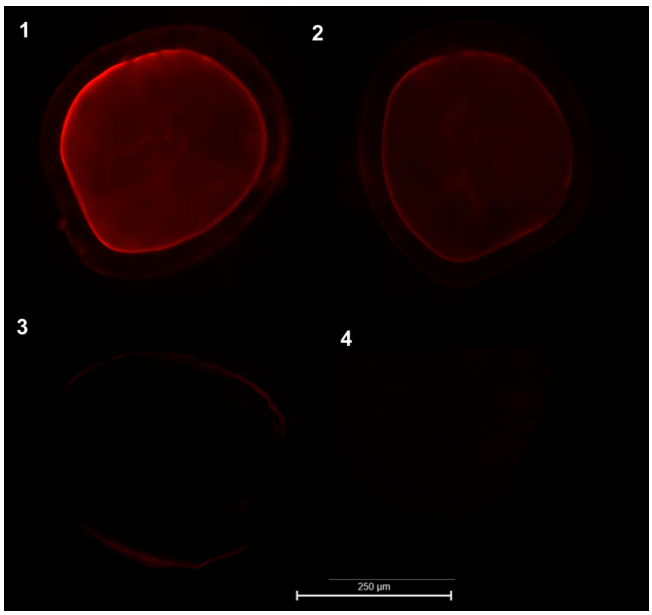

FIGURE 1 Saggitae otolith of a glass eel viewed in the darkroom under the epifluorescence microscope at 125× magnification: 1, brilliant; 2, sufficient; 3, questionable; 4, absent alizarin red S marks. Enlarge the page to 200% on a PC screen to see what is questionable about 3.

(Table 2). Different fish densities of glass eels in the marking bath were used in experiment 3. In experiment 5 the osmotic induction technique (Mohler, 2003) was applied before bathing the eels in the ARS solution. To keep the stress during marking as low as possible, the technique of osmotic induction was applied in experiment 6 in parallel to marking (the salinity of the marking bath was increased). In experiment 7, the marking bath concentration found in the previous experiments with marking success below 90% was used to test the ARS powders from different suppliers against each other. The experiments with lower quality marking success were used to compare

suppliers because differences are harder to detect in conditions leading to near 100% marking success.

The marking of the glass eels should not prolong the time between catching and restocking, and should also not increase handling. Therefore, if possible, the glass eels should be marked during transport, ideally after takeover from the glass eel fisher or from the supplier of the glass eels at the distribution point on the way to the stocking water. This practice can, however, lead to bathing times that exceed 3 h, therefore experiment 8 investigated whether a marking time longer than 3 h leads to impairments in the glass eels. The ARS from the supplier Waldeck was used in this experiment because it was the cheapest and achieved one of the best results regarding marking success found in the previous experiments. In experiment 8 an ARS concentration of 100 mg ARS L^{-1} was used because this was the lowest tested concentration with 100% marking success in the previous experiments.

2.4 | Statistical analysis

Statistical analyses were performed with the statistical program SPSS 9.0. (SPSS Inc.). Possible differences in environmental variables between the treatments within an experiment were tested using the Kruskal–Wallis test (*H* test) due to violation of normality ($p > 0.05$). Marking success and the percentage survival between treatments within an experiment were compared using a Pearson's chi-square test (x^2 test). If multiple tests were performed, the Bonferroni correction was applied to adjust *p* values. For all statistical hypotheses, the significance level was set at $\alpha < 0.05$.

2.5 | Ethics statement

German legislation concerning the care and use of laboratory animals was followed and ethical permission for the experiments was given by the State Office for Employment Protection, Consumer Protection and Health of the German Federal State of Brandenburg (reference number 2347-45-2017).

3 | RESULTS

The mean ± SD initial L_T and *M* of the delivered glass eels varied from 68 to 72 mm and 0.23 to 0.31 g between the experiments and was 69 ± 4 mm and 0.27 ± 0.06 g ($n = 800$) over all experiments (Table S1). The pH of the marking baths was between 7.2 and 8.1 at the beginning, and between 6.7 and 7.5 at the end of the marking in all experiments.

Water variables in the tanks during the experiments ranged from 9.6 to 20.9°C, oxygen from 8.6 to 10.9 mg L^{-1} (90% to 100% saturation), and pH from 7.6 to 8.7 (Table S2). In all experiments, no significant differences in environmental variables between the treatments of an experiment were found (*H* test, $df = 3$, $p > 0.05$).

3.1 | Mortality

During and after osmotic induction as well as during and after marking, no losses were observed in all experiments on the day of marking. Only in experiment 1, at the low marking temperature of 5°C, did the ARS appear to exert a stunning effect on the eels at concentrations of ≥100 mg L^{-1}. Approximately 20%–30% of the eels from the marking baths sank to the bottom after being placed in the aquaria and remained motionless there for several hours, despite the same good environmental parameters have existed in the marking bath as in the keeping tank for temperature adaptation before marking and aquaria for release after marking (e.g., similar water temperatures, pH value, oxygen saturation >90%). By the next day, however, most of the formerly immobile eels had recovered and were swimming around in the tank.

Some mortality occurred in some tanks in all experiments starting with the first day after marking. After 1 week with a few daily losses, a plateau was reached and further mortality was close to zero. From the third or fourth week of the experiments, the losses increased slightly again in some experiments and tanks with one to two animals per week and tank. In some experiments, however, individual tanks showed zero mortality until the end of the experiment (Table 3).

The mean ± SD mortality of marked and unmarked fish within the 28–49 days of experiments in the treatments ranged from 2 ± 3% to 27 ± 5% (Table 3). The mean mortality of fish did not differ significantly, either between the treatments (x^2 test, $df = 2$, $p > 0.05$) or between tanks within an experiment (x^2 test, $df = 15$, $p > 0.05$) in seven of the eight experiments at the end of the experiment. In experiment 3 (25, 50, and 100 g L^{-1} fish density) the mean losses at a fish density of 100 g L^{-1} were significantly higher than in the control group and the other fish densities (Table 3). Differences were also found between the individual experiments. In experiment 1 (5°C bath temperature), the mean losses over all treatments were significantly higher than in all other experiments except experiment 3.

3.2 | Marking success

In total, otoliths from 4284 marked fish were checked for a mark. The mean (range) number of fish checked for a mark was 43 (31–56) per tank and 171 (135–216) per group. Observed marking success rates ranged from 43% to 100% between tanks and from 46% to 99% between treatments 28 to 49 days after marking (Figure 2).

With bath temperatures of 5 and 10°C, sufficient marking (>95%) within 3 h bath duration was observed only in treatments with an ARS concentration of 150 mg L^{-1} (Figure 2). In contrast, at a bath temperature of 15°C and fish density of 25 g L^{-1}, sufficient marking was possible within 3 h bath duration with an ARS concentration of 100 mg L^{-1} (experiment 4, Figure 2). At a fish density of 100 g L^{-1}, a bathing temperature of 10°C with 150 mg L^{-1} ARS resulted in a significantly lower and insufficient marking success than at densities of 25 and 50 g L^{-1} (experiment 3).

The osmotic induction, whether applied before or in parallel, led to a better uptake of ARS at lower bathing concentrations than in the

TABLE 3 Mean (range) total mortality (%) at the end of the experiments for glass eels marked with alizarin red S (ARS) under different conditions.

Experiment no.	Group	Mortality[a]
1	Control	$16^{a} \pm 1$ (15–17)
	50 mg ARS	$15^{a} \pm 3$ (13–20)
	100 mg ARS	$19^{a} \pm 2$ (17–21)
	150 mg ARS	$21^{a} \pm 4$ (17–27)
2	Control	$6^{a} \pm 2$ (4–9)
	50 mg ARS	$7^{a} \pm 2$ (4–9)
	100 mg ARS	$5^{a} \pm 3$ (2–9)
	150 mg ARS	$5^{a} \pm 4$ (0–9)
3	Control (50 g L^{-1})	$12^{a} \pm 5$ (7–17)
	25 g L^{-1}	$9^{a} \pm 2$ (7–11)
	50 g L^{-1}	$12^{a} \pm 3$ (9–15)
	100 g L^{-1}	$27^{b} \pm 5$ (22–33)
4	Control	$9^{a} \pm 2$ (6–10)
	50 mg ARS	$10^{a} \pm 2$ (8–12)
	100 mg ARS	$8^{a} \pm 2$ (6–10)
	150 mg ARS	$7^{a} \pm 2$ (4–8)
5	Control	$5^{a} \pm 4$ (0–9)
	60 mg ARS	$4^{a} \pm 3$ (2–7)
	80 mg ARS	$3^{a} \pm 2$ (0–6)
	100 mg ARS	$2^{a} \pm 3$ (0–6)
6	Control	$10^{a} \pm 2$ (8–12)
	60 mg ARS	$7^{a} \pm 3$ (4–10)
	80 mg ARS	$10^{a} \pm 2$ (8–12)
	100 mg ARS	$8^{a} \pm 2$ (6–12)
7	Sigma-Aldrich	$12^{a} \pm 3$ (9–15)
	Carl Roth	$11^{a} \pm 1$ (11–13)
	Thermo Fisher	$9^{a} \pm 3$ (7–13)
	Waldeck	$9^{a} \pm 2$ (6–11)
8	Control	$9^{a} \pm 2$ (7–12)
	3 h	$9^{a} \pm 4$ (2–12)
	4 h	$7^{a} \pm 2$ (5–10)
	6 h	$7^{a} \pm 2$ (5–10)

Note: Numbers refer to the means of four tanks. The same letters indicate no significant differences (Pearson's chi-square test, $p > 0.05$).
[a]Percentage values rounded to integers.

same experiments without osmotic induction and 10°C bathing temperature. Thus, when osmotic induction was applied before marking, sufficient marking was possible within 3 h bathing time with an ARS concentration of only 80 mg L^{-1} and fish density of 25 g L^{-1} (experiment 5, Figure 2). When applying parallel osmotic induction for 3 h by a fish density of 50 g L^{-1}, sufficient marking with an ARS concentration of 100 mg L^{-1} is possible (experiment 6).

When the glass eels were marked at 10°C, first for 5 min in 1% salt water and then for 3 h with 80 mg L^{-1} ARS, the ARS from the supplier Carl Roth had the highest mean percentage of marked eels with a brilliant mark (61%) and the ARS from Sigma-Aldrich the lowest (10%) (experiment 7, Figure 2). The same applies to the mean percentage of marked eels with a sufficient mark (Carl Roth 92% and Sigma-Aldrich 59%).

In experiment 8, when glass eels were marked at 10°C, first for 5 min in 1% salt water and then with 80 mg L^{-1} ARS for 3, 4, or 6 h, the mean proportion of brilliant marks increased from 82% to 94% with increasing bath duration (Figure 2). However, the mean proportion of eels with sufficient marks was above 90% for all bathing times, increasing from 97% to 99%.

4 | DISCUSSION

Successful use of a marking method in mark–recapture and restocking experiments depends on not affecting either the survival or growth of marked fish due to the handling and marking procedures, and at least 95% marking success would be necessary for a successful recapture estimate (Buckley & Blankenship, 1990).

The pH of the marking baths was between 6.7 and 8.1 in all experiments and thus in the optimal range for the binding of ARS on the calcium of bones (Lievremont et al., 1982). The water temperature in the tanks during the experiments was influenced by outdoor conditions, but the observed water variables covered a normal range for eels (Schreckenbach et al., 1987).

4.1 | Mortality

In the present study, mortality was 0% in all treatments during and immediately after marking. This is consistent with other previous studies on the marking of glass eels with ARS (Simon et al., 2009; Simon & Dörner, 2005) and studies on other fish species (e.g., Bashey, 2004; Peterson & Carline, 1996; Secor et al., 1991; Skov et al., 2001). Mortality rates, however, can be greater at higher ARS concentrations (Beckman & Schulz, 1996; Bumguardner & King, 1996; Crook et al., 2007; MacFarlane et al., 2002), longer bathing times (van der Walt & Faragher, 2003), or higher temperatures (Brooks et al., 1994). The results of the present study reveal that bathing in ARS showed little or no effect on mortality under the given water quality characteristics, which were kept within normal ranges for the fish species marked (Schreckenbach et al., 1987).

In the present study, however, in all experiments mortality was observed within the first 24 h after marking. These results are different to prior studies involving marking glass eels with ARS, where first mortality was observed 7 and 55 days after marking (Simon et al., 2009; Simon & Dörner, 2005). The mortality observed in the present study is likely related to the catch, storage and transport by the glass eel fisher and trader, and handling during all these steps. All glass eels for the present study were obtained from a commercial eel-trade company from France. In France, 95% of glass eels are caught with push nets, where glass eels with larger lesions die in the first days after capture and post fishing mortality of glass eels can range from

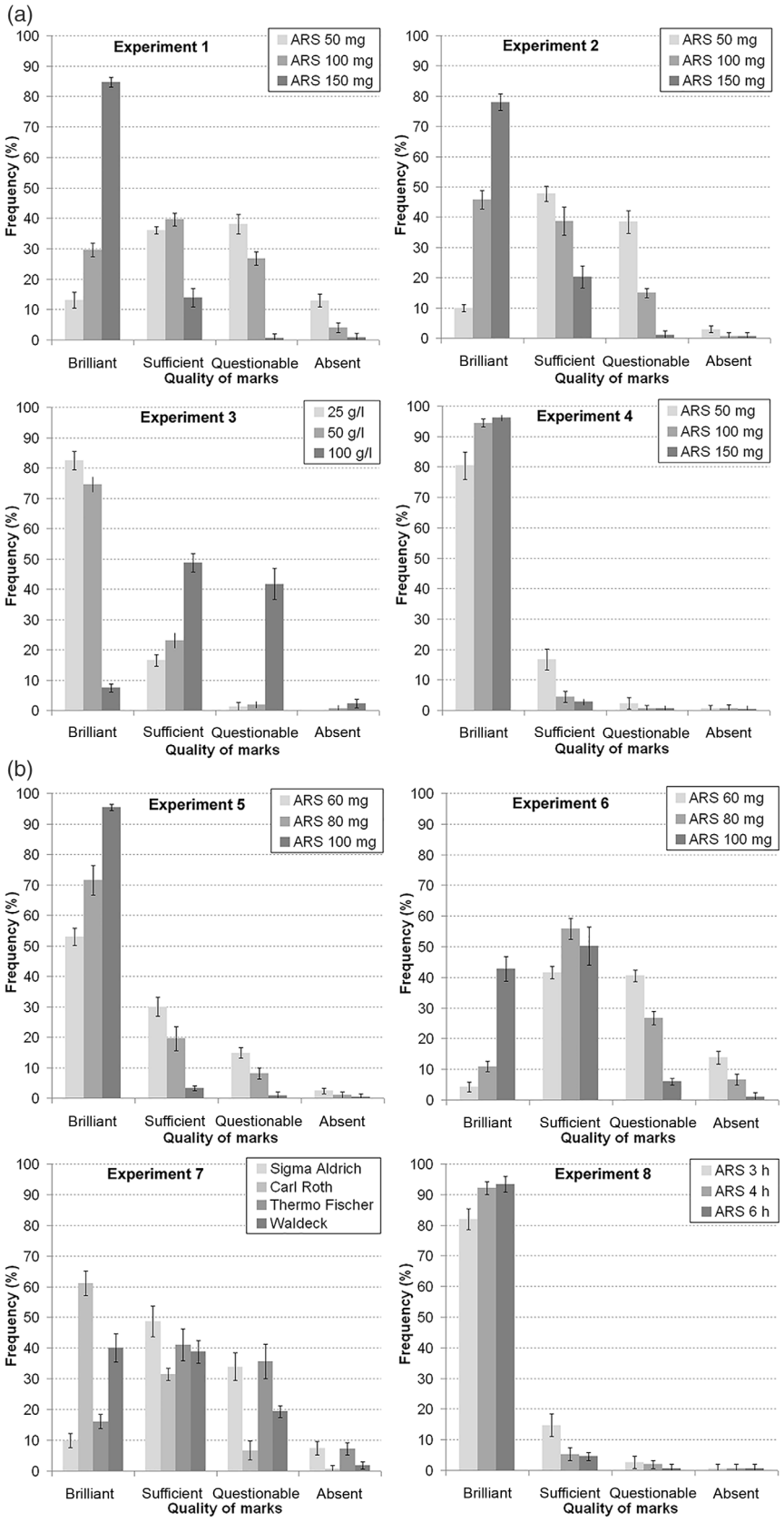

FIGURE 2 Comparison of the mean quality of otolith marks of glass eels investigated 4–7 weeks after immersion in alizarin red S (ARS) in eight experiments with different conditions. Results (values) refer to the means of four tanks.

0% to 67.2% after capture (Simon et al., 2022). In contrast, the prior studies of Simon and Dörner (2005) and Simon et al. (2009) purchased glass eels from the UK, where the glass eels were caught with hand nets (Forrest, 1976). In the hand-net fishing, no post-fishing mortality was observed within 2 days after capture (Briand et al., 2012). Furthermore, the glass eels for this study were kept by the French trader

for several days before they were delivered. In the studies by Simon and Dörner (2005) and Simon et al. (2009), on the other hand, the glass eels were delivered from UK to Germany within 24 h.

In seven of the eight experiments, no significant differences in mean mortality between the marked groups and the control group could be detected. Thus, osmotic induction (first or in parallel) and most of the marking concentrations used did not lead to any marking-related mortality. Based on previous research by Simon et al. (2009), marking was not expected to increase mortality in treatments with concentrations ≤150 mg L^{-1} ARS, 3-h bath duration, and a marking temperature of 10°C. This does not apply to all other marking methods in this study. At lower marking temperatures (5°C, experiment 1), the glass eels seemed to tolerate the marking and handling (landing net, etc.) worse, and the mean mortality was significantly higher (also in the control group) than in all other experiments with the exception of experiment 3. Thus, marking glass eels at water temperatures of 5°C and below is not recommended.

The same applies to a fish density of 100 g L^{-1} in the marking bath at 10°C bathing temperature (experiment 3). This fish density resulted in significantly higher mean mortality than the other fish densities and the control group in the experiment (Table 3). The higher mortality can be attributed to the higher concentration of metabolic end products of the eels in the bath. Thus, the ammonia content (NH_3) in the marking bath at the end of the experiment was higher at 100 g L^{-1} fish density with 0.10–0.12 mg L^{-1} NH_3 than at 50 g L^{-1} fish density with 0.007 mg L^{-1} NH_3 (J. Simon, unpublished data). Ammonia concentrations of 0.01–0.02 mg L^{-1} can have a chronic toxic effect and concentrations of 0.1 mg L^{-1} and higher can be acutely toxic (Gulyas & Fleit, 1990; Steffens, 1979). This can lead to damage to the mucous membrane or nerve and liver cells, and thus to the subsequent death of the glass eels. In contrast, a longer bathing time of 4 or 6 hours at a fish density of 50 g L^{-1} and 10°C bath temperature does not lead to higher mortality (experiment 8). High NH_3 concentrations can also be counteracted by decreasing the pH of the marking bath by adding, for example, hydrochloric acid to shift the dissociation equilibrium towards ammonium (NH_4), but this makes the marking procedure more complex.

Finally, the osmotic induction (first or in parallel) used in the present study did not lead to any marking-related mortality. The use of osmotic induction during marking, however, can cause stress and higher losses in some fish species than marking without osmotic induction (e.g., Ibáñez et al., 2013). Bathing eels in 1% artificial salt water probably does not serious differ for the eels than a bath in natural salt water (brackish water or seawater) (Wickström & Sjöberg, 2014). In some studies glass eels were bathed in 5% salt water for 3.5 min before marking with tetracycline chlorhydrate or OTC over 3.5 min without observed mortality (Alcobendas et al., 1991; Simon et al., 2009; Simon & Dörner, 2005). Furthermore, some glass eel fishers store captured glass eels in fish tanks with salt water after capture to reduce glass eel mortality (Simon et al., 2023a). Bathing eels in salt water is a common and regularly used method in aquaculture to improve their health status and growth (e.g., Mellergaard & Dalsgaard, 1987).

4.2 | Marking success

Observed marking success rates with ARS ranged from 98% to 100% between tanks and from 98% to 99% between treatments 28 to 49 days after marking in the treatments with concentrations of 150 mg ARS L^{-1}, fish densities ≤50 g L^{-1}, and bath temperatures of ≥10°C in the present study (Figure 2). Comparable marking success rates between 97.7% and 100% were observed by Simon and Wickström (2020) in three lakes 1–14 years after restocking with ARS marked glass eels. Other studies marking glass eels with ARS concentrations of 150 mg L^{-1} found 100% marking success in 10, 30, and 60 investigated eels, 14 days, 15 days, and 2 years after the marking event (Caraguel et al., 2015; Kullmann et al., 2018; Simon et al., 2009). In the present study, where 135–216 eels per experiment and marking group were investigated and in the study of Simon and Wickström (2020) where distinctly higher numbers of eels were investigated, marking success was not 100%. This shows that statistical analysis of the number of marked eels, mark retention of marks, and a conclusion of mark success rates of 100% based on the investigation of a low number of marked fishes should be evaluated with caution. The results of the present study suggest that a 100% marking success cannot be achieved in most cases. Even in the test groups with high bathing temperature, high bathing concentration, and long bathing time and thus a proportion of brilliantly marked eels of over 90%, there were still individual eels that did not show any marking (Figure 2). Marking success rates of 90%–100%, however, were reported for ARS-marked larvae and juveniles of other fish species (e.g., Sánchez-Lamadrid, 2001; Skov et al., 2001).

Otoliths in most fish species are composed of aragonite (Campana, 1999), but European eel otoliths can form an opaque mosaic structure with vaterite instead of aragonite (both are different crystal forms of calcium carbonate), which has a considerable impact on the incorporation of several elements (Tzeng et al., 2007). In the present study, out of the eels investigated for an otolith mark, there were only 24 fish (0.6%) showing a vaterite structure and abnormal size and structure in one or both of the two otoliths examined. Fifty per cent of these otoliths, however, showed a distinct ARS mark. Comparable results were observed by Simon and Wickström (2020).

Marking glass eels with ARS at 15°C water temperature leads to significantly better marking results than at 10°C (Figure 2). Nevertheless, marking glass eels at 15°C cannot be recommended in field conditions. Glass eels are caught at water temperatures of 7–12°C in estuaries (Simon et al., 2023a) and transport to receiving waters in boxes at mean water temperatures of 3.4°C (range 1.2–8.8°C; Simon et al., 2023b). Furthermore, water temperatures in most northern European countries are below 6°C at the time of restocking in winter and early spring. This means that the glass eels not only have to be adapted to a higher water temperature before marking, but also to a lower water temperature after marking before restocking. This is associated with additional stress for the eels as well as substantial effort. However, marking glass eels with ARS at 15°C can be used in aquaculture when glass eels are to be grown into farm-sourced eels for restocking. In aquaculture, glass eels are usually adapted to water

temperatures of 20–25°C before feeding begins because eels show their best growth at water temperatures between 23 and 26°C (Sadler, 1979; Seymour, 1989).

The marking success depends, among other things, on the metabolism of the fish during, but not after, marking (e.g., Liu et al., 2009; Lü et al., 2019). Furthermore, in experiments 7 and 8 there were practically no differences (only nuances, which may be due to preparation) between the marking quality of the two otoliths of a marked eel. This means that the marking is not random but dependent on the metabolic activity and other physiological processes of the fish. This was also shown by the fact that the marking quality increased significantly with the increasing water temperature of the marking bath. Therefore, a better uptake of the marking dye and thus mark quality can possibly be achieved by the targeted activation of the fish during marking, for example by a current in the marking bath, which forces them to swim slowly. This would again speak in favor of marking during transport to the stocking water. A weakness of this study is therefore that a different delivery of glass eels was used for each experiment. Depending on the quality of the glass eels supplied, there may be differences in the marking quality. Exhausted, weak fish form a mark more poorly than fresh, active fish. However, if in the present study the marking quality is compared for treatment groups within in the experiments (e.g., treatment group 150 mg L^{-1} ARS at 50 g L^{-1} fish density in experiments 2 and 3), the results do not differ significantly (x^2 test, $df = 1$, $p > 0.05$; Figure 2).

To keep the stress for the glass eels during marking as low as possible, the application of osmotic induction should not take place before the marking as in, for example, Mohler (2003), but parallel to the marking as in, for example, Baer and Rösch (2008). In this way, the fish are spared additional handling and adaption to a new environment, and the quality of the marking remains the same. In the present study, however, the application of the osmotic induction parallel to the marking (experiment 6) leads to significantly worse marking results than the application of the osmotic induction before the marking (experiment 5, Figure 2). The poorer results in experiment 6 are possibly mainly due to the higher fish density in the experiment, as the marking quality decreases with increasing fish density (experiment 3, Figure 2). In experiment 5 with the osmotic induction before, the fish density of 25 g L^{-1} was only half as high as in experiment 6 with parallel osmotic induction and a fish density of 50 g L^{-1}. It is possible that a fish density of 25 g L^{-1} in the marking bath with the osmotic induction applied in parallel can lead to good results similar to those with the application of the osmotic induction before the marking bath. This would have to be checked in a further experiment. Alcobendas et al. (1991) found no significant differences in the mark quality of otoliths of glass eels marked with tetracycline chlorhydrate or calcein between the two procedures (osmotic shock followed by immersion in the marking bath or simultaneous osmotic shock and marking bath).

Finally, when using lake water (or river water) for the marking bath, as in the present study, attention must be paid to the content of alkaline earth ions. If these (especially Ca) are present in high concentrations in the water, they already bind a considerable part of the ARS, which can reduce the marking success. Alternatively, partly deionized water could be used (e.g., 50% lake water and 50% deionized water) for the marking bath, but deionized water also causes costs. If only deionized water were used for the marking bath, then salt must be added and it has to be buffered with a buffer (e.g., sodium hydroxide, sodium bicarbonate, Tris buffer). This increases marking costs and means additional stress for the fish, as the water does not correspond to their natural environment.

4.3 | Marking costs

Direct immersion marking is the most commonly used method for all fluorescent dyes, especially for small fish (Lü et al., 2019). The marking process is relatively simple and fish recovery faster compared to the osmotic induction method prior or in parallel to immersion. In our experiments, however, we found that if osmotic induction is applied before marking, the marking concentration can be reduced from 150 to 100 mg L^{-1}ARS without affecting the marking success substantially while reducing costs for the ARS powder by 33%.

Exposure to 5% compared to 1.5% salt concentration during the osmotic induction can increase the absorption of the marking agent. This leads to marks of greater intensity and persistence as shown, for example, for Chinook salmon *Oncorhynchus tshawytscha* marked with calcein (Negus & Tureson, 2004). Crook et al. (2007), however, found that increasing the immersion time in the salt bath beyond 3.5 min may not necessarily improve the mark quality of ARS marks in golden perch fingerlings *Macquaria ambigua*. This should therefore be tested in further experiments, if even high salinity can further reduce marking concentration and thereby costs.

In addition, it should be examined whether a marking bath could possibly be used a second time to save costs. The results of the present study suggest, however, that at fish densities of 50 and 100 g L^{-1} in the dye bath and a dye bath concentration of 150 mg L^{-1}, reusing the bath is likely to result in insufficient marking (Figure 2). With a fish density of 25 g L^{-1} in the dye bath and the same dye bath concentration, this seems possible. In this case, however, it makes more economic sense to instead work with the higher density of 50 g L^{-1} in the dye bath rather than two passes of 25 g L^{-1} because of the extra time required.

To keep marking costs low, a fish density of 50 g L^{-1} of glass eels in the marking bath should be preferred. This means halving the necessary bath quantity, with a slightly lower but sufficient marking quality that is still >95% (experiment 3). A fish density of 100 g L^{-1} of glass eels, on the other hand, is not recommended, as it not only leads to insufficient marking of the eels but also to marking-related mortality.

The ARS from the supplier Sigma-Aldrich Chemie GmbH was the most expensive (Table 1), but it is certified by the Biological Stain Commission. The cheapest supplier in our study was Waldeck GmbH & Co. KG, but this is a supplier for dyestuffs and their solutions, not for chemicals. Irrespective of this, in our study the ARS from the suppliers Carl Roth GmbH + Co. KG and Waldeck GmbH & Co. KG achieved the best results with regard to marking success

(experiment 7, Figure 2). This means that a cheaper supplier of ARS does not necessarily have to be associated with disadvantages for marking success and offers a considerable savings potential. For example, for the marking of 10 kg of glass eels at a fish density of 50 g L^{-1}, the costs for the ARS would be 124.80 € from Sigma-Aldrich Chemie GmbH and only 12.00 € from Waldeck GmbH & Co. KG (Table 1).

Regardless of the available studies on successful marking methods of glass eels with ARS, a control group of fish should always be held back, reared for 2–4 weeks, and the successful marking checked. Recent studies have shown also that the use of a well-known supplier of chemicals can result in a failure of marking success. Wanke et al. (2016), for example, used ARS from Carl Roth GmbH + Co. KG for marking yolk-sac larvae of the vendace (*Coregonus albula*), which had an anesthetizing effect on the larvae. The larvae recovered completely after exposure to lake water, and during the following days there was no increase in mortality observed compared to unmarked larvae, but a failure of the marking procedure (few marked fish with low-quality marks) was found. In contrast, in our study we observed no anesthetizing effect on the glass eels and the highest mean percentage of marked glass eels with clearly brilliant marking compared to the other suppliers of ARS from the ARS from Carl Roth GmbH + Co. KG (Figure 2).

Besides glass eels, farm-sourced eels (i.e., glass eels reared to 5–10 g in an eel farm) are also widely restocked in Europe (ICES, 2021). Around 14 million farm eels were restocked annually in 2018 and 2019 (ICES, 2021), therefore the results of this study should be used to determine the optimal ARS concentration or marking method for farm eels as well. It is, however, more practical and more economical to mark the eels as glass eels before they are taken into the eel farm and feeding starts, rather than later as farm eels, due to the larger bath volumes and higher quantities of dye required. Furthermore, the marking methods presented here should next be tested in practice (marking of larger quantities on a larger scale). Finally, a technique for on-site removal of ARS from waste solutions should be developed, as disposal of bathing solutions represents an additional cost and hurdle for mass marking.

4.4 | Conclusion

In conclusion, two ways of reducing marking costs have been shown, which, when combined, result in a total cost reduction of 94% (Table 1). This creates the preconditions for making even larger marking campaigns cost-efficient.

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ORCID

Janek Simon https://orcid.org/0000-0002-6566-0072

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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